The Role of Slp-76 Phosphotyrosines in TCR Signal Transduction and T Cell Differentiation

Jennifer E. Smith-Garvin
University of Pennsylvania, smithje@mail.med.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations
Part of the Immunology and Infectious Disease Commons

Recommended Citation
http://repository.upenn.edu/edissertations/428

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/428
For more information, please contact libraryrepository@pobox.upenn.edu.
The Role of Slp-76 Phosphotyrosines in TCR Signal Transduction and T Cell Differentiation

Abstract
The cytosolic adapter protein src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) lacks enzymatic activity but nucleates a multi-molecular signaling complex that links early T cell receptor (TCR)-induced phosphorylation events into multiple downstream signaling pathways. The N-terminus of SLP-76 contains three tyrosines at residues 112, 128 and 145 that are phosphorylated following TCR ligation and, although the mechanisms are not entirely clear, they are required for optimal TCR signal transduction. TCR signals are required for T cell proliferation, cytokine production, and effector and memory differentiation. The experiments described in this dissertation have first tested the biochemical mechanisms by which the SLP-76 tyrosines transmit signals and second tested how alterations in the TCR signals transmitted through SLP-76 tyrosines influence T cell differentiation and effector function. Experiments were performed using two genomic knock-in (KI) mice that express tyrosine to phenylalanine mutations at residue 145 (Y145F) or 112 and 128 together (Y112/128F). Using biochemistry-, flow cytometry- and microscopy-based approaches we show that mutations in the tyrosines of SLP-76 result in graded defects in TCR-induced signals and function depending on the tyrosine(s) affected. Surprisingly, localization of SH2 domain containing effector proteins to mutant SLP-76-nucleated signaling complexes was not lost and therefore could not account for the observed signaling defects. Infection of SLP-76 KI mice with lymphocytic choriomeningitis virus (LCMV) resulted in normal CD8 expansion but graded enhancement of memory differentiation consistent with a model in which weaker TCR signals preferentially promote memory versus effector differentiation. Furthermore CD8+ effector and memory KI T cells failed to produce appropriate cytokine upon antigen restimulation. Similarly, in vitro polarized KI Th17 and Th2 cells failed to produce IL17a and IL4, respectively, following TCR restimulation. Taken together our data show that SLP-76 tyrosines are essential for optimal TCR signal transduction and, moreover, TCR signals sufficient to promote T cell differentiation are different than those required to elicit inflammatory cytokine production.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Immunology

First Advisor
Gary Koretzky

Keywords
T cell signaling, SLP-76, Tyrosine

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/428
Subject Categories
Immunology and Infectious Disease

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/428
THE ROLE OF SLP-76 PHOSPHOTYROSINES IN TCR SIGNAL TRANSDUCTION AND T CELL DIFFERENTIATION

Jennifer E. Smith-Garvin

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2010

Supervisor of Dissertation

________________________________________

Gary A. Koretzky, MD, PhD

Graduate Group Chairperson

________________________________________

Steven L. Reiner, MD

Dissertation Committee

Janis Burkhardt, PhD

Michael May, PhD

Bruce Freedman, VMD, PhD

David Allman, PhD
Acknowledgements

I would first like to thank Gary for his devotion to scientific mentoring, for his patience, his support and, most of all, for his unrelenting optimism. I would also like to thank Martha for having the patience to teach and mentor a completely inexperienced graduate student on a daily basis for many years. I thank the entire Koretzky lab, past and present for contributing to an incredibly productive, collaborative and functional environment. I thank Jeremy, Mercy, Tao and Jiyeon, in particular, for the generous sacrifices they made to help me study CD8 differentiation using an LCMV model while I was pregnant. I thank my collaborators John Wherry for help in designing and interpreting my CD8 experiments and Michael May for help with the FPLC experiments that did not make it into this dissertation. I thank Steve Krakowka for encouraging me to pursue a research career following vet school. I thank John Wolfe and Pete Felsburg for T32 training grant support. I thank Steve Reiner for accepting me into IGG despite my lack of experience and all of IGG for their support and fun times. I thank the members of my committee for their interest, advice and understanding. I thank the Flow Core for many long hours of sample collection with minimal clogging. I also thank the mice and the ULAR staff who keep them well. Finally I would like to thank my husband, Chris and our son, Oskar for putting things in perspective and for introducing me to the joys of family.
ABSTRACT

THE ROLE OF SLP-76 PHOSPHOTYROSINES IN TCR SIGNAL TRANSDUCTION AND T CELL DIFFERENTIATION

Jennifer E. Smith-Garvin

Supervisor - Gary A. Koretzky

The cytosolic adapter protein src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) lacks enzymatic activity but nucleates a multi-molecular signaling complex that links early T cell receptor (TCR)-induced phosphorylation events into multiple downstream signaling pathways. The N-terminus of SLP-76 contains three tyrosines at residues 112, 128 and 145 that are phosphorylated following TCR ligation and, although the mechanisms are not entirely clear, they are required for optimal TCR signal transduction. TCR signals are required for T cell proliferation, cytokine production, and effector and memory differentiation. The experiments described in this dissertation have first tested the biochemical mechanisms by which the SLP-76 tyrosines transmit signals and second tested how alterations in the TCR signals transmitted through SLP-76 tyrosines influence T cell differentiation and effector function. Experiments were performed using two genomic knock-in (KI) mice that express tyrosine to phenylalanine mutations at residue 145 (Y145F) or 112 and 128 together (Y112/128F). Using biochemistry-, flow cytometry- and microscopy-based approaches we show that mutations in the tyrosines of SLP-76 result in graded defects in TCR-induced signals and function depending on the tyrosine(s) affected. Surprisingly, localization of SH2 domain containing effector proteins to mutant SLP-76-nucleated
signaling complexes was not lost and therefore could not account for the observed signaling defects. Infection of SLP-76 KI mice with lymphocytic choriomeningitis virus (LCMV) resulted in normal CD8 expansion but graded enhancement of memory differentiation consistent with a model in which weaker TCR signals preferentially promote memory versus effector differentiation. Furthermore CD8⁺ effector and memory KI T cells failed to produce appropriate cytokine upon antigen restimulation. Similarly, in vitro polarized KI Th17 and Th2 cells failed to produce IL17a and IL4, respectively, following TCR restimulation. Taken together our data show that SLP-76 tyrosines are essential for optimal TCR signal transduction and, moreover, TCR signals sufficient to promote T cell differentiation are different than those required to elicit inflammatory cytokine production.
# Table of Contents

**ACKNOWLEDGEMENTS** .................................................................................................................. II

**ABSTRACT** ................................................................................................................................. ERROR! BOOKMARK NOT DEFINED.

**TABLE OF CONTENTS** .................................................................................................................. V

**LIST OF FIGURES** ......................................................................................................................... IX

**CHAPTER 1: INTRODUCTION** ........................................................................................................ 1

TCR SIGNAL TRANSDUCTION .............................................................................................................. 1

*SLP-76 and the Proximal TCR Signaling Complex* ........................................................................... 2

*PLCγ1 Activation and Signal Transduction* ......................................................................................... 6

*DAG-Mediated Signaling Pathways* .................................................................................................. 7

*Ca²⁺-Mediated Signaling Pathways* ................................................................................................... 8

*Actin and Cytoskeletal Responses* .................................................................................................... 9

**TCR SIGNALS AND T CELL DIFFERENTIATION** ......................................................................... 12

*Heterogeneity and differentiation of CD4 effector cells* ................................................................. 12

* T helper differentiation ..................................................................................................................... 13

*Transcription factors downstream of the TCR* .................................................................................. 15

*TCR signal strength and Th differentiation* ....................................................................................... 15

*The role of the proximal signaling complex in Th differentiation* .................................................... 16

*Heterogeneity and differentiation of CD8⁺ T cells* .......................................................................... 18

*CD8 effector and memory differentiation* ......................................................................................... 18

*Heterogeneity of effector and memory pools* .................................................................................. 19
TCR signals and the balance between terminal differentiation and memory differentiation.................................................................20

CONCLUSIONS................................................................................................................21

CHAPTER 2: THE ROLE OF SLP-76 PHOSPHOTYROSINES IN TCR SIGNAL TRANSDUCTION........................................................................................................................................25

ABSTRACT ........................................................................................................................................25

INTRODUCTION ..................................................................................................................................26

RESULTS ...........................................................................................................................................29

SLP-76 Y-F genomic KI mice .........................................................................................................29

Thymic selection in SLP-76 KI mice ............................................................................................29

Proximal TCR signaling is defective in SLP-76 mutant thymocytes .............................................29

SLP-76 KI mice generate peripheral T cells .................................................................................30

Proximal signaling defects in SLP-76 KI mice reveal a functional hierarchy for SLP-76 tyrosines..................................................................................................................................31

Itk and Vav1 functions are differentially affected in Y145F and Y112/128F SLP-76 KI mice ........................................................................................................................................32

Complementation of SLP-76 mutations in vivo ............................................................................33

Preserved binding of Vav1 and Itk to SLP-76 tyrosine mutants ....................................................34

SLP-76 tyrosines are required for optimal TCR signaling independent of their role in T cell development ...................................................................................................................................35

DISCUSSION .................................................................................................................................36

CHAPTER 3: TCR SIGNALS THROUGH SLP-76 TYROSINES REGULATE T CELL DIFFERENTIATION AND Effector FUNCTION ....................................................................................................................58

ABSTRACT ........................................................................................................................................58
INTRODUCTION .................................................................................................................. 59

RESULTS ............................................................................................................................ 61

Differential requirements for SLP-76 tyrosine signals in T helper lineage polarization ......................................................................................................................... 61

The requirement of SLP-76 tyrosines for IL17a competence and production is independent of their role in T cell thymic development ......................................................... 63

SLP-76 KI Th17 polarized T cells can upregulate the Th17 master regulator RORγT ........................................................................................................................................ 64

A subset of splenic CD4+ CD44hi T cells are poised to produce IL17a independent of SLP-76 tyrosines.................................................................................................................. 64

Th17 skewing conditions promote IL17a potential in WT and SLP-76 KI CD44hi CD4+ T cells. ................................................................................................................................. 65

CD8 expansion in response to acute LCMV infection is intact in SLP-76 KI mice .... 66

Altered effector function upon re-stimulation in SLP-76 KI CD8+ T cells following LCMV infection.......................................................................................................................... 67

Conditional SLP-76 KI T cells show defects in their response to LCMV challenge . 68

Poor polyfunctionality in KI mice is the result of defective responses to TCR re-stimulation ................................................................................................................................. 69

SLP-76 KI mice generate long-lived antigen-specific T cells with accelerated acquisition of a “mature” memory phenotype ............................................................................ 70

SLP-76 KI memory cells show intact expansion in response to rechallenge in vivo 72

DISCUSSION ......................................................................................................................... 73

CHAPTER 4: DISCUSSION ................................................................................................... 97

Introduction ......................................................................................................................... 97
New insights into the role of SLP-76 tyrosines in T cell signal transduction ..........97
Model for TCR signal transduction via the proximal signaling complex...............98
SLP-76 tyrosine-dependent and independent functions for Vav1 and Itk..............99
Hierarchical and non-hierarchical requirements for SLP-76 tyrosines...............101
Lineage specific requirements for SLP-76 tyrosine signals...............................102
Effect server versus Memory .............................................................................103
Conclusions .........................................................................................................105

CHAPTER 5: MATERIALS AND METHODS..............................................................106

Mice .....................................................................................................................106
Tamoxifen Treatment ..........................................................................................107
Flow Cytometry ....................................................................................................107
Calcium Flux .........................................................................................................108
CD69 upregulation ...............................................................................................108
CFSE/BRSE proliferation assays ........................................................................109
Immunoprecipitations and Western blots............................................................109
Constructs and retroviral transduction of T cells .............................................110
Immunofluorescence and cell imaging...............................................................111
T helper skewing assays .......................................................................................111
T helper restimulation assays ..............................................................................112
Infections .............................................................................................................112
LCMV peptide restimulation assays ..................................................................113
Statistical analyses ..............................................................................................113

WORKS CITED......................................................................................................114
List of Figures

FIGURE 1.1 SCHEMATIC OF THE ASSEMBLY AND CONSTITUENTS OF THE ADAPTOR-NUCLEATED PROXIMAL SIGNALING COMPLEX. ................................................................. 23

FIGURE 2.1 SCHEMATIC OF Y-F MUTANT SLP-76 MOLECULES EXPRESSED IN GENOMIC KI MICE ........................................................................................................... 41

FIGURE 2.2 THYMIC PROFILES OF SLP-76 KI MICE. .......................................................... 42

FIGURE 2.3. KNOCK-IN THYMOCYTES HAVE DEFECTS IN TCR INDUCED SIGNALING EVENTS. 43

FIGURE 2.4. SLP-76 KI MUTANTS GENERATE PERIPHERAL T CELLS. ................................. 44

FIGURE 2.5. SLP-76KI MUTANTS SHOW DEFECTS IN TCR INDUCED SIGNALING AND FUNCTION ................................................................................................................. 45

FIGURE 2.6 VAV1 AND ITK DEPENDENT DEFECTS IN SLP-76 KI THYMOCYTES ............... 47

FIGURE 2.7. Y145F KI MICE SHOW A PREPONDERANCE OF INNATE-LIKE LYMPHOCYTES. .... 48

FIGURE 2.8. SLP-76 MUTANTS CAN COMPLEMENT ONE ANOTHER IN TRANS ................. 50

FIGURE 2.9. PRESERVED BINDING OF VAV1 AND ITK TO SLP76 TYROSINE MUTANTS ........ 51

FIGURE 2.10. VAV1 AND ITK KD FORM CLUSTERS FOLLOWING TCR LIGATION IN Y145F AND Y112/128F KI T CELLS. ........................................................................ 52

FIGURE 2.11. SCHEMATIC OF GENETIC APPROACH TO THE GENERATION OF SLP-76 CONDITIONAL HETEROZYGOUS AND Y-F MUTANT MICE ................................. 54

FIGURE 2.12. CONDITIONAL SLP-76 KI T CELLS SHOW DEFECTS IN TCR INDUCED PROXIMAL SIGNAL TRANSDUCTION AND FUNCTIONAL RESPONSES IN VITRO. ........................................................ 56

FIGURE 3.1. IN VITRO TH1, TH2 AND TH17 LINEAGE POLARIZATION AND FUNCTION HAVE DIFFERENTIAL REQUIREMENTS FOR SLP-76 TYROSINES ................................. 78

FIGURE 3.2. TH17 POLARIZATION DEFECTS IN CONDITIONAL KI MICE ............................ 80
FIGURE 3.3. KI CD4 T cells upregulate RORγt expression under Th17 polarizing conditions..................................................................................................................................................81

FIGURE 3.4. A population of splenic CD44hi, CD4+ but not TCRγδ KI T cells is poised to produce IL17A directly ex vivo............................................................................................................................82

FIGURE 3.5. CD44hi CD4 KI T cells produce IL17A following in vitro Th17 polarization..............................................................................................................................83

FIGURE 3.6. SLP-76 KI mice have intact CD8 expansion in response to acute LCMV infection. ........................................................................................................................................................................84

FIGURE 3.7. SLP-76 KI T cells show defective effector function in response to acute LCMV infection.............................................................................................................................................85

FIGURE 3.8. LCMV infected cSLP-76 KI T cells show defective effector responses in vitro. ..........................................................................................................................................................87

FIGURE 3.9. Poor polyfunctionality in KI mice is the result of defective responses to TCR re-stimulation. .................................................................................................................................89

FIGURE 3.10. SLP-76 KI mice generate long-lived memory T cells with accelerated acquisition of a “mature” phenotype. ............................................................................................................91

FIGURE 3.11. SLP-76 KI LCMV-specific memory cells persist but show defective effector responses in vitro. ..........................................................................................................................93

FIGURE 3.12. SLP-76 KI memory cells expand in response to rechallenge in vivo. 95
Chapter 1: Introduction

The remarkable adaptability of the mammalian immune system results in immune responses that are tailored to specific pathogens and can become more efficient over time. T cells are key players in the adaptive wing of the immune system and have diverse functional capabilities that are determined by the nature of the inciting pathogen. Conventional naïve T cells can be divided into CD4+ helper T cells (Th) and CD8+ cytotoxic T lymphocytes (CTLs). To differentiate and acquire specific effector functions, both naïve CD4 and naïve CD8 T cells must integrate multiple signals from their external environment. Indeed, the T cell’s interpretation of the inflammatory context in which antigen presenting cells (APCs) present pathogen peptides to them dictates the character of the immune response. However, the nature of the T cell intrinsic signals propagated from peptide ligation of the TCR can also have a profound effect on T cell differentiation, but this process is less well understood. Below we discuss TCR signaling mechanisms and T cell differentiation and how the former may affect the latter during an infection.

TCR signal transduction

The functional αβ TCR is a complex consisting of an αβ TCR heterodimer, a γε CD3 heterodimer, a δε CD3 heterodimer and a ζζ CD3 homodimer (Kuhns et al., 2006). The extracellular domain of TCR heterodimer contains a variable domain responsible for binding antigenic peptide bound to an MHC molecule (pMHC). A disulfide bond in the extracellular constant region is responsible for the heterodimerization of the two chains. Three basic residues in the transmembrane domains interact with acidic residues in the CD3 dimers (Call et al., 2002). The intracellular domain of the TCR heterodimer is
minimal and it is the intracellular domains of the CD3 chains that contain the immunoreceptor tyrosine based activation motifs (ITAMs) necessary for the initiation of the intracellular signaling cascade. There are a total of ten ITAMs in the TCR complex with three ITAMs on each ζ chain and one on each δ, γ, ε chain. The biological relevance of the large number of ITAMs within the TCR complex is not yet fully understood, but they may play a role in thymocyte selection (Holst et al., 2008). ITAMs have no enzymatic activity but rather contain two tyrosines that upon phosphorylation recruit and activate cytosolic protein tyrosine kinases (PTKs) including Zap70 (Lin and Weiss, 2001). The Src family kinases (Lck and Fyn) are responsible for phosphorylation of ITAMs associated with the TCR (Lin and Weiss, 2001). While this phosphorylation step is essential, and is the first measurable step in the TCR signaling cascade, how it is regulated remains largely unknown. Indeed, the mechanics of TCR triggering – how ligation of the extracellular domain of the TCR complex results in the phosphorylation of the intracellular ITAMs – have been difficult to ascertain, although multiple models have been suggested with varying degrees of experimental support (Aivazian and Stern, 2000; Alarcon et al., 2006; Davis and van der Merwe, 2006; Ma et al., 2008; Minguet and Schamel, 2008; Varma, 2008). Below we will focus on the steps following the initiation of these earliest phosphorylation events.

**SLP-76 and the Proximal TCR Signaling Complex**

Following recruitment of Zap-70 to the CD3 ITAMs, a cascade of phosphorylation events ensues resulting in the assembly of a subcellular, adapter protein nucleated multi-molecular signaling complex (Figure 1.1). This complex is responsible for propagating the TCR/PTK signal into multiple and diverse distal signaling pathways.
Among the most important of the Zap-70 targets are the transmembrane adapter protein linker for the activation of T cells (LAT) and src homology 2 (SH2) domain-containing leukocyte phosphoprotein of (76 kDa SLP-76) (Bubeck Wardenburg et al., 1996; Zhang et al., 1998a). These two adapters form the backbone of the complex that organizes effector molecules in the correct spatio-temporal manner to allow for the activation of multiple signaling pathways. The importance of these adapters is underscored by studies showing that the loss of either LAT or SLP-76 results in a near complete loss of TCR signal transduction reminiscent of syk/Zap-70 or Lck/Fyn doubly deficient T cells (Clements et al., 1998; Koretzky et al., 2006; Sommers et al., 2004; Zhang et al., 1999b).

LAT contains nine tyrosines that are phosphorylated upon TCR engagement, which have been shown to bind the C-terminal SH2 domain of phospholipase Cγ1 (PLCγ1), the p85 subunit of phosphoinositide 3-kinase (PI3K), and the adapters growth factor receptor-bound protein 2 (GRB2) and GRB2-related adapter downstream of Shc (Gads) (reviewed in (Sommers et al., 2004)) (Figure 1.1). SLP-76 is then recruited to phosphorylated LAT via their mutual binding partner Gads (Liu et al., 1999). SLP-76 itself contains four modular domains: an N-terminal acidic domain with three phosphorylatable tyrosines that interact with the SH2 domains of the guanine nucleotide exchange factor (GEF) Vav1, the SH2/SH3 adaptor Nck, and IL-2-induced tyrosine kinase (Itk); a sterile alpha motif (SAM) domain; a proline rich region (PRR) that binds constitutively Gads, PLCγ1 and Itk; and a C-terminal SH2 region that can bind adhesion and degranulation-promoting adapter protein (ADAP) and hematopoietic progenitor kinase 1 (HPK1) (reviewed in (Koretzky et al., 2006)) (Shen et al., 2009). While LAT and SLP-76 serve to nucleate this large signaling complex, the effector molecules themselves are also important for stabilizing the complex. For example, the Tec family
kinase Itk is required in a kinase-independent manner for the recruitment of Vav1 to the APC contact site, while Vav1 is required for optimal SLP-76 phosphorylation and recruitment to LAT as well as for Itk activation (Dombroski et al., 2005; Reynolds et al., 2004; Reynolds et al., 2002). These and other data suggest that the formation of the complex is more complicated than the linear model most often invoked for simplicity. For example, PLCγ1 has been shown to directly bind to SLP-76, LAT, Vav1, as well as its activating kinase Itk (reviewed in Qi and August, 2007). It is thought that these interactions collectively are required to stabilize PLCγ1 in the correct conformation within the complex to allow for its optimal activity (Beach et al., 2007). Advancements in biochemical and structural techniques are needed to elucidate the precise allosteric and perhaps stochiometric changes within the multi-molecular complex that allow for signal transduction.

Of the effector molecules within the signaling complex, Itk, Vav1, and PLCγ1 appear to be the most important for T cell functions. Itk is a Tec family protein kinase that is critical for TCR-induced PLCγ1 phosphorylation and activation, cytoskeletal reorganization and proliferation (Labno et al., 2003; Schaeffer et al., 1999). Itk activation following TCR stimulation is complex and still poorly understood. The first step in Itk activation appears to be localization to the plasma membrane via a PH domain (Pletneva et al., 2006). This is dependent on PI3K activity and Vav1 is required for PI3K activation, which may explain the defect in Itk activation observed in the absence of Vav1 (Reynolds et al., 2004; Reynolds et al., 2002). Itk must also bind SLP-76 for kinase activation (Bogin et al., 2007). A proline cis-trans conversion within the SH2 domain of Itk is required for kinase activity and it has been hypothesized that
phosphotyrosine binding to the SH2 domain may stabilize Itk in the trans (active) conformation (Pletneva et al., 2006).

Vav1 is a GEF that can activate the small GTPases Rac and Cdc42. Vav1 is also important for PI3K, Erk, Jnk, PLCγ1 and Akt activation as well as cytoskeletal rearrangements (Charvet et al., 2006; Fischer et al., 1998a; Fischer et al., 1998b; Fujikawa et al., 2003; Holsinger et al., 1998; Katzav et al., 1989; Reynolds et al., 2004; Reynolds et al., 2002; Villalba et al., 2001; Wood et al., 2006). It is important to note that Vav1 has both GEF-dependent and -independent functions and the role, if any, of GEF activity in T cells is contested (Miletic et al., 2009; Saveliev et al., 2009). It is apparent however that the role of Vav1 in Ca2+- and ERK-mediated pathways are GEF independent (Miletic et al., 2009; Saveliev et al., 2009). Vav1 is phosphorylated following TCR ligation in both T cells and thymocytes but the role of phosphorylation has been difficult to ascertain as the tyrosines have both activating and inhibitory functions (Miletic et al., 2006). Furthermore, the role of SLP-76 in TCR-induced Vav1 phosphorylation has not been resolved as studies in Jurkat cell lines have shown that SLP-76 is not required for Vav1 phosphorylation (Dombroski et al., 2005) while T cells from SLP-76-/- mice reconstituted with a Y3F SLP-76 transgene do show a defect in Vav1 phosphorylation (Myung et al., 2001).

It is becoming increasingly apparent that the molecular interactions among the constituents of the proximal signaling complex serve more than to simply recruit and maintain the subcellular location of effector molecules but they may also influence the enzymatic activities of the effector molecules. For example, genomic knock-in mutation of tyrosines on Vav1 does not result in a loss of interaction with their proposed binding partners, but does result in abrogation of Vav1-dependent signaling (Miletic et al., 2006). The co-precipitation observed in these experiments is likely the result of tertiary
interactions with other Vav1 domains or other molecules within the larger signaling complex, however, the functional defects observed in this study suggests that SH2/phosphotyrosine interactions may play important regulatory roles for the activation of effector molecules. Indeed, structural studies have suggested that the interaction between the SH2 domain of Itk and a phosphotyrosine results in a conformational switch allowing kinase activity (Pletneva et al., 2006). Consistent with these data, it was shown in Jurkat T cell lines that an Itk/SLP-76 interaction is required for Itk kinase activity, although it has not yet been shown if it is specifically the SH2/phosphotyrosine interaction that mediates this kinase activity (Bogin et al., 2007). While the role of specific domain/domain interactions within the proximal signaling complex remain unclear, it is clear that an intact complex is required for optimal signal transduction from the TCR to multiple downstream pathways, including the activation of PLCγ1-dependent Ca\(^{2+}\) and DAG-induced responses as well as the cytoskeletal responses necessary for T cell function and differentiation.

**PLCγ1 Activation and Signal Transduction**

Following TCR ligation, PLCγ1 is found in the proximal signaling complex bound to SLP-76, Vav1, and LAT, where it is phosphorylated and activated by Itk. Activated PLCγ1 then hydrolyzes the membrane lipid PI(4,5)P\(_2\) (phosphatidylinositol 4,5 bisphosphate), producing the second messengers IP\(_3\) and DAG. These two messengers are essential for T cell function, and therefore the regulation of PLCγ1 activation has been the subject of intensive studies. Localization of PLCγ1 to the proximal signaling complex is dependent on LAT and the Gads-binding region of SLP-76 (Beach et al., 2007). Activation of PLCγ1 is dependent on Itk kinase activity that, in turn, is dependent on Vav1, Lck, ZAP-70, LAT, and SLP-76 (Berg et al., 2005; Liu et al., 1998; Reynolds et al.,
Following TCR ligation, Itk is recruited to the membrane through PH domain interactions with PIP$_3$, which has been locally generated by Lck-induced PI3K activity (reviewed in (Berg et al., 2005)). At the membrane, Lck phosphorylates Itk, and the SH2 and SH3 domains of Itk interact with phosphorylated tyrosine 145 and the PRR of SLP-76, respectively (Bunnell et al., 2000; Shan and Wange, 1999; Su et al., 1999). The role of Vav1 and ZAP-70 in Itk activation is not understood but may relate to their involvement in the phosphorylation of SLP-76 or in the activation of PI3K (Bubeck Wardenburg et al., 1996; Reynolds et al., 2004; Reynolds et al., 2002). A second Tec family kinase, Rlk, can also phosphorylate PLC$\gamma$1, resulting in a relatively mild defect in Itk-deficient mice and requiring the study of Rlk/Itk double deficient mice to better understand the role of Tec kinases in T cell activation (reviewed in (Berg et al., 2005)).

**DAG-Mediated Signaling Pathways**

TCR-induced PLC$\gamma$-dependent production of DAG results in the activation of two major pathways involving Ras and PKC$\theta$. Ras, a guanine nucleotide-binding protein, is only active in the GTP-bound state, and its activation is facilitated by GEFs and suppressed by GTPase activating proteins (GAPs). Two Ras GEFs are present in T cells, son of sevenless (SOS) and Ras guanyl nucleotide-releasing protein (RasGRP) (Ebinu et al., 2000; Egan et al., 1993). RasGRP is inducibly recruited to the membrane through a DAG-binding domain (Ebinu et al., 1998), where it is phosphorylated by PKC$\theta$ (Roose et al., 2005). SOS is constitutively bound to the adapter protein GRB2, and upon TCR stimulation, the GRB2 SH2 domain is recruited to and binds phosphorylated tyrosines on LAT, thereby bringing SOS into the proximal signaling complex where it can facilitate the localized activation of Ras (Finco et al., 1998). RasGRP-dependent RasGTP production enhances SOS activity, resulting in a positive feedback loop and robust TCR-induced
Ras activation (Roose et al., 2007). Ras activity is required for the activation of the serine-threonine kinase Raf-1 that leads to the activation of the mitogen-associated protein kinases (MAPKs) extracellular signal-regulated kinase 1 (Erk1) and Erk2. Erk activation results in transcriptional activation of Elk1 and signal transducer and activator of transcription 3 (STAT3) and Lck serine phosphorylation (reviewed in (Genot and Cantrell, 2000). Ras is also involved in activation of the activator protein-1 (AP-1) (c-Jun/c-Fos) transcription complex and upregulation of CD69 expression (D'Ambrosio et al., 1994).

The second major signaling pathway regulated by DAG is mediated by PKCθ, which is recruited to the plasma membrane following TCR ligation through a lipid-binding domain specific for DAG. Lck phosphorylation of PKCθ may also play a role in its localization and activation (reviewed in (Hayashi and Altman, 2007). Additionally, other proximal signaling molecules, including Vav1, PI3K, and 3-phosphoinositide-dependent kinase 1 (PDK1) have been implicated in regulating PKCθ localization, but details of their contributions are not completely defined (Hayashi and Altman, 2007). PKCθ activation is critical for the regulation of NFκB activity. The NFκB family of transcription factors consists of five members that upon activation and translocation to the nucleus activate multiple genes involved in the function, survival, and homeostasis of T cells (reviewed in (Schulze-Luehrmann and Ghosh, 2006).

**Ca^{2+}-Mediated Signaling Pathways**

Ca^{2+} ions are universal second messengers in eukaryotic cells. The IP₃ generated by TCR-stimulated PLCγ1 activity stimulates Ca^{2+}-permeable ion channel receptors (IP₃R) on the endoplasmic reticulum (ER) membrane, leading to the release of ER Ca^{2+} stores.
into the cytoplasm. Depletion of ER Ca\(^{2+}\) triggers a sustained influx of extracellular Ca\(^{2+}\) through the activation of plasma membrane Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels in a process known as store-operated Ca\(^{2+}\) entry (SOCE) (reviewed in Oh-Hora and Rao, 2008). While CRAC channels appear to be the dominant mode of Ca\(^{2+}\) entry in T cells, other Ca\(^{2+}\) channels exist; however, their relevance remains unclear (reviewed in Oh-Hora and Rao, 2008).

TCR-induced increases in intracellular Ca\(^{2+}\) levels result in the activation of Ca\(^{2+}\) and calmodulin-dependent transcription factors and signaling proteins, including the phosphatase calcineurin and the Ca\(^{2+}\)-calmodulin-dependent kinase (CaMK), that in turn activate a variety of transcription programs (reviewed in Savignac et al., 2007)). Activated calcineurin dephosphorylates members of the nuclear factor of activated T cells (NFAT) family, leading to their translocation to the nucleus. In the nucleus, NFATs can form cooperative transcriptional complexes with a variety of transcription factors, thereby integrating signaling pathways resulting in differential transcriptional patterns.

The most well studied interaction is NFAT/AP-1 that results in IL-2 production; however, the regulatory T cell lineage-specific transcription factor forkhead box protein 3 (FOXP3) also has been shown recently to cooperate with NFAT (Wu et al., 2006). Other pathways including those resulting in NF\(\kappa\)B and Jnk activation have been shown to be Ca\(^{2+}\)-dependent; however, these effectors also require additional signals and emphasize crosstalk between Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent T cell signaling pathways (Oh-Hora and Rao, 2008).

**Actin and Cytoskeletal Responses**

When a T cell is presented with cognate antigen by an APC, signals from the TCR initiate a program of actin cytoskeletal rearrangements that results in polarization and
activation of the T cell (reviewed in (Burkhardt et al., 2008). Actin reorganization is essential for T cell function, as actin polymerization inhibitors impede T cell/APC interactions (Henney and Bubbers, 1973) and abolish proximal TCR signals (Holsinger et al., 1998).

T cell/APC conjugation results in morphological changes, as the stimulated T cell rounds up and accumulates filamentous actin (F-actin) at the stimulatory interface. These changes are thought to be dependent on TCR-induced signals. For example, plasma membrane fluidity is increased, in part, by the TCR- and Vav1-dependent transient dephosphorylation of ERM (ezrin, radixin, and moesin) proteins, resulting in the loss of their ability to link the plasma membrane to the actin cytoskeleton (Faure et al., 2004). Ca^{2+} signaling and integrin activation downstream of the TCR results in additional modifications of actin-associated proteins that may play roles in altering plasma membrane rigidity (Burkhardt et al., 2008).

Accumulation of F-actin at the T cell/APC interface is the result of TCR-induced localized activation of multiple actin regulatory and polymerizing pathways, the best studied of which involves the actin-related proteins 2/3 (Arp2/3) complex, although Arp2/3-independent pathways also contribute to this process (Gomez et al., 2007). Activation of Arp2/3 requires its interaction with nucleation-promoting factors (NPF) including Wiskott-Aldrich syndrome protein (WASp), WASp family verprolin homologous protein (Wave2), and hematopoietic cell lineage specific protein 1 (HS1). WASp is recruited to the site of TCR activation through its interaction with the SLP-76-associated adapter protein Nck, where it is activated via Vav1-dependent stimulation of the Rho-family GTPase Cdc42 (Zeng et al., 2003). Like WASp, Wave2 activation is dependent on Vav1; however, in this case it is through Vav1-mediated stimulation of a second Rho-family GTPase, Rac1 (Burkhardt et al., 2008). Given the proposed reliance of WASp
and Wave2 on Vav1-mediated GTPase activation, it is interesting that actin-dependent processes that are defective in Vav1-deficient T cells can be rescued with the expression of a GEF-inactive Vav1 mutant, suggesting that other Rac and Cdc42 GEFs may be able to support TCR-induced actin changes (Miletic et al., 2006). This result also suggests that other Vav-1 functions are important for TCR-induced actin changes. Consistent with this is the observation that through its protein interaction domains, Vav1 may contribute to Wave2 and WASp activation through the recruitment of Dynamin2, a GTPase known to be important for TCR-induced actin dynamics (Gomez et al., 2005).

pMHC/TCR ligation also results in the formation of intracellular microclusters that contain the TCR complex and associated signaling molecules including LAT and SLP-76 (Bunnell et al., 2002). These clusters initiate and sustain Ca\(^{2+}\) signals (Yokosuka et al., 2005). The clusters persist for a short time, after which they converge towards the center of the T cell/APC interface at the center of the IS (Varma et al., 2006; Yokosuka et al., 2005). Formation and translocation of the clusters is dependent on F-actin dynamics, and new clusters continue to form even after the mature IS is established (Campi et al., 2005; Varma et al., 2006; Yokosuka et al., 2005). Integrins play a key role in sustaining microclusters, emphasizing the importance of integrin activation for T cell function (Nguyen et al., 2008).

TCR signaling cascades and pathways downstream of actin reorganization are intertwined and difficult to tease apart, as many of the effector molecules involved have multiple enzymatic and adapter functions. WASp, Wave2, and Vav1 signals play roles in TCR-induced signaling that appear to be independent of their roles in actin responses (Nolz et al., 2006; Reynolds et al., 2002; Zhang et al., 1999a). Therefore, loss of different actin regulators may result in complex TCR signaling defects (Billadeau et al., 2007). Future studies are required to fully understand the feedforward and feedback
mechanisms that define the interdependence between cytoskeletal dynamics and T cell activation.

**TCR signals and T cell differentiation**

While much is known about how the nature and strength of TCR signals can affect the positive and negative selection of thymocytes and thus the emergence of naïve T cells, much less is known about the role of TCR signals in the differentiation of naïve T cells into effector and memory cells. Indeed the focus of differentiation research has been on the cytokines and transcription factors that play key regulatory roles in determining the fate decisions and functional capabilities of peripheral T cells. However, it is well accepted that TCR signals are indispensable for the initiation of an effector differentiation program in naïve T cells and, in most cases, again for effector function in antigen experienced T cells. Understanding how alterations in TCR signals can affect the quality of T cell differentiation and, in turn, the character of the immune responses will be essential for vaccination and therapeutic strategies to become more specific.

**Heterogeneity and differentiation of CD4 effector cells**

Recent studies have shown that the CD4 T cell pool that emerges from the thymus is more heterogeneous than previously thought. Emerging CD4 T cells can have innate-like or inducible effector functions. CD4 T cells with innate-like function emerge from the thymus already capable of effector function and include the well described NKT cells and natural T regulatory cells (nTreg) (Bendelac et al., 1994; Sakaguchi et al., 1995) as well as CD4+ innate-like lymphocytes (ILLS)(Hu and August, 2008) and a recently described subset of thymically-derived Th17 cells (Marks et al., 2009). However, most CD4 T cells that emerge from the thymus are of the naïve, “conventional” variety in that they require
signals from their environment for the induction of distinct transcriptional patterns and
the acquisition of effector abilities. Potential fates of conventional CD4 T cells include T
helper cells (Th), T follicular helper cells (Tfh), inducible T regulatory cells (Tregs) as well
as central and effector memory cells (Tcm, Tem). Th cells can be further divided into
Th1, Th2, Th17 and perhaps the newly described Th9 and Th22 cells based on their
cytokine signatures and the inflammatory milieu that induces their differentiation
(reviewed in (Zhu et al.)). Whether the Th, Tfh, Treg and memory T cells are distinct
lineages or rather different phenotypic states of the same lineage has been difficult to
ascertain. Furthermore, the plasticity of the Th subsets has become an area of intense
investigation as it becomes apparent that different fate potentials are not completely lost
as cells differentiate (O'Shea and Paul). While the lineage relationships and plasticity
between CD4 T cell fates raise very intriguing questions, for the purposes of this
dissertation, we will focus on the mechanisms of differentiation of the Th subsets; Th1,
Th2 and Th17. Most data suggests that, given the right combination of signals, any
single naïve CD4 T cell has the potential to become any one of these Th subsets
although this has yet to be definitively shown for Th17 cells (Sad and Mosmann, 1994).

*T helper differentiation*

Upon differentiation each Th cell type acquires a signature cytokine expression pattern.
Differentiation is induced by a particular cytokine milieu that activates signaling
transducer and activator of transcription (STAT) proteins which, in combination with
transcription factors activated via TCR signals, result in the activation of a lineage
specific master transcription factor. Activation of the master transcription factors is
important for initiation of further transcriptional programs that ultimately reinforce lineage
commitment while suppressing the transcriptional programs associated with other
lineages (Hermann-Kleiter and Baier, ; O'Shea and Paul, ; Zhu et al.). Th differentiation can be divided into two phases: induction and polarization. During induction TCR-and cytokine-stimulated T cells begin to produce signature cytokines. These cytokines then act in an autocrine manner and provide a positive feedback loop that further enhances cytokine and transcriptional activity, culminating in full Th polarization and acquisition of Th effector competence. Competent Th cells are then poised to rapidly produce their signature cytokines in response to restimulation with cognate antigen (Zhu et al.).

Th1 cells are defined by their ability to produce their signature cytokine, IFN$\gamma$, although they also produce IL2 and TNF$\alpha$ (Killar et al., 1987; Mosmann et al., 1986). Optimally, Th1 cells are generated in response to infection with intracellular pathogens. Th1 cells are important in cellular immune responses and they promote the recruitment, maturation and function of macrophages and CTLs that can subsequently kill infected cells. An aberrant or dysregulated Th1 response can contribute to autoimmune pathologies. Th1 differentiation is induced by IFN$\gamma$ and IL12 signals that activate STAT1 and STAT4 respectively. These cytokines act in combination with the TCR-induced transcription factors NFAT, AP-1 and NFkB activate the Th1 master transcription factor T-bet (Afkarian et al., 2002; Schulz et al., 2009).

Th2 cells produce IL4, IL5, IL6, IL9 and IL13 and are important for clearance of helminthes and the promotion of humoral responses (Killar et al., 1987; Mosmann et al., 1986). Th2 cells are important in the pathogenesis of asthma and allergies as well. The cytokines IL4 and IL2 initiate Th2 differentiation by activating STAT6 and STAT5 respectively. STAT5 and STAT6 cooperate with TCR-induced NFAT, AP-1 and NFkB activity to drive further IL4 transcription and activation of the Th2 master regulator
GATA3 (Ansel et al., 2006). GATA3 itself cooperates with NFAT to further promote Th2 lineage commitment (Avni et al., 2002).

Th17 cells produce IL17a, IL17f and IL22, are important for the clearance or extracellular bacteria and fungi and they recruit neutrophils to sites of infection (Aggarwal et al., 2003; Mangan et al., 2006). Like Th1 cells, Th17 cells can contribute to autoimmune pathologies (Weaver and Murphy, 2007). They are induced by TGF-β, IL-6 and IL21 cytokines and maintained via signals from IL23. STAT3 activation downstream of the IL6 receptor cooperates with TCR-activated NFAT and AP-1 to upregulate of RORγt. In turn, RORγt cooperates with NFAT proteins to upregulate expression of Th17 cytokines (Korn et al., 2009).

Transcription factors downstream of the TCR

Downstream of the TCR, NFAT and NFκB transcription factors are important regulators of induction, polarization and function of T helper lineages (Das et al., 2001; Hermann-Kleiter and Baier). As mentioned previously, Ca²⁺ mediated pathways induce nuclear localization of NFAT family members while the DAG/PKCθ pathway is thought to be the dominant pathway involved in NFκB activation. The ratio of expression of NFAT family members and the transcriptional binding partners with which NFATs and NFκB associate determine the transcriptional program initiated. The availability of transcriptional partners is influenced by signals transduced from cytokine receptors.

TCR signal strength and Th differentiation

Integration of signals received through the TCR and cytokine receptors is clearly essential for optimal Th differentiation. However, the mechanisms by which alterations in signals downstream of the TCR can affect the differentiation and effector functions of
CD4+ T cells remain unclear. Initial studies using altered peptide ligands (APLs) to prime CD4+ T cells both in vivo and in vitro showed that stronger pMHC:TCR interactions or higher doses of peptide presentation result in increased ERK activation and preferentially induce Th1 over Th2 differentiation programs (Jorritsma et al., 2003; Kumar et al., 1995; Pfeiffer et al., 1995; Tao et al., 1997). These and other studies support a model in which, in the absence of exogenous Th skewing cytokines, sustained ERK activation suppresses GATA3 production required to establish Th2 differentiation, and T cells preferentially undergo a Th1 pattern of differentiation (Yamane et al., 2005). The mechanism by which ERK signals can suppress GATA3 has not been defined. Furthermore, it is unclear whether Th1 differentiation in this system is a default program or an actively induced program. If and how ERK activation levels regulate Th differentiation in the presence of inflammatory skewing cytokines or during Th17 differentiation has not been addressed either.

The role of the proximal signaling complex in Th differentiation

Because of the effects of TCR signaling strength on CD4 differentiation it is not surprising that several components of the proximal signaling complex have been implicated in Th differentiation. Mutation of a key tyrosine in LAT confers a Th2 type lymphoproliferative disorder that is presumably due to loss of a yet to be defined inhibitory function (Aguado et al., 2002). Conversely, expression of a mislocalized SLP-76 mutant results in a TCR-hyproresponsive T cell population that appears to be skewed toward Th1 and Th17 lineages. Again, the mechanism is unclear (Sonnenberg et al., 2009).

Several non-adaptor components of the proximal signaling complex have been shown to be important for T cell differentiation. The role of Itk in Th differentiation has
been extensively studied. Most recently, CD4 T cells from mice lacking Itk were shown to have defects in the ability to acquire IL17a but not IL17F or IL22 competence following Th17 skewing and this defect was attributed to diminished NFATc1 nuclear translocation (Gomez-Rodriguez et al., 2009). A series of previous studies have shown that Itk-/- T cells can differentiate into competent Th2 cells but they fail to produce WT levels of inflammatory cytokines upon restimulation. This has been attributed to a Ca\(^{2+}\)-dependent defect in NFATc1 nuclear localization following restimulation (Fowell et al., 1999). Conversely, mice expressing a hyperactive mutation of Itk develop Th2 mediated allergic disease (Colgan et al., 2004). Furthermore, in the absence of exogenous cytokines, Itk deficient T cells preferentially acquire a Th1 phenotype due to enhanced T bet expression (Miller et al., 2004). A similar phenotype is observed in Vav1-deficient T cells. Enhanced Th1 and diminished Th2 responses in Vav1-deficient cells have been linked to decreased expression of the Th2-specific transcription factor c-Maf, which is dependent on Ca\(^{2+}\) and NFAT signals (Tanaka et al., 2005). Despite the similarities between the two phenotypes there are important differences as well. First, Vav1-/- T cells and not Itk-/- T cells acquire Th1 function even during Th2 polarizing conditions with exogenous cytokines. Second, while both Itk-/- T cells and Vav1-/- T cells have defects in IL4 production, production of other Th2 cytokines is preserved in the Vav1-/- T cells but not in the Itk-/- T cells. Thus, while both Vav1- and Itk-deficient T cells have defects in Ca\(^{2+}\) mobilization, they display different polarization defects, suggesting that additional Vav1- and/or Itk-specific pathways are playing a role in the observed phenotypes. This underscores the fact that in studies in which proximal signaling molecules are manipulated it is difficult to distinguish between the effects of specific signaling pathways and the effects of generalized diminished signal strength.
Heterogeneity and differentiation of CD8+ T cells

Like the CD4+ T cell pool, the CD8+ pool is heterogeneous, although differences between effector populations are not as clear. Non-conventional CD8 T cells with innate-like functions include H2-M3-restricted T cells and other cell types selected on MHC class 1b molecules (Berg, 2007). However, the majority of the CD8+ pool that emerges from the thymus are conventional CD8 T cells that must encounter cognate antigen in order to acquire effector functions.

CD8 effector and memory differentiation

In a CD8+-mediated immune response, CD8+ effector cells produce inflammatory cytokines and develop cytolytic activity against infected target cells after which a small number of memory cells survive with the ability to rapidly regain effector function in the event of a rechallenge. During this process, a relatively homogeneous pool of naïve CD8 T cells differentiates into heterogeneous pools of effector and memory CD8 T cells (Obar and Lefrancois). To initiate a differentiation program, naïve T cells integrate signals from their external environment. These signals are provided by peptide:MHC complexes, co-stimulatory molecules and cytokines. Manipulations of these signals have been shown to influence the quality and kinetics of CD8 T cell memory differentiation (Joshi and Kaech, 2008; Kaech and Ahmed, 2001; Obar and Lefrancois). The molecular signals that orchestrate the function and diversification of effector and memory CD8+ T cell subsets downstream of these receptors are just beginning to be elucidated and how TCR signals affect CD8+ effector function and dictate CD8+ T cell fate choices is not fully understood (Intlekofer et al., 2005; Kotturi et al., 2008; Lefrancois et al., 2003; Munitic et al., 2009; Ndhlovu et al., 2010; Rutishauser et al., 2009; Sarkar et al., 2007; Takemoto et al., 2006; Teixeiro et al., 2009; Zehn et al., 2009).
Heterogeneity of effector and memory pools

The CD8 effector pool can be divided into terminally differentiated, short-lived KLRG-1$^{hi}$, IL7r$^{\alpha}$lo effector cells (SLECs) and less differentiated KLRG-1$^{lo}$, IL7r$^{\alpha}$hi memory precursor cells (MPECs) (Joshi et al., 2007; Joshi and Kaech, 2008; Kaech et al., 2003; Sarkar et al., 2008). SLECs typically produce the effector cytokine IFN$\gamma$ and may also co-produce TNF$\alpha$ in response to antigen but only memory precursors can produce IL2 in addition to IFN$\gamma$ and TNF$\alpha$ (Precopio et al., 2007; Sarkar et al., 2008). The memory pool itself is a heterogeneous population that matures over time. Memory cells are commonly divided into Effector Memory and Central Memory (Tem, Tcm) based on their anatomical location and expression of surface markers, including CD62L that is expressed at higher levels on Tcm (Bachmann et al., 2005; Sallusto et al., 1999). While the number of memory cells remains constant after antigen clearance, the ratio of CD62Lhi to CD62Llo cells increases over time; the rate of this transition can be affected by the strength and duration of the initial antigenic stimulus (Sarkar et al., 2007; Wherry et al., 2003b). Furthermore, the CD62Lhi memory pool itself matures over time and gains enhanced proliferative capabilities (Slifka and Whitton, 2001). Recently it was shown that cells with the most robust proliferative potential have increased surface expression of the activation markers CXCR3 and CD27, and the frequency of these cells increases over time post infection (Hikono et al., 2007). Importantly, the relative frequencies of memory subsets can vary depending on the nature or persistence of the inciting antigen (Obar et al., 2004; Obar et al., 2006; Sheridan et al., 2006; Wherry et al., 2003a), and since Tem and Tcm are functionally distinct in their ability to proliferate and regain effector function
(Bachmann et al., 2005; Roberts et al., 2005; Sallusto et al., 1999), understanding their development is important in predicting the quality of a secondary response.

**TCR signals and the balance between terminal differentiation and memory differentiation**

While multiple cytokines and transcription factors have been shown to affect the balance between terminal differentiation and memory, the role of TCR signals has been more difficult to ascertain (Araki et al., 2009; Intlekofer et al., 2005; Joshi et al., 2007; Joshi and Kaech, 2008; Kaech and Wherry, 2007; Rutishauser et al., 2009; Takemoto et al., 2006). Adjustments in the quantity, quality and duration of antigen presentation can affect the magnitude and kinetics of memory responses but they do not appear to affect the quality of these responses (Badovinac et al., 2007; Badovinac and Harty, 2007; D'Souza and Hedrick, 2006; Joshi and Kaech, 2008; Kaech and Wherry, 2007; Lefrancois et al., 2003; Ndhlovu et al., 2010; Wherry et al., 1999; Wherry et al., 2003b; Williams and Bevan, 2004; Zehn et al., 2009). For example, when mice containing OT-1 TCR transgenic T cells are exposed to *Listeria monocytogenes* (LM) expressing different APLs, the duration of T cell expansion is proportional to the OT-1 TCR stimulating potency of the inciting APL (Zehn et al., 2009). While fewer cells respond to the weak APLs, the immune response is complete in that functional effector and memory cells are produced. Prior studies have suggested that the strength and the duration of the TCR signal can influence not only the magnitude of clonal expansion but also the ratio of terminal differentiation to memory differentiation (Badovinac and Harty, 2007; D'Souza and Hedrick, 2006; Kaech and Wherry, 2007; Sarkar et al., 2007; Wherry et al., 2003a; Wherry et al., 1999; Williams and Bevan, 2004; Ndhlovu et al., 2010). These studies have shown that strong or lengthy/repeated TCR signals favor terminal differentiation at the expense of memory differentiation. Thus, if strong/repeated TCR signals result in
greater clonal expansion and a higher frequency of SLECs one would expect that those T cells expressing TCRs that are specific to the more dominant epitopes presented, or that bind to viral peptides with high avidity, will form the bulk of the primary effector pool. Comparatively, T cells with lower avidity for their cognate peptide, or T cells specific for less dominant epitopes, will undergo less clonal expansion, generate fewer SLECs, but they will generate a stable population of MPECs. Taken together, one could predict that T cells with a variety of avidities to a variety of epitopes contribute to the stable memory pool even though the bulk of the primary effector response is much less polyclonal.

Furthermore, recent data suggest that specific TCR signals can differentially affect the long-term fate of CD8 T cells as T cells with defective NFκB activation were capable of normal primary expansion and terminal differentiation but not memory development (Teixeiro et al., 2009). While previous studies have shown a similar phenotype when the Il12-mTor-Tbet/Eomes pathway is altered (Araki et al., 2009; Intlekofer et al., 2005; Joshi et al., 2007; Rao et al., ; Takemoto et al., 2006), this was the first study to completely separate effector from memory downstream of the TCR. However, the mechanism by which NFκb signals contribute to memory formation remains to be elucidated.

Conclusions

SLP-76 nucleates a proximal signaling complex that transduces signals initiated from MHC:peptide ligation of the TCR into multiple downstream pathways, including those mediated by Ca^{2+} and Erk. The protein-binding domains of SLP-76 differentially affect the processes and functions dependent on these downstream pathways. Two important T cell functions that are dependent on TCR-induced signals are effector and memory fate choices and the elicitation of effector function. For an optimal immune response, the effector functions of the antigen-experienced T-cell pool must be tailored for the
specific inciting pathogen. T cell-mediated immunity is further enhanced by the
generation of antigen-specific long-lived memory cells that retain the ability to rapidly
invoke effector function in the event of a rechallenge. In the studies presented in this
dissertation, we first establish a role for the N-terminal phosphotyrosines of SLP-76 in
the transduction of TCR signals to Ca^{2+}- and Erk-dependent pathways using genomic
knock-in mice expressing tyrosine to phenylalanine mutant SLP-76 molecules. We next
use these mice to begin to elucidate the role of TCR signals in T cell fate choices and
effector functions both in vitro and in vivo.
Figure 1.1 Schematic of the assembly and constituents of the adaptor-nucleated proximal signaling complex.

Following TCR triggering, Lck activates Zap70 which phosphorylates (p) tyrosine residues on LAT. LAT then recruits GADS and its constitutive binding partner SLP-76.
Zap70 mediated phosphorylation of SLP-76 results in the recruitment of multiple SH2 domain-containing effector molecules (circles) and adaptor proteins (octagons). SH3 domains (hatching) also link effectors to adaptors and contribute to stabilization of the complex.
Chapter 2: The role of SLP-76 phosphotyrosines in TCR signal transduction

Abstract

Molecular studies have shown that the three N-terminal phosphotyrosines of SLP-76 serve to recruit SH2 domain containing proteins essential to TCR signal transduction. To determine the relevance of these tyrosines in vivo, we characterized two genomic knock-in mice that express tyrosine to phenylalanine mutations at residues 145 (Y145F) or 112 and 128 together (Y112/128F), tyrosines purported to associate with Itk and Vav1/Nck respectively. Both positive and negative selection are defective in the KI mice, however they do generate peripheral CD4+ and CD8+ T cells. Substantial defects in TCR signal transduction and T cell function are observed in the KI T cells and analysis of mice conditionally expressing the KI mutations reveal that these defects are independent of the role of the tyrosines in T cell development. Surprisingly, despite defects in Vav1- and Itk-dependent pathways, mutation of SLP-76 tyrosines did not result in abrogation of the recruitment of Vav1 or Itk to the signaling complex or to TCR-induced microclusters in primary T cells. Taken together, these data support a model in which phosphorylation of SLP-76 tyrosines is not essential for the localization of their binding partners, but may be required for specific functions of their binding partners. Furthermore, T cells from compound heterozygous mice that express both SLP-76 mutant proteins showed a rescued phenotype, suggesting that Y145 and Y112/128 can promote the function of
their binding partners independently of one another on separate SLP-76 molecules, and that multiple SLP-76 molecules can function cooperatively.

**Introduction**

Following TCR-induced phosphorylation, the plasma membrane-associated adaptor protein LAT recruits SLP-76 through their mutual binding partner GADS and together these adaptors nucleate a proximal multi-molecular signaling complex (Liu et al., 2003; Liu et al., 1999; Motto et al., 1996; Zhang et al., 1998a; Zhang et al., 1998b) (see Figure 1.1). Since SLP-76/- mice lack peripheral T cells, our knowledge of the role of SLP-76 for mature T cell function has been based on studies using transgenic systems and cell lines (Fang and Koretzky, 1999; Fang et al., 1996; Jordan et al., 2006; Myung et al., 2001; Yablonski et al., 1998). SLP-76 deficient Jurkat T cells (J14 cells) show striking signaling defects following TCR ligation. These defects are apparent in PLCγ1 activation, Ca++ mobilization, Ras/MAPK activation and NFAT activation (Yablonski et al., 1998).

SLP-76 is composed of four modular domains; an N-terminal acidic domain, a SAM domain, a proline rich region and a C-terminal SH2 domain (reviewed in (Koretzky et al., 2006)) (Shen et al., 2009). Both in vivo and in vitro studies using SLP-76 constructs with mutations in each of the three domains have shown that the acidic domain is the most important for optimal T cell function (Kumar et al., 2002; Myung et al., 2001). The acidic region of SLP-76 contains three tyrosines at residues 112, 128 and 145 that are phosphorylated upon TCR stimulation (Bubeck Wardenburg et al., 1998; Fang et al., 1996). Analysis of J14 T cells reconstituted with single, double and triple tyrosine to phenylalanine (Y-F) mutants of SLP-76 has shown a hierarchy of functional importance of these tyrosines (Jordan et al., 2006). Tyrosine 145 appears to
be the most important single tyrosine in TCR signal transduction as measured by activation of downstream signaling molecules, Ca\textsuperscript{2+} mobilization, and upregulation of activation markers. Mutation of tyrosines 112 and 128 together also confers a significant defect in T cell function and, interestingly, ablates phosphorylation of tyrosine 145. However, the phenotype of the cells expressing the double 112,128 Y-F (Y112/128F) mutation is not as severe as those cells expressing the triple Y-F (Y3F) mutation or even the single 145 Y-F (Y145F) mutation. The mechanisms by which the SLP-76 tyrosines transduce TCR initiated signals into multiple distal signaling pathways are thought to be related to their phosphorylation-induced interactions with SH2 domain containing effector proteins. Biochemical analyses have implicated phosphotyrosines 112 and 128, each within a tyrosine-glutamic acid-serine-proline (YESP) motif, in binding the guanine nucleotide exchange factor (GEF), Vav1 and the SH2/SH3 adaptor protein, Nck (Bubeck Wardenburg et al., 1998; Wu et al., 1996; Wunderlich et al., 1999). Phosphotyrosine 145 has a tyrosine-glutamic acid-proline-proline (YEPP) motif and has been predicted by phospho-peptide mapping studies to interact with the kinase, Itk (Bunnell et al., 2000). In addition to binding to SLP-76 through its SH2 domain, the SH3 domain of Itk has been shown to interact with the proline rich domain of SLP-76, although the importance of this interaction has not been well defined (Bunnell et al., 2000).

The functional roles of the interactions between SLP-76, Vav1, Nck and Itk have been difficult to tease apart, as all of these molecules appear to have independent and interdependent, enzymatic and/or non-enzymatic and overlapping functions downstream of the TCR. For example Itk, in a kinase-independent manner, and the SLP-76 tyrosines are both required for the recruitment of Vav1 to the proximal signaling complex (Dombroski et al., 2005; Labno et al., 2003). Vav1, in turn, is required for stabilization of the complex, optimal phosphorylation of SLP-76 and Itk kinase activity (Reynolds et al.,
Furthermore, the optimal localization and activation of PLCγ1, a key effector molecule downstream of the TCR, is dependent on many components of the proximal signaling complex including SLP-76, Vav1 and Itk (Qi and August, 2007). Downstream of the TCR all three of these molecules are also required for the activation of ERK pathways, cytoskeletal reorganization events, integrin mediated processes and, in thymocytes, optimal selection (reviewed in (Smith-Garvin et al., 2009)). Taken together these data suggest that multiple protein-protein interactions contribute to the assembly and function of the proximal signaling complex and that, given the interplay between the complex constituents, this process is not necessarily linear.

Unraveling the overlapping roles of adaptor and effector molecules within the TCR-proximal signaling complex has been technically difficult. Extensive knowledge of the contribution of signaling molecules has been gained in vivo by the use of genetic “knock-out” mice, however loss of an entire molecule can complicate functional analysis, especially when that molecule is involved in multiple protein-protein interactions and the stabilization of multi-molecular complexes. Teasing apart specific roles of domains within the molecules has been traditionally approached with the use of cell lines and overexpression studies. Here, we combine qualities of both systems by using genomic “knock-in” mice to focus on how abrogation of one (Y145F) or two (Y112/128F) phosphotyrosines of SLP-76 in vivo can influence the outcome of signal transduction downstream of the TCR. We find that signal transduction is indeed impaired when SLP-76 tyrosines are mutated but, surprisingly, this occurs without grossly disrupting the assembly of the proximal signaling complex.
Results

SLP-76 Y-F genomic KI mice

To determine the contribution of SLP-76 tyrosines for TCR signal transduction in vivo, we used two genomic KI mice with tyrosine to phenylalanine mutations at residues 145 or 112/128 (Figure 2.1). SLP-76 protein expression as measured by flow cytometry was not affected by the genomic manipulation (data not shown). Both Y145F and Y112/128F mice were generated at Mendelian ratios, and there was no evidence of the perinatal lethality (Clements et al., 1998) or vascular defects (Abtahian et al., 2003) that are observed in SLP-76-deficient mice.

Thymic selection in SLP-76 KI mice

KI mice have defects in thymic selection that were well characterized by our lab (Jordan et al., 2008). Briefly, both Y145F and Y112/128F KI mice have mild decreases in thymic cellularity and show a partial block at the DN2 to DN3 stage of thymocyte development (Figure 2.2), the stage at which SLP-76 deficient mice display a complete block (Clements et al., 1998). Both lineages generate single positive (SP) thymocytes, however when compared to WT, the mutant lineages have a decreased ratio of CD4 to CD8 SP thymocytes. Further studies using TCR transgenic backgrounds revealed defects in both positive and negative selection with the Y145F KI mice showing a more severe phenotype than the Y112/128F KI mice (data not shown).

Proximal TCR signaling is defective in SLP-76 mutant thymocytes

Mutation of tyrosine residues within the N-terminus of SLP-76 is predicted to lead to loss of SLP-76 phosphorylation. Indeed, mutation of Y112 and Y128 ablated nearly all
detectable SLP-76 phosphorylation (Figure 2.3A); mutation of Y145, however, resulted in little diminishment of total phosphorylation. These data are consistent with previous studies in Jurkat cells in which Y145 was shown to be phosphorylated, but optimal phosphorylation occurred only when Y112 or Y128 were intact (Jordan et al., 2006). It is unclear why the Y112/128F mutant does not have a more severe phenotype than the Y145F mouse. It is possible that little phosphorylation is required for Y145 function or that Y145 supports phosphorylation-independent functions. SLP-76 phosphorylation was also analyzed using a pY128 specific antibody. Stimulated Y112/128F thymocytes failed to react with the pY128 antibody, whereas WT and Y145F thymocytes were capable of phosphorylating this site (Figure 2.3B). To probe whether events upstream of SLP-76 were intact, we assessed TCR-induced phosphorylation of tyrosine 191 on LAT and found this process to be normal in both mutants (Figure 2.3B). Global loss of tyrosine phosphorylation was not observed in either mutant; rather loss of phosphorylation of a 95kDa species was noted in Y112/128F thymocytes (Figure 2.3C).

To address the biochemical mechanism(s) underlying the selection defects observed in SLP-76 mutant mice, we analyzed PLCγ1 phosphorylation and ERK phosphorylation after TCR stimulation. In unfractionated thymocytes, PLCγ1 and ERK phosphorylation were markedly diminished in both mutant mice after TCR stimulation (Figure 2.3D, E). Sorted double positive thymocytes showed similar defects in ERK phosphorylation following TCR and CD4 co-receptor cross-linking (Figure 2.3F).

**SLP-76 KI mice generate peripheral T cells**

Despite defects in thymocyte selection and function, peripheral T cells were found in spleen and lymph nodes in normal numbers and ratios (Figure 2.4A, data not shown). Both CD4⁺ and CD8⁺ T cells from KI mice showed decreased surface expression of CD5
consistent with decreased TCR signal strength (Figure 2.4B, right panel). CD5 expression was consistently lower on the surface Y145F KI T cells when compared to Y112/128F KI T cells. TCR expression was decreased on Y145F KI T cells but not Y112/128F KI T cells.

*Proximal signaling defects in SLP-76 KI mice reveal a functional hierarchy for SLP-76 tyrosines*

Similar to what was observed in thymocytes, SLP-76 KI peripheral T cells show defects in phosphorylation of PLCγ1 and ERK, but not in LAT which is upstream of SLP-76 (Figure 2.5A). TCR signal propagation in CD4⁺ and CD8⁺ T cell populations was further examined by flow cytometric analysis of Ca²⁺ mobilization following TCR and CD4 or CD8 co-cross-linking and was found to be severely impaired in both Y145F and Y112/128F T cells (Figure 2.5B).

To determine if the biochemical signaling defects observed in the SLP-76 KI T cells translate into functional defects, we examined early and late responses in Y145F and Y112/128F T cells following TCR stimulation in vitro. Following TCR stimulation, Y145F and, to a lesser extent, Y112/128F T cells did not upregulate CD69, an early activation marker, to the level of WT T cells (Figure 2.5C, left panel). This hierarchical requirement for the SLP-76 tyrosines was also observed during TCR-induced proliferation (Figure 2.5C, right panel). These data indicate that signals transmitted from the TCR in Y112/128F T cells are weaker than those in WT T cells and those in Y145F T cells are weaker still.
Itk and Vav1 functions are differentially affected in Y145F and Y112/128F SLP-76 KI mice

As tyrosines 112 and 128 of SLP-76 have been predicted to interact with Vav1 and Y145 has been predicted to interact with Itk, we next sought to determine if Vav1 and Itk functions were differentially affected in the two mutant lineages. We first looked at TCR-inducible Vav1 phosphorylation and found it to be nearly absent in Y112/128F KI thymocytes but not in Y145F KI thymocytes consistent with a 112 and 128 tyrosine specific role for SLP-76 in Vav1 activation (Figure 2.6A). Interestingly, Vav1 phosphorylation was not consistently decreased in peripheral Y112/128F KI T cells. This could suggest a compensatory pathway in Y112/128F T cells that survive thymic selection processes or general differences in signaling pathways between thymocytes and T cells (data not shown).

Itk-deficient mice are unique in the fact that they preferentially generate CD8 and CD4 innate-like-lymphocytes (ILLS) at the expense of conventional T cells (Atherly et al., 2006b; Hu and August, 2008). ILLS can be identified by high surface expression of the activation markers CD44 and CD122. Y145F KI mice, but not Y112/128F, phenocopy the CD8 thymocyte distribution in Itk/-/- mice, consistent with a Y145-specific role for SLP-76 in Itk-mediated pathways (Figure 2.6b). CD122hiCD44hiCD8+ ILLS also represent the majority of CD8+ T cells in the periphery of Y145F KI but not Y112/128F KI mice (Figure 2.7A, top panel). Y145F mice have an abundance of CD122hiCD44hiCD4+ cells in the periphery as well (Figure 2.7A, bottom panel). Both CD44hi CD4+ and CD8+ cells in the Y145F mice produce IFNγ when stimulated with PMA and ionomycin ex vivo, consistent with an ILL phenotype (Figure 2.7B)(Berg, 2007); however, CD44hi T cells from SLP-76 KI mice show defects in TCR-induced Ca2+ mobilization similar to those
observed in their CD44lo counterparts (Figure 2.7C). Thus, although ILLs have the potential to produce copious amounts of IFNγ in the presence of mutated SLP-76 protein, WT SLP-76 expression is still required for coupling the TCR to early signaling events.

Taken together, these data suggest that while tyrosines 112 and 128 together and 145 alone contribute similarly to PLCγ1 and ERK pathways, they are differentially required for Vav1 phosphorylation (at least in thymocytes) and phenotypes associated with Itk deficiency, respectively.

**Complementation of SLP-76 mutations in vivo**

Because Y112/128F and Y145F thymocytes show distinct differences in their abilities to support Vav1 and Itk functions, we asked whether co-expression of these mutants could complement one another functionally. To this end, we generated “double mutant” mice bearing one Y112/128F allele and one Y145F allele. Double mutant mice were compared to WT and SLP-76 single mutant mice in multiple phenotypic and functional assays. In all cases, responses of the double mutant cells were greater than either single mutant cells and in some cases identical to that observed for cells from WT mice. Functionally, the ability of double mutant thymocytes to phosphorylate PLCγ1 and ERK, and to mobilize Ca2+ was similar to or only slightly reduced from that observed in WT thymocytes (Figure 2.8A,B). We did not observe a dominant negative effect of expressing the Y112-128F and Y145F mutations simultaneously, inasmuch as Vav1 phosphorylation, which was nearly normal in Y145F thymocytes but defective in Y112/128F thymocytes, was normal in the double mutant (data not shown). Similarly, double mutant CD8SP thymocytes were not CD122+CD44hi, a phenotype of Y145F but not Y112/128F mice (data not shown). Thus, based on phenotypic and functional data,
these two distinct mutants of SLP-76 can complement each other in trans in developing thymocytes.

Preserved binding of Vav1 and Itk to SLP-76 tyrosine mutants

We next sought to characterize the biochemical mechanisms by which the tyrosines of SLP-76 promote the transduction of TCR-induced signals through Vav1 and Itk. Adaptor proteins are traditionally thought to contribute to signaling via recruitment of effector molecules through their protein interaction domains. Co-immunoprecipitation experiments revealed that in WT primary thymocytes and T cells, SLP-76 and Vav1 are constitutively associated regardless of the phosphorylation status of SLP-76 (Figure 2.9A, data not shown). Furthermore this association was not abrogated in the Y112/128F KI thymocytes or T cells (Figure 2.9A, data not shown). A similar co-precipitation pattern was observed between Itk and SLP-76 in WT and KI mice (Figure 2.9B). Co-immunoprecipitation experiments, however, may lack the sensitivity to distinguish differences in stability of associations between these molecules. Furthermore, tertiary interactions could also account for the co-precipitation.

T cell activation requires not only the assembly of the proximal signaling complex but also the subcellular localization of the complex and its constituents to signaling clusters or the T cell/APC interface. We looked for recruitment of GFP-tagged Vav1 and Itk constructs to TCR-induced signaling clusters in WT and KI T cells using confocal microscopy (Figure 2.10). A kinase dead Itk (Itk KD) construct was used due to cytotoxicity induced by overexpression of kinase-sufficient Itk (data not shown). When CD4+ T cells from WT or KI mice were left unstimulated the Itk KD was difficult visualize and Vav1 was distributed evenly the cytoplasm. Staining with fluorescently-labeled phalloidin, a toxin that specifically binds filamentous actin, revealed all T cells to be
uniformly small and round with smooth edges characteristic of resting T cells (Figure 3.10 left panels). Following a 10 minute incubation on an anti-CD3, anti-CD28 coated surface, Phalloidin staining showed that the footprints of T cells from both WT and KI mice had spread and the cells had ruffled edges consistent with activation (Figure 2.10, right panels). Furthermore, well-defined clusters of GFP-tagged Itk KD and Vav1 could be observed in cells from both WT and KI mice (Figure 2.10, right panels). Clusters could not be visualized in cells expressing GFP in an empty vector (data not shown). Thus, despite their roles in TCR signaling and Vav1 and Itk dependent functions, these data suggest that the SLP-76 phosphotyrosines are not required for the recruitment Vav1 and Itk to the SLP-76-nucleated complex or for the localization of Vav1 and Itk to TCR-induced microclusters.

**SLP-76 tyrosines are required for optimal TCR signaling independent of their role in T cell development**

We have previously shown that the SLP-76 tyrosines are required for optimal T cell selection in the thymus, therefore it is possible that the signaling defects observed in the peripheral KI T cells are the result of abnormal development. To determine if the observed signaling defects in Y145F and Y112/128F KI mice were dependent on the role of SLP-76 tyrosines in thymic selection, we generated conditional KI (cKI) mice using a previously described strategy in which T cells in mice heterozygous for mutant SLP-76 and WT SLP-76 are allowed to develop normally in the thymus before WT SLP-76 is excised using drug-inducible cre recombinase activity (Figure 2.11)(Sonnenberg et al., 2009). Conditional mice have one WT SLP-76 allele that is flanked by lox-p sites and the other allele is either Y145F SLP-76 (cY145F), Y112/128F SLP-76 (cY112/128F) or, as a control, WT SLP-76 (cSLP-76+/−). These mice also express a transgene for cre
recombinase-human estrogen receptor (Cre-ER\textsuperscript{T2}) under the ubiquitin promoter that allows for cre recombinase activity only in the presence of the estrogen analogue tamoxifen. Additionally, enhanced yellow fluorescent protein (YFP) knocked-in to the Rosa-26 locus is preceded by a lox-p-flanked tpA transcriptional stop codon such that YFP can serve as a reporter for those cells that have Cre-ER\textsuperscript{T2} activity.

Following oral tamoxifen treatment, YFP\textsuperscript{+} CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are found in the periphery of all three strains of conditional mice (Figure 2.12A). YFP\textsuperscript{+} cKI T cells have levels of TCR\textgreek{b} on their surface similar to that of YFP\textsuperscript{+} cSLP-76\textsuperscript{+/−} T cells; however, they have decreased levels of CD5 expression (Figure 2.12B). cKI and cSLP-76\textsuperscript{+/−} mice have similar frequencies of peripheral CD122\textsuperscript{hi}CD44\textsuperscript{hi} T cells (Figure 2.12C).

Deletion of WT SLP-76 in mature peripheral KI T cells confers similar TCR signaling abnormalities to those observed in T cells that develop only in the presence of the mutant SLP-76 proteins. YFP\textsuperscript{+} cKI T cells are defective in their ability to phosphorylate PLC\textgreek{y}1 and ERK1/2 following TCR ligation, and this defect is more pronounced in cY145F T cells than in cY112/128F T cells (Figure 2.12D). Functionally, T cells from both strains of cKI mice show defective TCR-induced upregulation of CD69 and proliferation (Figure 2.12E). These data suggest that the SLP-76 tyrosines are required in a hierarchical manner for TCR signal transduction and function, independent of their role in thymic selection.

**Discussion**

In these studies we modified SLP-76 to explore mechanisms by which it supports signaling and to gain insights into how this adaptor coordinates formation and activity of a signaling complex. Expression of SLP-76 harboring mutations at tyrosines 112 and 128 or 145 revealed that these tyrosine “units” are important to support preTCR/TCR
signal transduction in thymocytes and peripheral T cells. Ca^{2+} mobilization and Erk activation were severely impaired and resulted in functional defects in vitro as measured by upregulation of activation markers and proliferation. Consistent with previous studies in Jurkat T cell lines, a hierarchy of importance for the tyrosines was observed such that Y145F mutation conferred more severe functional defects than Y112/128F mutation in some (ERK and PCLγ1 phosphorylation, CD69 upregulation, proliferation) but not all assays (Ca^{2+} mobilization). Analysis of mice conditionally expressing SLP-76 mutants revealed that the functional requirements and hierarchy of SLP-76 tyrosines are independent of their role in thymic selection.

While mutation of Y112/128 or Y145 tyrosines resulted in similar functional defects, our data suggest that proximal signals are differentially affected such that Y112/128 support Vav1 dependent signals and Y145 supports Itk dependent signals. Evidence for this is especially apparent in the thymus where mutation of Y112/128 but not Y145 signals results in complete loss of detectable TCR-induced Vav1 phosphorylation and mutation of Y145 but not Y112/128 results in the enhanced generation of thymic ILLs (similar to Itk-/- mice). However there maybe interplay between the tyrosine units as well, for example, Vav1 is required for optimal Itk activation and loss of Vav1 phosphorylation could potentially lead to defects in Itk activity (Reynolds et al., 2002). Furthermore, these studies and previous studies in Jurkats have suggested that Y112/128 are required for Y145 phosphorylation which others and we suggest is important for Itk kinase activity (Jordan et al., 2006; Qi and August, 2007). Additionally, we did observe a mild but consistent defect in Vav1 phosphorylation in the Y145F KI thymocytes that further supports the idea of cross talk between SLP-76 tyrosines, Vav1 and Itk within the complex.
A simple model for how the N-terminal tyrosines of SLP-76 support TCR signaling is that, following phosphorylation, each tyrosine binds its respective SH2-domain-containing partner thereby orienting it in a spatio-temporal manner that is conducive to its function. Indeed we predicted, based on previous studies in Jurkat cells, that mutation of tyrosines 112 and 128 or 145 would abolish the SLP-76 and Vav1 or SLP-76 and Itk associations, respectively. However, we see constitutive association between SLP-76 and these two proteins in primary murine T cells and these associations do not appear to be dependent upon the N-terminal tyrosines of SLP-76. We speculate that although these tyrosines are likely still important for binding to Itk and Vav1, their contributions are not appreciated in primary T cells due to tertiary protein-protein interactions. Consistent with these data, we do not see an appreciable increase in the binding of Vav1 and Itk to SLP-76 even in WT thymocytes after TCR stimulation although there is robust inducible SLP-76 phosphorylation. Furthermore, we do not see abrogation of the subcellular localization of exogenously expressed Vav1 and Itk constructs into microclusters following TCR stimulation in the KI T cells although more rigorous analysis will be required to confirm our findings. Taken together these data suggest that SLP-76 phosphotyrosines are dispensable for the assembly and localization of Itk and Vav1 within the proximal T cell signaling complex, at least to the extent that we can measure by immunoprecipitation and confocal studies.

Co-expression of both SLP-76 mutants resulted in partial and in some instances full rescue the single mutant phenotypes. These data demonstrate that the tyrosines of SLP-76 can function independently and that the co-operatively between mutant SLP-76 molecules occurs early following TCR stimulation, as Ca^{2+} signaling and activation of proximal signaling molecules are restored in double mutant mice. While SLP-76 itself has not been shown to oligomerize, other proteins in the proximal signaling complex,
including LAT (through its associated adaptor GRB2), have been shown to oligomerize after TCR stimulation (Houtman et al., 2006). Thus, it is likely that the complex that forms, or is stabilized, subsequent to TCR ligation includes at least two SLP-76 molecules. The partial rescue seen in some assays leads us to speculate that the trans complex is not as stable as a cis complex or that recruitment or activation of other key proteins to the signaling complex may be inefficient. This idea is consistent with data from Itk knock-down Jurkat cells and Vav1-deficient thymocytes, in which the absence of Itk or Vav1 results in the destabilization of the SLP-76 and Vav1 and SLP-76 and PLCγ1 associations, respectively (Dombroski et al., 2005; Reynolds et al., 2002).

Taken together we show that in vivo mutation of tyrosine 145 or tyrosines 112 and 128 to phenylalanines confers defects in peripheral T cell signal transduction following TCR ligation that result in activation and proliferation defects in vitro. Many of the same signaling pathways are affected by the two mutations, however Vav1 phosphorylation was defective in Y112/128F cells and not Y145F cells and Y145F but not Y112/128F mice phenocopy ITK deficient mice. Interestingly, co-expression of both mutations rescues all of these pathways to varying extents suggesting that multiple SLP-76 molecules can co-operate to transduce TCR signals. Some of the signaling and functional defects are more severe in the Y145F compared to the Y112/128F KI T cells suggesting that Y145 is more important for signaling transduction that Y112/128. As shown in cKI mice, these TCR-dependent defects, as well as the hierarchical requirements for the SLP-76 tyrosines are independent of their previously described role in T cell thymic development (Jordan et al., 2008). Furthermore, Vav1 and Itk localize to signaling clusters and co-precipitate with SLP-76 even in the absence of their phosphotyrosine binding motifs suggesting that SLP-76 has regulatory roles beyond simple scaffolding mechanisms. As Y112/128 and Y145 appear to be required for the
optimal function of Vav1 and Itk respectively it is possible that SH2/pTyr interactions with
the signaling complex result in stability of the complex and potentially conformation
changes within the molecules that enhance their activation.
Figure 2.1 Schematic of Y-F mutant SLP-76 molecules expressed in genomic KI mice.

Following TCR stimulation WT SLP-76 (above) has three phosphorylated tyrosines at positions 112, 128 and 145. Tyrosines 112 and 128 interact with Vav1 and Nck while Itk interacts with tyrosine 145. Itk can also interact with the proline rich region of SLP-76. Mutation of tyrosines 112 and 128 (bottom left) and mutation of tyrosine 145 (bottom right) to phenylalanines results in failure of phosphorylation of these residues following TCR stimulation. Y=tyrosine, F=phenylalanine, P=phosphate, PRR=proline rich region, SH2=Src homology domain 2.
Figure 2.2 Thymic profiles of SLP-76 KI mice.

Thymocytes from WT, Y145F, and Y112-128F mice were stained with anti-CD4 and anti-CD8 to determine DN, DP, and SP populations. Contour plots (top row) are representative of 25 mice. DN1-DN4 populations were determined by analyzing CD25 versus c-kit expression on thy1.2^+ lineage^- (lineage^- gate consisted of antibodies directed to CD8, B220, DX5, NK1.1, TCRγδ, Mac1, and Gr1) thymocytes (n=5). Data adapted from Martha Jordan (Jordan et al., 2008).
Figure 2.3. Knock-in thymocytes have defects in TCR induced signaling events.

A. SLP-76 immunoprecipitation of thymocytes from WT and Y112/128F (left) or WT and Y145F (right) that were stimulated with anti-CD3 for the indicated times or with pervanadate (PV) for 3 minutes. Precipitates were probed with 4g10 anti-phosphotyrosine (anti-pY) and anti-SLP-76 for control (n=3) B-E. Lysates from WT, Y145F and Y112/128F thymocytes stimulated with either anti-CD3 for the indicated times or pervanadate for 3 minutes were analyzed by western blot for the presence of phospho-SLP-76 (Y128), phospho-LAT (Y191) and total PLCγ1 for control (n=5) (B), anti-pY and total PLCγ1 as a control (C), phospho-PLCγ1(Y783) and total PLCγ1 (D) or phosphorylated ERK1/2 and total ERK (E). F. Lysates from purified WT, Y145F and Y112/128F DP thymocytes left unstimulated or stimulated with anti-CD3 and anti-CD4 for the indicated times were probed for pERK1/2 and total PLCγ1 as a control (n=2)
Figure 2.4. SLP-76 KI mutants generate peripheral T cells.

A) Contour plots show surface expression of CD4 and CD8 on lymphocytes from wild-type (WT), Y145F and Y112/128F spleens. B) Surface expression of TCRβ and CD5 on CD4+ and CD8+ lymphocyte gated splenocytes from WT, Y145F and Y112/128F mice. Data are representative of greater than 20 mice analyzed.
Figure 2.5. SLP-76KI mutants show defects in TCR induced signaling and function.
A. Spleen and lymph node cells from WT and KI mice were stimulated with anti-CD3 for the indicated times, lysed, and probed by western blot with anti-pPLCγ1 (Y783), anti-pERK1/2 and anti-pLAT (Y190). Anti-Erk2 was used for a loading control (n=3). B. Cytoplasmic Ca^{2+} influx was measured in WT, Y145F and Y112/128F CD4+ and CD8+ gated lymph node cells by flow cytometry following CD3, CD4 and CD8 cross linking (n=4). C. Splenocytes from WT, Y145F and Y112/128F mice were cultured in the presence of anti-CD3 overnight or for 72 hours then measured for CD69 upregulation (left panel) and CFSE dilution (right panel) respectively (n>5).
Figure 2.6 Vav1 and Itk dependent defects in SLP-76 KI thymocytes.

A. Vav1 was immunoprecipitated from thymocyte lysates, and phosphorylation of Vav1 (arrow) was determined by immunoblotting with anti-phospho-tyrosine antibody (4G10). Blot was stripped and probed for total Vav1 as a loading control (n=3).

B. Thymocytes from WT, Y112-128F, Y145F and Itk−/− mice were stained with anti-CD4 and anti-CD8. CD8SP cells were evaluated for expression of CD44 and CD122 (n>7). Data for B. courtesy of Martha S. Jordan.
Figure 2.7. Y145F KI mice show a preponderance of innate-like lymphocytes.
A. Contour plots show CD44 and CD122 surface expression on WT, Y145F and Y112/128F splenocytes gated on either CD8+ (top panel) or CD4+ (bottom panel) lymphocytes. Numbers represent the percent of cells in each gate. B. Splenocytes from naïve WT, Y145F and Y112/128F mice were incubated directly ex vivo with PMA and ionomycin for 5 hours and then fixed, permeabalized and stained. Contour plots show CD44 and IFNγ expression in cells gated on CD8+ or CD4+ lymphocytes. C. Cytoplasmic Ca^{2+} levels were measured in WT, Y145F and Y112/128F CD4+ CD44hi and CD8+ CD44hi gated lymph node cells by flow cytometry following CD3, CD4 and CD8 cross-linking.
Figure 2.8. SLP-76 mutants can complement one another *in trans*.

A. Lysates from anti-CD3 stimulated WT, Y112-128F, Y145F, and Double mutant thymocytes were analyzed by immunoblot for phosphorylation of PLCγ1 and ERK (n=3).

B. Ca²⁺ mobilization was induced by cross-linking CD3 and CD4. The tracings represent the relative amount of Ca²⁺ in the cytoplasm of CD4SP thymocytes from WT (thin black line), the Double mutant (thick grey line), or Y112/1284F (thick grey line) and Y145F (thick black line) mice (n=4).
Figure 2.9. Preserved binding of Vav1 and Itk to SLP-76 tyrosine mutants.

A. SLP-76 was immunoprecipitated from thymocyte lysates. Vav1 co-immunoprecipitated with SLP-76 in lysates from unstimulated cells and cells stimulated for 1 min and 5 min (n=3). SLP-76 phosphorylation was monitored by phospho-tyrosine immunoblotting and total SLP-76 protein was used as a loading control. B. SLP-76 co-immunoprecipitated with Itk from thymocyte lysates under resting and TCR stimulated conditions (n=2)
Figure 2.10. Vav1 and Itk KD form clusters following TCR ligation in Y145F and Y112/128F KI T cells.
WT, Y145F and Y112/128F T cells transfected with GFP-tagged Vav1 (top) or kinase dead ITK (ItkKD)(bottom) were incubated on cover slips coated with poly-l-lysine (unstimulated) or 10µg/ml 2C11 for 10 minutes then fixed, permeabilized and stained with phalloidin and analyzed by confocal microscopy. Images are representative of 3 independent experiments with 5-10 cells analyzed per condition/per experiment.
Figure 2.11. Schematic of genetic approach to the generation of SLP-76 conditional heterozygous and Y-F mutant mice.

Mice express one WT SLP-76 allele that is flanked by LoxP sites (>) and one WT SLP-76 allele (cSLP-76+/−) or one tyrosine mutant SLP-76 allele (cKI). An eYFP transgene is present and preceded by a stop codon flanked by LoxP sites. A CreT2 transgene is expressed under the ubiquitin promoter (UBC). Exposure to Tamoxifen results in nuclear localization of CreT2 protein where it can splice DNA at LoxP sites resulting in deletion of the LoxP flanked allele of WT SLP-76 and deletion of the stop codon preceding the eYFP transgene. Expression of eYFP protein can therefore be used to
identify cells with Cre recombinase activity that have lost the ability to generate WT SLP-76 protein (cKI) or can generate WT SLP-76 form one allele (cSLP-76+/−). Adapted from Wiehagan et al. 2010, submitted.
Figure 2.12. Conditional SLP-76 KI T cells show defects in TCR induced proximal signal transduction and functional responses in vitro.

A. Contour plots display CD4 and CD8 surface expression on YFP+ splenocytes from tamoxifen-treated cSLP-76+/-, cY145F and cY112/128F mice (n=10-12). B. Surface expression of TCRβ and CD5 on CD4+ YFP+ and CD8+ YFP+ gated splenocytes from cSLP-76+/-, cY145F and cY112/128F mice (n=5-7). C. Contour plots show surface expression of CD44 and CD122 on CD4+YFP+ and CD8+YFP+ splenocytes from cSLP-76+/-, cY145 and cY112/128F mice. Numbers represent the percent of cells in each gate. D. YFP+ T cells from cSLP-76+/-, cY145 and cY112/128F mice were stimulated with anti-CD3 for the indicated times, lysed and probed by western blot with anti-pPLCγ1 (Y783) and anti-pERK1/2 and actin as a loading control (n=2). E. CD69 upregulation
(right panel, n=5-7) and BRSE dilution (left panel, n=3-5) in splenocytes from cSLP+/−, cY145F and cY112/128F splenocytes was measured by flow cytometry following overnight and 72 hour stimulation with anti-CD3 respectively. Histograms are gated on CD8+YFP+ and CD4+YFP+ lymphocytes.
Chapter 3: TCR signals through SLP-76 tyrosines regulate T cell differentiation and Effector Function

Abstract

Using Y145F and Y112/128F SLP-76 mutant KI mice we show that altering proximal TCR signaling can differentially affect T cell fate choices independent of the external inflammatory environment. In vitro T helper polarization studies reveal defects in Th2 and Th17 but not Th1 differentiation in the absence of SLP-76 tyrosines. Conventionally induced Th2 cells are dependent on SLP-76 tyrosines for IL4 production but not competence. Induced Th17 cells, on the other hand, are dependent on SLP-76 tyrosine signals for both IL17a production and Il17a competence. Furthermore, these studies have uncovered a previously undefined subset of CD44hi CD4+ T cells that acquire the potential to produce IL17a in vivo, independent of SLP-76 tyrosines. To define the effects of dampened TCR signals on CD8+ T cell effector and memory differentiation, we infected WT and SLP-76 KI mice with the Armstrong strain of Lymphocytic Choriomeningitis Virus (LCMV). Data from these studies support a model in which altered TCR signals can determine the rate of memory versus effector cell differentiation, independent of initial T cell expansion. Furthermore, we show that TCR signals sufficient to promote CD8+ T cell differentiation are different than those required to elicit inflammatory cytokine production, reminiscent of our observations in the in vitro Th2 polarization studies.
Introduction

The heterogeneity of possible T cell effector responses reflects the heterogeneity of possible offending pathogens and is essential for an effective adaptive immune system. Integration of multiple extracellular signals including those from cytokines, pathogen associated molecular patterns, co-stimulatory receptors and peptide:MHC complexes is required for the establishment of a T cell response that is specific to the nature of the inciting pathogen. Naïve CD4+ T cells can differentiate into various T helper (Th) lineages including Th1, Th2 or Th17 effector cells as well as regulatory T cells, follicular helper T cells, memory cells and other less well-defined cell types (Zhu et al., 2010). Naïve CD8+ T cells differentiate into effector cytotoxic T lymphocytes (CTLs) and they either undergo terminal effector differentiation or they acquire a memory phenotype (Kaech and Wherry, 2007). While many studies have focused on the role of cytokines for T cell effector choices and function, the role of TCR signals on these processes is still not fully understood.

It is apparent that altering distinct TCR signals can have a profound effect on the effector functions of T cells. For example, mutations in the TCR proximal signaling adaptor LAT result in aberrant cytokine production and lymphoproliferative disease (Sommers et al., 2002). Furthermore, studies using altered peptide ligands (APLs) have shown both in vitro and in vivo that the strength of the interaction between the TCR and pMHC can influence lineage choices. In CD4+ T cells, these studies focused on Th2 versus Th1 differentiation and showed that, in the absence of exogenous cytokines, strong interactions induce higher levels of ERK phosphorylation which correlates with inhibition of Th2 differentiation and acquisition of Th1 effector function (Jorritsma et al., 2003; Kumar et al., 1995; Pfeiffer et al., 1995; Tao et al., 1997). The role of TCR signal
strength in the presence of Th polarizing cytokines has not been established. However, it is established that, downstream of the TCR, translocation and activation of NFAT and NFκB are important for inducing the distinct patterns of gene expression that define Th cell effector lineages (Hermann-Kleiter and Baier, 2010; Zhu et al., 2010). Activity of NFAT and NFκB are dependent on TCR-induced Ca^{2+} mobilization and DAG signals respectively, as well on their transcriptional binding partners that are differentially expressed in response to distinct cytokine signals. Following differentiation, bona fide Th2 and Th17 cells, in particular, require further TCR-induced NFAT transcriptional activity to produce their signature cytokines (Gomez-Rodriguez et al., 2009; Hermann-Kleiter and Baier, 2010; Kosaka et al., 2006).

CD8^{+} T cell differentiation and function has largely been tested with in vivo viral and bacterial infection models, which have revealed a typical pattern of the CD8^{+} response that begins with a robust expansion phase and, following antigen clearance, a contraction phase and finally a memory maintenance phase. Naïve CD8^{+} T cells acquire effector function upon infection. The pool of these effector cells is composed of terminally differentiated short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). Following antigen clearance the SLECs die by apoptosis and a subset of the MPECs survive and become long-lived memory cells. Over time the memory pool itself undergoes a process of functional maturation and conversion of the prominent cell type from effector memory (Tem) to central memory (Tcm) (Obar and Lefrancois, 2010; Wherry et al., 2003b). The role of TCR signaling in these processes has been, for the most part, tested indirectly by manipulating the immunogen or by comparing responses to epitopes that are recognized with different affinities (Kaech and Ahmed, 2001; Lefrancois et al., 2003; Ndhlouv et al., Sarkar et al., 2007; Wherry et al., 2003a; Wherry et al., 1999; Wherry et al., 2003b; Zehn et al., 2009). These studies have shown that
weak externally supplied signals diminish the magnitude of initial expansion, hasten T cell contraction following infection and increase the rate of acquisition of Tcm cells. More recently, infection of mice bearing a mutation in the TCRβ chain, which decreases NFkB activation, provided evidence that distinct TCR signals can direct effector versus memory fates as these mice showed intact effector differentiation but severely defective memory CD8 T cell formation following Listeria monocytogenes (LM) infection (Teixeiro et al., 2009).

Here we explore the role of TCR signals in the differentiation and function of CD4+ T helper cells and CD8+ effector and memory T cells using Y145F and Y112/128F SLP-76 KI mice. Using in vitro Th polarization assays we establish a role for SLP-76 tyrosine signals in Th2 and Th17 function. Using the Armstrong strain of lymphocytic choriomeningitis virus (LCMV) as a model of CD8+ T cell effector and memory differentiation, we show that SLP-76 mutant CD8+ T cells undergo primary clonal expansion, contraction and memory development, despite striking defects in cytokine effector function. Our findings indicate that TCR signals required to initiate a program of T cell differentiation are quantitatively and/or qualitatively different than those required to elicit cytokine responses upon re-stimulation.

**Results**

*Differential requirements for SLP-76 tyrosine signals in T helper lineage polarization*

To begin to address the effects of altered TCR signals on the quality of immune responses we first sought to determine if the dampened signals in SLP-76 KI T cells could support differentiation into Th subsets in vitro. Naïve CD4 T cells were purified and
cultured on a CD3/CD28 stimulating surface in the presence of: IL12 and anti-IL4 for Th1 skewing; IL4, anti-IL12 and anti-IFNγ for Th2 skewing; TGFβ, IL6, IL23 and anti-IFNγ for Th17 skewing. Following culture, T cells were restimulated with CD3 and CD28 and analyzed for production of signature Th cytokines (Figure 3.1, A, B, C; top panels). SLP-76 KI T cells produced IFNγ at similar frequencies and levels to WT T cells following Th1 polarization (Figure 3.1A, top panel). However following Th2 and Th17 polarization many fewer SLP-76 KI T cells produced IL4 and IL17a, respectively, compared to their WT counterparts (Figure 3.1B,C; top panels). Furthermore, the mean fluorescence intensity (MFI) of IL4 or IL17a of the populations of SLP-76 KI T cells that did produce cytokine was less than that of the WT cytokine producers, suggesting that SLP-76 KI cells tend to produce less cytokine on a per cell basis (Figure 3.1B,C; top panels).

The lack of cytokine production in Th2- and Th17-skewed SLP-76 KI T cells following TCR restimulation could be attributed to either defects in the ability of SLP-76 KI cells to differentiate into competent Th cells or defects in the ability of competent SLP-76 KI Th cells to elicit TCR-induced recall functions. To distinguish between these two possibilities we restimulated skewed WT and SLP-76 KI T cells with PMA and Ionomycin in order to by-pass proximal TCR signals and looked for the production of signature Th cytokines. As expected, there was no difference in the frequency of IFNγ producers between Th1-skewed WT and SLP-76 KI T cells following PMA and Ionomycin treatment (Figure 3.1A; bottom panel). There was also no difference in IL-4 production between Th2-skewed WT and SLP-76 KI T cells, suggesting that the SLP-76 KI T cells can undergo Th2 differentiation and produce IL4 competent cells but they have a defect in the ability to elicit IL4 production following TCR restimulation (Figure 3.1B; bottom panel). Conversely, severe defects were observed in IL17a production in Th17 skewed KI cells even when the proximal TCR signaling machinery was by-passed, suggesting
that these cells have a defect in the ability to acquire IL17a competence (Figure 3.1C). Despite the SLP-76 tyrosine hierarchy observed in previous signaling and functional assays (see Chapter 2), there were no significant differences between Y145F KI and Y112/128F KI responses under any of the Th skewing conditions.

**The requirement of SLP-76 tyrosines for IL17a competence and production is independent of their role in T cell thymic development**

Since SLP-76 tyrosines plays a role in T cell thymic development (see chapter 2) it is possible that inability of naïve SLP-76 KI T cells to acquire IL17a competence could be due to a developmental defect. We therefore looked at the ability of naïve SLP-76 cKI T cells to acquire and produce IL17a following culture under Th17 polarizing conditions. As previously described in chapter 2, SLP-76 cKI T cells express WT SLP-76 during thymic development but it is later deleted with Tamoxifen-inducible Cre activity, leaving expression of only the mutant form of SLP-76. Following tamoxifen treatment, sorted naïve, YFP+ CD4+ T cells from SLP-76 cKI and cSLP-76+/− mice were cultured under Th17 skewing conditions and then restimulated with either anti-TCR/CD28 or PMA and Ionomycin. Similar to what was observed in non-conditional SLP-76 KI T cells, SLP-76 cKI T cells failed to produce WT levels of IL17a regardless of the restimulating conditions (Figure 3.4). These data suggest that TCR-induced signals through the SLP-76 tyrosines are required for naïve CD4 T cell acquisition of IL17a competence independent of their role in thymic development.
**SLP-76 KI Th17 polarized T cells can upregulate the Th17 master regulator RORγT**

We next looked at expression RORγT transcription following Th17 skewing in WT and SLP-76 KI T cells to determine if SLP-76 KI T cells have a defect in the ability to upregulate this master regulator of Th17 differentiation and Il17a production. The overwhelming majority of both SLP-76 KI and WT T cells expressed RORγT following Th17 skewing and all cells that produced IL17a following TCR/CD28 or PMA and ionomycin stimulation expressed RORγT (Figure 3.3). Thus, TCR signals through SLP-76 tyrosines are required for the acquisition of IL17a competence but not for acquisition of other characteristics of Th17 differentiation including RORγT upregulation, consistent with the phenotype recently observed in Itk deficient T cells (Gomez-Rodriguez et al., 2009). Future studies are required to determine if SLP-76 KI Th17 cells, like Itk-/- Th17 cells, are competent to produce other cytokines associated with Th17 function including IL17f and IL22.

**A subset of splenic CD4⁺ CD44hi T cells are poised to produce IL17a independent of SLP-76 tyrosines**

As other T cell types have been shown to have the potential to produce IL17a including iNKT cells, CD8 T cells and γδ T cells (reviewed in (O'Brien et al., 2009)), we next sought to determine if the defect in the ability to acquire competence to produce IL17a was a global defect amongst T cells in the SLP-76 KI mice. To this end, we stimulated bulk splenocytes from WT and SLP-76 KI mice directly *ex vivo* with PMA and ionomycin and looked for the production of IL17a. Among CD5⁺ WT T cells we found a small population of CD44hi cells that produced IL17a (Figure 3.4, top panel). Surprisingly,
similar populations were found in Y145F KI mice and Y112/128F KI mice and, in fact, the frequency of these cells was greater in the KI mice than in the WT mice (Figure 3.4, top panel). Of the CD5+, IL17a producers almost 30% were found to be γδ T cells in the WT mice but only around 1% of CD5+, IL17a producers were γδ T cells in either strain of SLP-76 KI mice (Figure 3.4, middle panel). There was no difference in the frequency of γδ T cells in the SLP-76 KI mice when compared to WT mice (data not shown). It is possible that the CD44 hi IL17a producing cells we observed were NKT cells, however, previous reports have shown that IL17a producing NKT cells are CD4- (Michel et al., 2008) and most of the γδ T cells that produced IL17a were CD4+ in WT and SLP-76 KI mice (data not shown). In the total CD4+ T cell population, the frequency of IL17a producers was considerably greater in both strains of KI mice when compared to WT (Figure 3.4, bottom panel). The greater frequency of this population of CD4+ T cells in the KI mice may be due to altered thymic selection and studies using cKI mice are underway to test this.

**Th17 skewing conditions promote IL17a potential in WT and SLP-76 KI CD44 hi CD4+ T cells.**

We cultured CD62L lo CD44 hi CD4+ T cells from WT and SLP-76 KI mice under Th17 polarizing conditions to determine if they could acquire, or further acquire, IL17a potential. Restimulation with anti-TCR/CD28 or PMA and Ionomycin induced almost 10% or 20% of WT cultured cells to produce IL17a, respectively (Figure 3.5, left panels). Surprisingly, cultured T cells from both strains of SLP-76 KI mice produced IL17a not only in response to PMA and Ionomycin but also in response to anti-TCR/CD28. It is still unclear whether culturing conditions promoted the expansion of existing T cells with IL17a potential (which could account for the greater frequency of these cells in the SLP-
76 KI cells cultures) or de novo induction of IL17a T cells or a combination of the two. Taken together these data suggest that unlike induced Th17 cells and γδ T cells, CD44\textsuperscript{hi} CD4\textsuperscript{+} T cells can acquire IL17a competence independent of SLP-76 tyrosines and furthermore they can produce IL17a in response to TCR stimulation independent of SLP-76 tyrosines.

**CD8 expansion in response to acute LCMV infection is intact in SLP-76KI mice**

Having established a role for SLP-76 tyrosine signals in T cell differentiation \textit{in vitro}, we next sought to examine the role of these signals for a complete \textit{in vivo} immune response. We chose LCMV infection as it is also a well-studied model of CD8 differentiation. WT, Y145F and Y112/128F mice were infected with the Armstrong strain of LCMV that induces an acute infection in WT mice. Analysis of peripheral blood lymphocytes (PBLs) 5 days before infection (baseline) shows that CD8\textsuperscript{+} T cells were present at similar frequencies within the blood in the three different strains of mice (Figure 3.6A). In WT mice at day 4 post-infection (p.i.), the percent of CD8\textsuperscript{+} T cells in the blood was decreased from baseline. It is unclear why this decrease was more dramatic in KI mice (Figure 3.6A). Regardless, by the peak of CD8\textsuperscript{+} T cell expansion at day 8 p.i. and later at day 15 p.i., the percent of circulating CD8\textsuperscript{+} T cells was similar between the three strains of mice (Figure 3.6A). Absolute numbers of CD8\textsuperscript{+} T cells were calculated from spleens on day 8 p.i. and confirmed an intact CD8\textsuperscript{+} T cell expansion in the KI mice (Figure 3.6B). In the Y145F mice, splenic CD8\textsuperscript{+} T cells were present in higher numbers, although the differences were not significant in all experiments (Figure 3.6B). Thus, although both Y112/128F and Y145F T cells demonstrate a significant
proliferative defect \textit{in vitro} in response to TCR engagement, an \textit{in vivo} viral challenge is sufficient to induce substantial T cell expansion comparable to that induced in WT mice. We next examined the quality of the T cell response to LCMV in WT and KI mice. Expansion of T cells reactive to specific dominant and subdominant LCMV epitopes were examined using 3 different H2D\textsuperscript{b}: peptide tetramers: NP396, GP33 and GP276 (Figure 3.6C). While expansion of CD8\textsuperscript{+} T cells reactive to the more dominant epitopes NP396 and GP33 was intact in both strains of KI mice, very few cells reactive to the subdominant epitope, GP276, were observed in the Y145F KI mice (van der Most et al., 1996). Y112/128F KI mice showed a lesser but consistent decrease in the absolute numbers of CD8\textsuperscript{+} splenocytes reactive to GP276. Thus, expansion in response to particular epitopes may be differentially sensitive to the effectiveness of the TCR signal based on the level of epitope dominance. Alternatively, the decrease of GP276-reactive T cells observed in the SLP-76 KI mice could be due to decreased expansion or perturbation in selection of cells responsive to this epitope during thymic development or a combination of these possibilities.

\textit{Altered effector function upon re-stimulation in SLP-76 KI CD8\textsuperscript{+} T cells following LCMV infection}

The effector function of CD8\textsuperscript{+} T cells from day 8 p.i. WT and SLP-76 KI mice was analyzed \textit{ex vivo} by measuring cytokine production in splenocytes in response to incubation with NP396, GP33 and GP276 peptides. Consistent with tetramer reactivity, fewer SLP-76 KI T cells produced cytokines in response to GP276 peptide compared to WT cells (Figure 3.7A). The amount of IFN\textgamma produced by each cell, as measured by the mean fluorescence intensity (MFI), was lower in T cells from both strains of KI mice in response to all three peptides (Figure 3.7B). While similar frequencies of CD8\textsuperscript{+} cells from
the three strains of mice produced IFN\(\gamma\) in response to NP396 and GP33, the ratio of cells that produced TNF\(\alpha\) and IFN\(\gamma\) to cells that produced IFN\(\gamma\) alone was much lower in the Y145F mice but normal in the Y112/128F mice (Figures 3.7A, C, D). This ratio was decreased in Y112/128F T cells only in response to the subdominant epitope GP276 (Figure 3.7C). Despite the weaker cytokine responses, viral titers from liver and spleen were below the level of detection for all mice by day8 (data not shown).

*Conditional SLP-76 KI T cells show defects in their response to LCMV challenge*

To determine if the altered CD8 repertoire and T cell selection observed in the KI mice, especially the Y145F KI mice could account for either the surprising robustness of the SLP-76 KI response or the effector defects in the responding KI T cells, tamoxifen-treated cKI and cSLP-76\(^{+/−}\) mice were infected with LCMV Armstrong. At day 8 p.i., the frequency of YFP\(^+\)CD8\(^+\) T cells reactive to all three LCMV tetramers was not significantly different in either cKI mouse strain, when compared to cSLP-76\(^{+/−}\) mice (Figure 3.8A). This was in contrast to the near absence of GP276 tetramer-reactive T cells observed in Y145F KI mice (Figure 3.7). When challenged with LCMV peptides, cKI T cells behaved similarly to KI T cells (Figures 3.7, 3.8B,C). The ratio of double IFN\(\gamma\), TNF\(\alpha\) producers to single IFN\(\gamma\) producers was reduced in the YFP\(^+\) cY145F T cells when stimulated with NP396, GP33 or GP276 peptides (Figure 3.8B, left panel, 3.8C). While slight decreases in this ratio were consistently observed in YFP\(^+\) cY112/128F T cells in response to peptides, the differences were not significant (Figure 3.8B, left panel). The MFI of IFN\(\gamma\) in peptide-responsive cKI T cells was less than that in cSLP\(^{+/−}\) T cells, and this difference was more pronounced in the Y145F cKI cells compared to the Y112/128F cKI cells.
(Figure 3.8B, right panel). These experiments indicate that the altered thymic development associated with SLP-76 KI mice was not responsible for the diminished ability of T cells from these mice to respond to LCMV peptide re-stimulation. However, the contribution of GP276-reactive T cells to an LCMV response in Y145F mice was impacted by developmental events. Moreover, these experiments indicate that the ILLs neither diminished nor augmented the anti-LCMV response measured in the non-conditional Y145F mice.

**Poor polyfunctionality in KI mice is the result of defective responses to TCR re-stimulation**

Terminally differentiated cells lose cytokine polyfunctionality while cells that retain polyfunctionality, in particular IL2 production, are more likely to survive and differentiate into memory cells (Joshi et al., 2007; Precopio et al., 2007; Sarkar et al., 2008). To test whether the poor polyfunctionality seen in KI mice was the result of enhanced terminal differentiation or defective ability to respond to subsequent TCR stimulation, day 8 p.i. splenocytes were stimulated with PMA plus ionomycin to bypass proximal TCR signaling events. PMA/ionomycin stimulation revealed that KI effector CD8\(^+\) T cells with TNF\(\alpha\) and IL2 polyfunctional potential were present in frequencies similar to or greater than those observed in WT splenocytes, suggesting that the KI effector cells were not preferentially skewed toward terminal differentiation but potentially towards a memory precursor cell phenotype (Figure 3.9A). Effector cell surface marker expression has also been used to distinguish terminally differentiated cells from those with memory potential. IL7r\(\alpha\) surface re-expression and low levels of KLRG-1 expression have been associated with memory precursors (MPECs), while terminally differentiated short-lived effector cells (SLECs) express low levels of IL7r\(\alpha\) and high levels of KLRG-1 (Kaech et al., 2003;
Sarkar et al., 2008). Analysis of peripheral blood on day 15 p.i. revealed that the frequency of SLEC among H2D^b:GP33-reactive KI T cells was significantly less than that of the WT T cell population whereas the frequency of MPECs was greater (Figure 3.9B). However, SLEC and MPEC designations do not take into account the increased frequency of cells expressing high levels of both KLRG-1 and IL7rα in KI mice (Figure 3.9B). These cells are present in WT mice, but their contribution to memory is not clear.

**SLP-76 KI mice generate long-lived antigen-specific T cells with accelerated acquisition of a “mature” memory phenotype**

To determine if virus-specific KI T cells can differentiate into memory cells, we monitored KI and WT mice for the persistence of H2D^b: GP33-reactive CD8^+ T cells in the blood of infected animals. The kinetics of expansion and contraction of total CD8^+ T cells and H2D^b: GP33-reactive CD8^+ T cells were similar between KI and WT mice (Figure 3.10A). After 5 weeks, the frequency of CD8^+ and H2D^b: GP33-reactive CD8^+ T cells in all mice plateaued and remained stable for the duration of the experiments up to 130 days (Figure 3.10A and data not shown). We next examined H2D^b: GP33-reactive CD8^+ T cells for acquisition of CD62L and enhanced IL7rα expression, two phenotypic indicators of Tcm cells. CD62L and IL7rα upregulation occurred at a significantly faster rate in both KI mice, and the frequency of CD62L^hi and IL7rα^hi LCMV-specific cells at most time points after day 8 was greater in the KI compared to WT mice (Figure 3.10B). These differences were more pronounced in Y145F versus Y112/128F mice.

Throughout the immune response, circulating LCMV-specific KI CD8^+ T cells expressed higher levels of CD27 and CXCR3 (Figure 3.10C), two activation markers associated
with functional maturation in memory cells (Hikono et al., 2007; Slifka and Whitton, 2001). Thus, SLP-76 tyrosine mutant T cells, with a dampened ability to transduce TCR signals, show accelerated acquisition of a Tcm surface phenotype. Furthermore, the frequency of circulating antigen-specific cells expressing surface markers consistent with functionally mature memory cells is greater in both KI strains compared to WT mice. To determine whether long-lived antigen-specific SLP-76 KI T cells could function as memory cells, splenocytes from LCMV-infected mice were examined 70 days or later p.i. for their ability to exert an effector response to GP33 peptide ex vivo. Splenic H2D$^b$: GP33-reactive CD8$^+$ T cells were found in similar numbers in Y112/128F and WT mice but were increased in Y145F mice (Figure 3.11A). Consistent with circulating T cells, splenic H2D$^b$: GP33-reactive CD8$^+$ T from both KI mice had increased surface expression of CD62L, IL7r$\alpha$, CXCR3 and CD27, characteristics of Tcm or more mature memory cells, when compared to splenic T cells from WT mice (Figure 3.11B). Upon peptide stimulation, memory cells from both KI mice made less IFN$\gamma$, as measured by MFI, and were less polyfunctional (Figure 3.11C). At day 70 p.i., diminished polyfunctionality was observed in Y112/128F T cells, which was in contrast to day 8 of the primary response when they responded similarly to WT cells (Figures 3.11C, 3.7). Tcm and CD27$^{hi}$ cells have been shown to produce more IL-2 than Tem or CD27$^{lo}$ cells following antigen exposure (Hikono et al., 2007; Roberts et al., 2005), but despite the increased frequency of Tcm phenotype and CD27$^{hi}$ cells present in KI versus WT mice, KI T cells produced significantly less IL-2 (Figure 3.11D). These data indicate that although present, memory CD8$^+$ T cells from SLP-76 KI mice fail to mount a proper cytokine response to re-stimulation with cognate antigen.
**SLP-76 Ki memory cells can expand in response to rechallenge in vivo**

To determine if memory Ki T cells can function *in vivo*, CD8 purified cells from LCMV immune Ki and WT mice were transferred into WT congeneric hosts, which were then infected with a strain of *Listeria monocytogenes* that expresses GP33 (LM: GP33). Seven to eight days p.i., host spleens were analyzed for the presence and function of donor and host H2D\(^b\): GP33-reactive T cells. Expansion of host CD8\(^+\) T cells was similar regardless of the origin of the donor cells (data not shown). Despite the increased frequencies of more mature memory phenotype cells in the Ki memory pools, their response to rechallenge was not enhanced. Indeed, pooled data from all day 7 p.i. experiments suggests that a mild proliferative defect may be present in the Y145F Ki memory cells (Figure 3.12A) as in some experiments, Y145F Ki cells failed to expand to the extent of WT cells. The frequency of donor Y145F Ki T cells, but not Y112/128F Ki T cells, that responded to GP33 peptide (as measured by their ability to produce IFN\(\gamma\)) was less than that of WT donor cells despite similar frequencies of H2D\(^b\):GP33-reactive T cells (Figure 3.12B). These results suggest that fewer secondary effector GP33-specific Y145FKi T cells responded to peptide. The GP33 epitope also contains a peptide capable of presentation by H2K\(^b\) (Hudrisier et al., 1997). Therefore, the diminished response observed in GP33-stimulated Y145F cultures might also reflect defective H2K\(^b\) responsiveness. Consistent with our observations in primary effectors and long-lived memory cells, the Ki effector T cells following secondary challenge showed altered cytokine responses in both polyfunctionality and in the MFI of IFN\(\gamma\) when compared to donor WT T cells following GP33 peptide stimulation (Figure 3.12C). Furthermore, these defects were not observed when the donor cells were stimulated with PMA and ionomycin (Figure 3.12C). These data demonstrate that despite
accelerated acquisition of a mature memory phenotype and the ability to expand upon *in vivo* rechallenge, memory KI CD8⁺ T cells are defective in their ability to liberate effector cytokine responses downstream of the TCR.

**Discussion**

Using SLP-76 KI mice we have shown that defects in proximal TCR signaling events can result in varying degrees of defective T cell differentiation and effector functions depending on the T cell lineage examined. While not yet complete, initial studies suggest that the decreased signals in the mutant SLP-76 cells are strong enough to support naïve CD4 differentiation into Th1, Th2 and Th17 cells *in vitro*. However, the elaboration of IL4 and IL17a, but not IFNγ, recall responses were severely dampened in Th skewed SLP-76 KI T cells. Furthermore KI Th17 cells lacked the competence to produce IL-17a upon restimulation even if the proximal signaling complex was by-passed. Taken together, in our *in vitro* system, TCR signals through SLP-76 tyrosines contribute to two phases of CD4 function; first in the acquisition of cytokine competency during Th differentiation (Th17) and second in the elicitation of cytokine effector responses (Th2, Th17). Similar to what was observed in Th2 cells, CD8⁺ T cells from SLP-76 KI mice undergo differentiation into effector cells competent to produce signature cytokines, but have defects in the ability to elicit these cytokines upon antigen encounter. Therefore we have shown that the TCR signals required for differentiation are quantitatively or qualitatively different than those required for effector function.

We have not yet defined the pathway(s) by which the SLP-76 tyrosines contribute to Th differentiation and function. However, both Y145F and Y112/128F KI T cells display similar defects in TCR-induced Ca²⁺ mobilization, a pathway required for nuclear localization of NFATs, which in turn, are essential for multiple transcriptional
events during Th differentiation and subsequent effector cytokine production (Hermann-Kleiter and Baier, 2010). Furthermore, both Itk and Vav1, SLP-76 tyrosine binding partners, have roles in Th effector cytokine production that have been attributed to their requirement for Ca\(^{2+}\) mobilization and NFAT translocation following TCR stimulation. Cytokine defects that have been observed in Vav1- and Itk-deficient cells and are similar to the defects observed in the SLP-76 KI mice (Au-Yeung et al., 2006; Fowell et al., 1999; Gomez-Rodriguez et al., 2009; Miller et al., 2004; Tanaka et al., 2005).

One of the most intriguing findings described in this chapter, however, is the discovery of a population of splenic CD44\(^{hi}\) CD4\(^{+}\) T cells that are poised to produce IL17a even in the SLP-76 KI mice. It is possible that these cells are thymically derived, as a recent study has identified a population of natural IL17-producing CD44\(^{hi}\) CD4\(^{+}\) T cells that are selected in the thymus on self-ligand (Marks et al., 2009). This study also suggested that IL6 and TGF\(\beta\), cytokines known to induce naïve T cells to adopt a Th17 fate, can also regulate the development of natural Th17 cells in the thymus. We found that sorted CD44\(^{hi}\) CD62L\(^{lo}\) CD4\(^{+}\) T cells from both WT and SLP-76 KI mice become enriched for IL17a producing cells following culture with Th17 skewing cytokines in vitro. Furthermore, Th17 skewed CD44\(^{hi}\) CD4\(^{+}\) T cells from both WT and SLP-76 KI T cells can produce IL17 in response to TCR restimulation. IL17a production has been shown to require high levels of Ca\(^{2+}\) mobilization and TCR stimulation (Gomez-Rodriguez et al., 2009). CD44\(^{hi}\) CD4\(^{+}\) T cells from SLP-76 KI mice show defects in their ability to mobilize Ca\(^{2+}\) following TCR ligation ex vivo, suggesting that they do have defective TCR signal propagation, however we could not directly test Ca\(^{2+}\) mobilization in the IL17a poised subpopulation alone (see chapter 2). Taken together these data suggest that in the CD44\(^{hi}\) cells, either IL17a production is more sensitive to lower Ca\(^{2+}\) signals or IL17a production utilizes Ca\(^{2+}\) independent signaling machinery.
The experiments described in this chapter have shown that proximal TCR signals not only contribute to CD4\(^+\) effector functions, but they also contribute effector versus memory fate choices and effector functions in the CD8\(^+\) lineage. We show that while the magnitude of the CD8\(^+\)-mediated immune response is preserved in mice in which key SLP-76 tyrosines are mutated, differentiation and function of the reactive T cells is altered. Our data indicate that dampened proximal TCR signals can be sufficient for generating a complete CD8 immune response but skew the response towards a memory phenotype at the expense of effector cells. Moreover, this study demonstrates that the intracellular signals (either qualitative or quantitative) that drive memory T cell expansion and persistence can be separated from those that regulate cytokine production following re-stimulation with MHC:peptide.

SLP-76 mutant mice show a surprisingly normal magnitude of CD8 T cell expansion in response to acute LCMV infection despite profound \textit{in vitro} proliferative defects. This is also in contrast to previous studies that have shown decreased expansion in the context of diminished signals to or from the TCR, including studies in mice deficient for Itk (Atherly et al., 2006a; Zehn et al., 2009). It is possible that Itk has kinase- or SLP-76-independent functions that are important for proliferative responses \textit{in vivo}, perhaps downstream of inflammatory signals that are not accounted for in the \textit{in vitro} studies. It is also possible that the overall strength of the TCR signal in SLP-76 KI T cells falls above the threshold for optimal proliferation and that strength of TCR signal in Itk deficient cells and in other models of diminished TCR signal fall below this threshold.

Unlike our observations in Th effector function, the graded diminishment in the strength of proximal TCR signaling observed in the two strains of SLP-76 KI mice result in graded effects on CD8\(^+\) T cell differentiation. For example enhancement of memory
precursor differentiation (MPEC) at the expense of terminal differentiation (SLECs) was more pronounced in the Y145F KI compared to the Y112/128F KI. Graded dampening in TCR signaling in KI T cells also correlated with an increased number and rate of accumulation of GP33-specific Tcm cells (based on surface phenotype). These data are consistent with previous studies suggesting that the transition from a CD62Llo to a CD62Lhi (Tem to Tcm) CD8 T cell memory pool occurs faster in the face of weak initial antigen stimuli (Sarkar et al., 2007; Wherry et al., 2003b). Furthermore, the KI memory pool is enriched for cells expressing high levels of CD27 and CXCR3, two activation markers associated with increased functional avidity (Hikono et al., 2007; Roberts et al., 2005; Roberts and Woodland, 2004). Taken together these data suggest that the transition of the memory pool to a more mature and Tcm-dominant phenotype, is dependent on TCR signals and that strong TCR signals are required for terminal differentiation.

Effector responses in CD8+ T cells are also linked to TCR signal strength as cytokine recall is less affected in the Y112/128F KI compared to the Y145F KI T cells. CD8+ effector and memory cells from SLP-76 KI mice show defects in their ability to co-produce multiple cytokines and IL2, respectively, following MHC:peptide stimulation. However when primary or secondary effector cells were stimulated with PMA plus ionomycin to bypass the proximal TCR signaling machinery, KI cells were fully capable of polyfunctionality suggesting that their differentiation is intact. Therefore like our observations in Th2 differentiation and function, signals through mutant SLP-76 proteins can support TCR dependent CD8+ effector and memory differentiation but fail to support optimal cytokine production in response to subsequent TCR stimulation.

By manipulating a key intracellular signaling molecule, we have demonstrated that the quality of effector functions of CD4+ and CD8+ T cells can be influenced by the
signals downstream of the TCR despite intact effector differentiation. Furthermore, while TCR signals have long been shown to differentially affect lineage choices in the thymus, we show that TCR signals can differentially affect the acquisition of distinct lineage fates in the periphery as well. For example, in our model, the loss of SLP-76 tyrosines had no effect on acquisition of Th1 and Th2 competency \textit{in vitro}, but Th17 competency was diminished. \textit{In vivo} studies support this notion as the TCR signals generated in SLP-76 KI mice support CD8\textsuperscript{+} memory differentiation at the expense of effector differentiation.
Figure 3.1. Th1, Th2 and Th17 lineage polarization and function have differential requirements for SLP-76 tyrosines.
Sorted, naïve CD62L^{hi}, CD44^{lo} CD4^{+} T cells were stimulated under Th1 (A), Th2 (B) or Th17(C) skewing conditions for 5,6 and 3 days respectively and then restimulated with anti-CD3 and anti-CD28 (top) or PMA and ionomycin (bottom) in the presence of Brefeldin A for 5 hours and then analyzed by flow cytometry for the presence of IFN\textsubscript{\gamma}, IL4 and IL17a respectively. Numbers represent the frequency of cells within each gate. Contour plots are gated on CD4^{+} lymphocytes. Data are representative of at least 4 independent experiments.
Figure 3.2. Th17 polarization defects in conditional KI mice.

Sorted CD62L$^{hi}$, CD44$^{lo}$, CD4$^{+}$, YFP$^{+}$ T cells were sorted from cSLP$^{+/c}$, cY145F and cY112/128F KI mice and cultured under Th17 skewing conditions for 3 days and then restimulated with anti-CD3 and anti-CD28 (top) or PMA and ionomycin (bottom) in the presence of Brefeldin A for 5 hours and then analyzed by flow cytometry for the presence of IL17a. Numbers represent the frequency of cells within each gate. Contour plots are gated on CD4$^{+}$ lymphocytes. Data are representative of four independent experiments.
Figure 3.3. KI CD4 T cells upregulate RORγt expression under Th17 polarizing conditions.

CD62L<sup>hi</sup> CD44<sup>lo</sup> CD4<sup>+</sup>T cells were sorted from WT, Y145F and Y112/128F KI mice and cultured under Th17 polarizing conditions for 3 days and then restimulated with TCR and CD28 (above) or PMA and Ionomycin (below) in the presence of Brefeldin A for 5 hours and then analyzed by flow cytometry for the presence of IL17a and RORγt. Numbers represent the percent of cells within the gate. Contour plots are gated on CD4+ lymphocytes. Data are representative of three independent experiments.
Figure 3.4. A population of splenic CD44hi, CD4+ but not TCRγδ KI T cells is poised to produce IL17a directly *ex vivo*.

Splenocytes were stimulated with PMA and ionomycin directly *ex vivo* in the presence of Brefeldin A for 5 hours and then analyzed by flow cytometry. A) Contour plots are gated on CD5⁺ lymphocytes, numbers represent the percent of cells expressing high levels of CD44 and producing IL17a. B) Contour plots show and numbers represent percent TCRγδ usage amongst cells in gates from (A). C) Contour plots are gated on CD5⁺, TCRγδ⁺, CD4⁺ cells. Numbers represent percent of these cells that are CD44hi and express IL17a. Data are representative of 2 independent experiments with 1-2 mice per group.
Figure 3.5. CD44hi CD4 KI T cells produce IL17a following in vitro Th17 polarization.

CD62Llo CD44hi CD4+ T cells were sorted from WT, Y145F and Y112/128F KI mice and cultured under Th17 polarizing conditions for 3 days and then restimulated with anti-TCR/CD28 (above) or PMA and Ionomycin (below) in the presence of Brefeldin A for 5 hours and then analyzed by flow cytometry for the presence of IL17a. Numbers represent the frequency of cells within the gate. Contour plots are gated on CD4+ lymphocytes. Data are representative of five independent experiments.
Figure 3.6. SLP-76 KI mice have intact CD8 expansion in response to acute LCMV infection.

A. PBLs were isolated from serial bleeds from WT and KI mice at the indicated time points before and after LCMV infection and were analyzed by flow cytometry. CD8^+ T cells (avg ±SEM) are represented as a percent of the live lymphocyte gate (data are representative of 3 independent experiments each with 3-5 mice per group). Absolute numbers of B. CD8^+ (avg ±SEM) and C. tetramer reactive (avg ±SEM) splenocytes were calculated from day 8 infected WT and KI mice (representative of 2 independent experiments each with 5 mice per group). Significant p-values, when present, comparing KI to WT are depicted as stars: ***p<0.001, **p=0.001-0.01, *p=0.01-0.05.
Figure 3.7. SLP-76 KI T cells show defective effector function in response to acute LCMV infection.
A-D. Splenocytes from day 8 infected WT, Y145F and Y112/128F KI mice were cultured in vitro with the indicated stimuli and analyzed by flow cytometry (representative of 2 independent experiments each with 5 mice per group). A. Percent of CD8 cells that produced IFNγ was calculated (avg ±SEM). B. The MFI of IFNγ staining in all CD8+ T cells producing IFNγ is shown (avg ±SEM). C. CD8+ responders were quantified as a ratio (avg ±SEM) of IFNγ, TNFα double producers to IFNγ single producers. D. Representative contour plots show IFNγ and TNFα expression in CD8+ splenocytes. Numbers indicate the percent of CD8+, TNFα IFNγ double producers over the percent of total CD8+IFNγ producers (representative of 2 independent experiments each with 5 mice per group). Significant p-values, when present, comparing KI to WT are depicted as stars: ***p<0.001, **p=0.001-0.01, *p=0.01-0.05.
Figure 3.8. LCMV infected cSLP-76 KI T cells show defective effector responses in vitro.
Mice were infected with LCMV Armstrong 7-10 days following tamoxifen treatment. A) Tetramer reactivity among splenocytes from day 8 infected cSLP+/-, cY145F and cY112/128F mice is represented as a percent of CD8+YFP+ lymphocytes (representative of 2 independent experiments each with 5 mice per group). B-C) Splenocytes from day 8 infected cSLP+/-, cY145F and cY112/128F mice were incubated in vitro with the indicated peptides and analyzed by flow cytometry for the presence of TNFα and IFNγ.

B) Contour plots are gated on YFP+CD8+ lymphocytes (representative of 2 independent experiments each with 5 mice per group). Numbers indicate the percent of CD8+, TNFα IFNγ double producers over the percent of total CD8+IFNγ producers. C) Cytokine ratios (top panel) and MFI (bottom panel) were determined among CD8+ YFP+ splenocytes as in Figure 5.2 (representative of 2 independent experiments each with 5 mice per group). Bar graphs show averages ± SEM. Significant p-values, when present comparing KI to WT are depicted as stars: ***p<0.001, **p=0.001-0.01, *p=0.01-0.05.
Figure 3.9. Poor polyfunctionality in KI mice is the result of defective responses to TCR re-stimulation.

A. Splenocytes from day 8 p.i. WT, Y145F and Y112/128F mice were stimulated with PMA plus ionomycin. Representative contour plots show IFNγ and TNFα (top panel) and
IFNγ and IL2 (bottom panel) expression in CD8⁺ cells. Numbers indicate the percent of CD8⁺, IFNγ, TNFα (top panel) or IFNγ, IL2 (bottom panel) double producers over the percent of total CD8⁺ IFNγ producers. B. Dot plots show IL7rα and KLRG-1 surface expression on H2Db:GP33-reactive CD8⁺ PBLs from WT, Y145F and Y112/128F mice 15 days p.i. (top panel). Numbers indicate the percentage of CD8⁺ H2Db:GP33-reactive lymphocytes within each gate. Upper left and bottom right gates from top panel were used to quantify the percent of SLECs (left, bottom panel) and MPECs (right, bottom panel) respectively. For graphs each point represents an individual mouse; bars represent avg±SEM. Data are representative of 3 independent experiments with 5-10 mice per group. Significant p-values, when present, comparing KI to WT are indicated by asterisks: ***p<0.001, **p=0.001-0.01, *p=0.01-0.05.
Figure 3.10. SLP-76 KI mice generate long-lived memory T cells with accelerated acquisition of a “mature” phenotype.

WT, Y145F and Y112/128F mice were infected with LCMV Armstrong and monitored by longitudinal bleeds for 60-130 days. Data are representative of 3 independent experiments with 5-10 mice per group. A) The percent of CD8⁺ T cells (left panel) and H2Dk:GP33 reactive, CD8⁺ T cells (right panel) among PBLs from WT, Y145F and
Y112/128F mice at indicated days p.i. are shown. B) The percent of CD62L<sup>hi</sup> cells (left panel) and IL7r<sup>α</sup><sub>hi</sub> cells (right panel) among H2D<sup>b</sup>: GP33 reactive CD8<sup>+</sup> PBLs from WT, Y145F and Y112/128F mice at indicated days p.i. are shown. Average percentage of cells ± SEM is shown. C) CD27 and CXCR3 surface expression on WT, Y145F and Y112/128F H2D<sup>b</sup>: GP33 reactive, CD8<sup>+</sup> PBLs from day 15 (left panel) and day 70 (right panel) p.i.. Time points with significant differences between Y145F KI and WT (top stars) and Y112/128F KI and WT (bottom stars) are depicted: ***p<0.001, **p=0.001-0.01, *p=0.01-0.05. Slopes of the graphic representation of CD62L expression over time were compared between WT and Y145F and WT and Y112/128F by nonlinear regression; p<0.0001 for both comparisons. Slopes of the graphic representation of IL7r<sub>α</sub> expression over time were compared between WT and Y145F and WT and Y112/128F by nonlinear regression; p=0.0185 and 0.0023 respectively.
Figure 3.11. SLP-76 KI LCMV-specific memory cells persist but show defective effector responses in vitro.

A. Absolute numbers of H2D<sup>b</sup>: GP33 reactive splenic CD8<sup>+</sup> T cells is shown ± SEM. Each point represents an individual mouse. B. Representative histograms show surface expression of CD62L, IL7<sub>α</sub>, CD27, CXCR3 and KLRG-1 surface expression H2D<sup>b</sup>:
GP33⁺, CD8⁺, CD44⁺ splenocytes from WT, Y145F and Y112/128F mice >day 70 p.i. are shown (representative of 4 independent experiments each with 3-5 mice per group). C. Splenocytes from WT, Y145F and Y112/128F mice day >70 p.i. were incubated ex vivo with GP33 peptide. Contour plots show TNFα and IFNγ expression on CD8⁺ gated lymphocytes (left panel). Numbers indicate the percent of CD8⁺, TNFα IFNγ double producers over the percent of total CD8⁺IFNγ producers. The MFI of IFNγ in all CD8⁺ cells producing IFNγ is shown (leftmost graph). Peptide-responsive splenocytes were quantified as a ratio of CD8⁺ IFNγ, TNFα double producers to CD8⁺ IFNγ single producers (center graph). Percent of CD8⁺ splenocytes that produced IL2 is shown (rightmost graph). All graphs show average±SEM, significant p-values, when present, comparing KI to WT are depicted as stars: ***p<0.001, **p=0.001-0.01, *p=0.01-0.05.
Figure 3.12. SLP-76 KI memory cells expand in response to rechallenge in vivo.

Equal numbers of CD8⁺ T cells from LCMV immune WT, Y145F and Y112/128F mice were transferred into congenic hosts. Host mice were subsequently infected with
LM:GP33 and analyzed 7-8 days later. A. Fold increase of donor-derived H2D\textsuperscript{b}:GP33-reactive cells in spleens from host mice that received WT, Y145F and Y112/128F donor cells after 7 days p.i. Each point represents an individual mouse; bars indicate average ±SEM. Data are pooled from three independent experiments each with 2-7 recipients per group. B. Percentage H2D\textsuperscript{b}:GP33-reactive cells of donor CD8\textsuperscript{+} T cells from spleens of day7-8 post-re-challenge mice (avg±SEM)(left panel). Percentage of donor CD8\textsuperscript{+} T cells that produced IFN\textgamma in response to GP33 peptide stimulation is graphed (avg±SEM)(right panel). Data are pooled from 4 independent experiments, each with 2-7 recipients per group. C. Splenocytes from recipient mice receiving WT, Y145F and Y112/128F on day 7 following LM:GP33 infection were stimulated with GP33 or PMA plus ionomycin. Representative contour plots depict TNF\alpha and IFN\gamma expression in CD45.2\textsuperscript{+}CD8\textsuperscript{+} lymphocytes that were stimulated with GP33 peptide (top panel). MFI of IFN\gamma in response to GP33 is shown (leftmost graph). Numbers indicate the percentage TNF\alpha IFN\gamma double producers over the percentage of total IFN\gamma producers within the CD45.2\textsuperscript{+}CD8\textsuperscript{+} gate. Peptide- or PMA/ionomycin-responsive splenocytes were quantified as of the frequency of CD45.2\textsuperscript{+}CD8\textsuperscript{+} IFN\gamma, producers that co-produced TNF\alpha (center graph), or IL2 (rightmost graph) (representative of 4 independent experiments each with 2-7 recipients per group). Significant p-values, when present, comparing KI to WT are indicated by asterisks: ***p<0.001, **p=0.001-0.01, *p=0.01-0.05.
Chapter 4: Discussion

Introduction

In the experiments described in this dissertation we have explored how the adaptor protein, SLP-76, transduces TCR signals into multiple downstream pathways. Using genomic knock-in mice expressing tyrosine to phenylalanine mutant SLP-76 proteins, we have shown that the SLP-76 tyrosines have an important role in the mobilization of Ca^{2+} and the activation of Erk and that this role is independent of their role in T cell thymic development. Signaling defects in SLP-76 KI T cells translate into functional defects both in vitro and in vivo. We therefore took advantage of the dampened TCR signals observed in the KI mice to test the role of TCR signal strength on CD4^{+} and CD8^{+} T cell differentiation and function. Below, we discuss possible biochemical mechanisms underlying the requirements for SLP-76 tyrosines, and TCR signals in general, for T cell differentiation and function.

New insights into the role of SLP-76 tyrosines in T cell signal transduction

Despite striking defects in TCR signal transduction, T cells from Y145F and Y112/128F SLP-76 KI mice showed no defect in TCR-induced subcellular localization of Vav1 and Itk or in SLP-76/Itk and SLP-76/Vav1 co-precipitation. Previous phospho-peptide mapping studies had predicted that these proteins inducibly associate with SLP-76 in a phosphorylation-dependent manner. Indeed, in our hands, J14 cells expressing WT or
Y-F mutant SLP-76 protein showed inducible SLP-76/Vav1 co-precipitation that was dependent on the presence and phosphorylation of tyrosine 112 and 128 (data not shown). We cannot account for the disparity between Jurkat cell lines and primary murine T cells in this case, but TCR signaling may not be normal in Jurkats because they lack PTEN and as a result have altered lipid metabolism (Shan et al., 2000). This results in constitutive association of Itk to the Jurkat cell membrane, which could very well alter the mechanics of early T cell signaling (Shan et al., 2000). Regardless of these differences, in primary cells the co-precipitation experiments in combination with microcluster experiments strongly suggest that the SLP-76 tyrosines are dispensable for TCR-induced localization of Vav1 and Itk. These data are consistent with a model in which SLP-76 has regulatory functions for Vav1 and Itk beyond simply binding them in a scaffolding manner.

**Model for TCR signal transduction via the proximal signaling complex**

We propose a model in which Itk and Vav1 are constitutively associated with SLP-76 in a multi-molecular complex via direct or tertiary interactions and, following TCR-induced phosphorylation of SLP-76, SH2/phosphotyrosine-dependent conformational changes within the complex alter the activity of Vav1 and Itk. This model is supported by studies that have shown that Itk and SLP-76 can associate directly through the SH3 domain of Itk (Bunnell et al., 2000) and that Itk kinase activity is dependent on its association with a phosphotyrosine (Pletneva et al., 2006) and on its interaction with SLP-76 (Bogin et al., 2007). Furthermore, Vav1 and SLP-76 could associate constitutively through tertiary interactions as they have both been proposed to interact directly with Itk and PLCγ1 (Labno et al., 2003; Reynolds et al., 2002). We can also speculate that lack of phosphorylation at Y112/128 may prevent a conformational change in Vav1 that is
necessary for its GEF activity and/or for access by its kinase. Lack of Vav1 phosphorylation may impact PLC\(\gamma\)1 activation by affecting the Vav1-regulated, PI3K-dependent activation of Itk or stabilization of the interaction between PLC\(\gamma\)1 and SLP-76 (Reynolds et al., 2002). However, the roles of Vav1 phosphorylation, adaptor function and GEF function for TCR signal propagation are just beginning to be teased apart. For example, recent studies using GEF-inactive Vav1 proteins have suggested that GEF function is important for the role of Vav1 in thymic selection but not for its role in Ca\(^{2+}\)- or actin-mediated pathways in mature T cells (Miletic et al., 2009; Saveliev et al., 2009).

While Vav1 phosphorylation was nearly absent in Y112/128F thymocytes, we did observe some TCR induced Vav1 phosphorylation in peripheral Y112/128F T cells suggesting the requirements for Vav1/SLP-76 interactions may be different in these two cell types. It is also possible that only cells capable of Vav1 phosphorylation are able to undergo positive selection and enter the periphery and that our observations are the result of compensatory mechanisms during selection. TCR-induced Vav1 phosphorylation needs to be examined in cKI mice to address this possibility. Further studies are also required in thymocytes and T cells to determine if tyrosines 112 and 128 contribute to Vav1 GEF activity or adaptor activity or both.

**SLP-76 tyrosine-dependent and -independent functions for Vav1 and Itk**

Based on our data, we speculate that Vav1 and Itk may depend on SLP-76 tyrosines for their enzymatic and/or phosphorylation-mediated functions; it is also possible that they have SLP-76 tyrosine-independent functions. Although Y145F KI mice show a strikingly similar phenotype to Itk-/- deficient mice (suggestive that most Itk functions are dependent on Y145), Itk is required in a kinase-independent manner to recruit Vav1 to SLP-76 and the T cell/APC contact site, a requirement clearly not affected by the loss of
Y145 (Dombroski et al., 2005). It is unclear whether Vav1 GEF or adaptor functions or both are dependent on SLP-76 tyrosines. TCR-mediated actin polymerization is Vav1 GEF independent and phalloidin-staining intensity of activated thymocytes did reveal a defect in actin polymerization that was similar in thymocytes from both strains of KI mice (Jordan et al., 2008). Taken together, these data suggest that SLP-76 tyrosines contribute to some non-enzymatic Vav1 functions (Miletic et al., 2009; Saveliev et al., 2009). Vav1 GEF-dependent functions in mature T cells include Rac1 activation, Akt activation and activation of integrins (Saveliev et al., 2009). We have yet to measure these processes in the SLP-76 KI T cells. Vav1 is also required, in an unknown manner, for optimal TCR-induced SLP-76 phosphorylation and, in the absence of Y112/128 we fail to appreciate any SLP-76 phosphorylation, suggesting that a Vav1/Y112/128 interaction is required for phosphorylation of tyrosine 145 (Reynolds et al., 2002). This was confirmed using pY145 specific antibody (that is no longer available) in Jurkat cell lines (Jordan et al., 2006). In addition to the described PI3K-dependent pathway, Vav1 might contribute to Itk activation simply through its requirement for Y145 phosphorylation. Itk kinase assays are underway to determine the role of SLP-76 tyrosines in the enzymatic activity of Itk. Furthermore, we have not yet addressed the role of Nck in SLP-76 tyrosine-mediated signaling; Nck can bind Y112 and Y128 and is essential for TCR-induced cytoskeletal rearrangements (Lettau et al., 2010; Zeng et al., 2003).

If most Itk functions are dependent on Y145F of SLP-76 then it is not surprising that the Th2 and Th17 defects observed in Y145F KI mice are very similar to those reported for Itk-/- mice (Au-Yeung et al., 2006; Gomez-Rodriguez et al., 2009). Vav1-deficient T cells also display a defect in IL4 production but they show enhanced IFNγ production following both Th2 and Th1 skewing conditions, a phenotype that was not
observed in Y112/128F KI mice (Tanaka et al., 2005). Given the differences between Vav1-/- and Y112/128F Th1 and Th2 phenotypes, it is possible that Vav1 has SLP-76 tyrosine-independent functions in Th differentiation and/or function. While Itk-/- and Vav1-/- T cells display differences in their ability to differentiate into Th1 and Th2 lineages and exert effector cytokines, we have not yet observed any differences between Y145F and Y112/128F KI in these processes. Thus it is further possible that the role of Vav1 in Th differentiation is mostly independent of SLP-76 tyrosines and that the defects observed in Y112/128F KI T cell differentiation can be attributed to defects in phosphorylation of Y145 and subsequent Itk activation. Additionally, Vav1 has been implicated in Ca\(^{2+}\)-independent manner for the regulation of IL4 production downstream of CD28. Therefore, differences observed in Th differentiation between Y112/128F KI and Vav1-/- T cells could be accounted for if Vav1 is required in a SLP-76-independent manner for CD28-mediated signals (Fang and Koretzky, 1999; Hehner et al., 2000; Raab et al., 2001).

**Hierarchical and non-hierarchical requirements for SLP-76 tyrosines**

Our early studies in Th differentiation have revealed that Y145 and Y112/128 signals are equally required for Th effector functions, including Th2 and IL17 production. This is despite the hierarchical requirement for the tyrosines for proliferation, CD69 upregulation and phosphorylation of PLC\(\gamma\)1 and ERK. Conversely, thorough examination of CD8\(^{+}\) differentiation and function in the SLP-76 KI mice revealed graded defects between Y145F and Y112/128F T cell effector responses that are consistent with this hierarchy. The graded defects in CD8 effector function and non-graded defects in CD4 effector function could reflect either differences in signaling thresholds downstream of TCR signals or differences in requirements for distinct pathways. For example, Erk and
PLCγ1 phosphorylation are more defective in the Y145F KI T cells compared to Y112/128F KI T cells but TCR-induced Ca²⁺ mobilization was equally defective in T cells from both strains of mutant mice. Indeed, levels of Ca²⁺ mobilization have been shown to play a key role in IL-4 and IL-17 responses through activation of NFAT pathways (Fowell et al., 1999; Gomez-Rodriguez et al., 2009). Perhaps CD8⁺ fate choices are more dependent on TCR signaling pathways affected by the hierarchy.

Lineage-specific requirements for SLP-76 tyrosine signals

One of the most intriguing findings in these and other studies in the lab has been the differential requirements for SLP-76 tyrosines in different cell lineages. For example, in platelets, co expression of both SLP-76 mutants does not rescue the defects observed in the single mutants as happens with T cells (Bezman et al., 2008). These differences might be reflective of differences in the stimulating receptor involved, in particular the number ITAMs involved and the predicted complex size (TCR signaling utilizes ten ITAMs versus two used by the GPVI receptor). However, we have also noticed differences in the requirements for SLP-76 tyrosines between different T cell lineages. Most apparent is the SLP-76 tyrosine-independent, TCR-induced production of IL17a by a subset of CD44⁺CD4⁺ T cells, which is in contrast to the near absent production of IL17a in response to TCR stimulation by Th17 skewed naive KI T cells. Further studies are required to determine if the CD44⁺ T cells use a Ca²⁺-independent pathway. Our LCMV studies revealed subtler, but equally intriguing, differences in SLP-76 tyrosine dependence between different CD8⁺ T cell subsets. For example, at day 8 p.i. the ability of Y112/128F effector cells to produce multiple cytokines in response to GP33 peptide was not appreciably different when compared to WT effector cells. However, Y112/128F memory and secondary effector cells both showed significantly altered cytokine
responses to peptide re-stimulation and this was most striking in the secondary effector cells. This may be reflective of the role of TCR signaling machinery in functional avidity maturation such that the TCR threshold requirements for effector function change as CD8 T cells differentiate (Slifka and Whitton, 2001). The KI T cells became less sensitive to TCR signals where functional avidity maturation predicts that T cells become more sensitive to TCR signals. It is possible that in KI T cells the mechanisms required for functional maturation are dysregulated. Despite the increased frequencies of more “mature” memory phenotype cells (as measured by expression of surface markers) in the KI memory pools, their response to rechallenge was not enhanced. In all experiments KI memory cells proliferated to an equal or lesser extent and we therefore cannot rule out a proliferative defect in secondary expansion. Thus it is possible that TCR signals through Y145F SLP-76 are sufficient for optimal expansion in primary effector cells but not in memory cells. Differences in TCR signaling machinery between CD8 T cell subsets is supported by studies that have shown that resting memory CD8 T cells express higher levels of Lck and phosphorylated LAT when compared to naive cells or memory cells (Bachmann, 1999 #36; Kersh, 2003 #5; Slifka, 2001 #4; Slifka, 2001 #4). Furthermore, studies in CD4 T cells have shown differential expression of SLP-76 and different usage of key TCR-signaling molecules in memory, effector and naïve cells (Hussain et al., 2002).

**Effector versus Memory**

One of the most important findings in the studies presented in this dissertation is that TCR signals can direct the balance of CD8$^+$ effector versus memory differentiation independent of the magnitude of the primary response. Previous models of decreased TCR stimulation have suggested that higher TCR signals favor terminal differentiation
over memory differentiation, but in these studies the magnitude and duration of the response was decreased (Joshi and Kaech, 2008; Zehn et al., 2009). Further studies are required to determine if, over time post infection, as terminally differentiated T cells continue to be lost from the antigen-specific pool, the SLP-76 KI mice have a larger population of memory cells when compared to WT mice. Indeed, in our studies at 70-110 days post infection there is a trend towards a larger population of antigen-specific cells in the spleen of Y145F KI mice, but this has not yet reached significance. If this trend continues, our data would continue to support the “decreasing potential” model of CD8⁺ differentiation such that all CD8⁺ T cells undergo a memory precursor stage and strong or successive TCR stimulation is required for terminal differentiation. This is in contrast to the “dedifferentiating model” in which all cells first go through a terminal differentiation effector phase and then, after antigen clearance a few cells acquire memory potential and undergo further functional maturation (Joshi and Kaech, 2008).

Asymmetric division has also been suggested to play a role in memory versus effector fates (Chang et al., 2007). Furthermore, unpublished data have shown that Y145F KI T cells fail to undergo asymmetric division (Chang et al. unpublished data). How this defect might contribute to fate decisions is unclear but in the same studies Y145F KI T cells showed a defect in the asymmetric division of T-bet and the proteosome, which correlated with a failure to appropriately downregulate T-bet. As high T-bet levels favor terminal differentiation over memory differentiation, one might expect the Y145F KI mice to favor terminal differentiation, but as we have shown, the opposite is true; therefore additional factors must be playing a role in our observed phenotype (Intlekofer et al., 2005). Further studies on how of Tbet asymmetry, Tbet protein levels, and asymmetric division of proteosomal components impact the CD8⁺ T cell memory program are required to gain a full understanding of our findings.
TCR signal strength could affect the balance of memory and terminal differentiation not only through direct transcriptional mechanisms but also through indirect mechanisms. Dampened TCR signals could result in the false perception of truncated antigen exposure, such that even if the kinetics of antigen clearance are similar in KI and WT mice, KI cells may not “see” very low levels of antigen during the later phases of the primary response. Indeed, T cell memory differentiation is hastened if the level of antigen or the duration of antigen exposure is decreased (Wherry et al., 2003b). Finally, as alluded to previously, specific signals downstream of SLP-76 may play a role in the skewed differentiation of KI T cells, including the Itk-dependent regulation of T-bet (Miller et al., 2004). It is still unknown if Y112 and Y128 play a role in the regulation of T-bet activity. Furthermore, the regulation of T-bet expression has been shown to be controlled via mTor signals downstream of the IL12r and the role of TCR signals in this process has not been defined (Joshi et al., 2007; Rao et al., 2010).

Conclusions

Our studies in CD4+ and CD8+ differentiation have caveats that need to be addressed but they also raise many intriguing questions. First, future studies in the LCMV model are required to confirm a T cell intrinsic role for SLP-76 tyrosines in T cell differentiation. SLP-76 is expressed in hematopoietic lineages including dendritic cells and other innate cells that could contribute to the inflammatory milieu, which is a potent regulator of T cell differentiation. Furthermore, we have already acknowledged a functional defect in CD4+ T cells expressing mutant SLP-76 proteins. We must therefore consider altered help as a potential confounding factor to our studies, although converse to our observations in SLP-76 KI mice, helpless CD8+ T cells preferentially undergo terminal differentiation at the expense of memory differentiation (Janssen et al., 2005).
These concerns can be addressed easily with adoptive transfer experiments, and ideally with cKI cells. Of more relevance to understanding the mechanisms of memory is our finding that different CD8+ T cell subsets may have different TCR sensitivities. Uncovering the mechanisms that induce functional avidity maturation and the mechanisms that support heightened TCR responsiveness is an important future direction. Further studies are also required to characterize the CD44hi natural Th17 cells. In particular, it will be important to ask how they can effectively produce IL17a following TCR ligation despite mutation of a critical TCR signaling molecule.

Understanding the role of distinct TCR signals and TCR signal strength in regulating different types of T cell responses is of importance when designing immunomodulating therapies, as aberrant or misguided immune responses to pathogens could have catastrophic consequences (Intlekofer et al., 2005). Here we have shown that TCR signals can affect the balance of effector versus memory cells, an important concept to consider for vaccine design. Furthermore we have shown that TCR signals strong enough to induce effector differentiation may not be strong enough to support effector function.

Chapter 5: Materials and Methods

Mice

SLP-76 KI mice were generated as described by Martha S. Jordan (Jordan et al., 2008) and were backcrossed to C57BL/6 mice 2-5 times for thymic studies and 5-10 times for most peripheral studies. WT mice were either KI littermate controls or C57BL/6 mice purchased from Jackson Laboratories. cKI and cSLP+/- mice were backcrossed to C57B/L6 mice 5-6 times. Mice bearing floxed copies of WT SLP-76 were generated as
previously described (Maltzman et al., 2005). The Cre\textsuperscript{T2} transgenic mice and the ROSA-YFP reporter were kind gifts from Dr. E. Brown (University of Pennsylvania) (Ruzankina et al., 2007) and Dr. F. Constantini (Columbia University) respectively. Itk-deficient thymi were the kind gift of Pamela Schwartzberg. Animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and all experiments were carried out following the guidelines of the University of Pennsylvania IACUC.

**Tamoxifen Treatment**

Tamoxifen (Sigma) was prepared by mixing in 1g/mL of ethanol. This mixture was then diluted to 20mg/mL with corn oil and dissolved by incubating at 37°C for several hours with constant mixing. To induce deletion of WT SLP-76, cKI and cSLP-76\textsuperscript{+/−} were treated for 5 days with 200µg tamoxifen/g bodyweight for 5 consecutive days by oral gavage. Mice were bled 3 days following the last day of treatment and successful treatment was confirmed by the presence of YFP+ cells.

**Flow Cytometry**

Splenocytes were harvested and following red blood cell lysis with BioWhittaker ACK lysis buffer (Lonza) stained with antibodies in FACS buffer (PBS containing 2% FBS and 0.002% azide) for 30 min. Thymocytes were prepared in the same manner but without red blood cell lysis. Peripheral blood was collected into 4% Na citrate, and PBLs were isolated on a density gradient with Ficoll-paque PLUS (GE Healthcare). Intracellular staining was performed using BD Cytfix/Cytoperm kits (BD Biosciences) according to the manufacturer’s instructions. Samples were collected on a FACSCalibur or LSRII (BD Biosciences), and analysis was performed using FlowJo software (Tree Star).
Fractionated thymic populations, naïve and activated phenotype CD4⁺ T cell populations, and YFP⁺ peripheral T cells were sorted on a BD FACSAria where indicated. H2Db tetramers were a gift of E. John Wherry (Wistar Institute, Philadelphia PA) (Wherry et al., 2003b). Antibodies for flow cytometry were purchased from commercial sources. BD PharMingen: CD4 (RM4-4), CD4 (RM4-5), CD8α, CD8β, IFNγ, CD122, CD44, CD25, CD5, CD3, CD69, IL-2, CD122, IL4, CD5, TCRγδ. Ebiosciences: KLRG-1, CD27, CD127, CD44, TCRβ, IL17a, RORγT. Biolegend: CXCR3, TNFα. Invitrogen or Caltag: CD62L.

**Calcium mobilization**

Lymph node cells were loaded with in Indo-1 (Molecular Probes) and stained with biotinylated anti-CD3 (2C11), biotinylated anti-CD4 (RM4-4), biotinylated anti-CD8β, FITC anti-CD44, PE anti-CD8α and PercypCy5.5 anti-CD4 (RM4-5) (all from BD Biosciences) in the presence of 4mM probenecid at 30°C for 30 minutes. Cells were washed, resuspended in serum-free RPMI and warmed to 37°C. Baseline Ca²⁺ levels were measured for 30 seconds before addition of 12.5µg/ml streptavidin (Molecular Probes). Ionomycin (1µg) was added 30 seconds before completion of the collection as a control. Ca²⁺ levels in the cytoplasm were measured as the ratio of the fluorescence of Ca²⁺-bound to Ca²⁺-unbound Indo-1. Data was collected on an LSRI (BD Biosciences) and analyzed using FlowJo software (TreeStar).

**CD69 upregulation**

Splenocytes were incubated at 37°C overnight in the presence of 0.1µg/ml of soluble anti-CD3 (2C11) and then analyzed by flow cytometry.
CFSE/BRSE proliferation assays

Splenocytes were loaded with 10µM Carboxyfluorescein succinimidy ester (CFSE) or 2.5µM Bodipy Red succinimidyl ester (BRSE) (both from Molecular Probe) and incubated at 37°C for 72 hours in the presence of 0.01µg/ml soluble anti-CD3 (2C11) and then analyzed by flow cytometry.

Immunoprecipitations and Western blots

Splenocytes and lymph node cells were isolated using Thy1.2 positive selection MACs MicroBeads (Miltenyi) according to the manufacturer’s instructions. For conditional mice, splenocytes were negatively selected using B220 MACs MicroBeads (Miltenyi) then sorted for YFP+Thy1.2+ expression using a FACS Aria (BD Biosciences). For thymic analysis, bulk thymocytes were used except for the experiment in Figure 2.3F in which DP thymocytes were sorted using a FACS Aria. Following isolation, cells were rested at 37°C in IMDM (no serum) for 30 to 60 min, after which time they were stimulated with 5µg/ml of anti-CD3 (500A2; Pharmingen) at 37°C for various amounts of time. DP thymocytes were first incubated with biotinylated anti-CD4 and anti-CD3 (2C11; BD Pharmingen) for 30 minutes on ice, washed and then cross-linked with 25µg/ml streptavidin at 37°C for various amounts of time. Ice-cold PBS was added to stop the stimulations. Cells were pelleted and lysed: 1% NP-40, 150mM NaCl, 50mM Tris HCl (pH 7.5), 1mM Na2VO4, 5mM NaF, 1mM PMSF, Protease Inhibitor Cocktail (Sigma), 5mM Na pyrophosphate with or without 10% glycerol. For immunoprecipitations, lysates were incubated, rotating at 4°, with either anti-SLP-76 (eBioscience) or anti-Vav1 (Cell Signaling) for 2 to 3 h after which 50µl of anti-mouse or anti-rabbit Trueblot beads (eBioscience) were added and the mixture was incubated for an additional hour. Beads
were washed 4 times with lysis buffer and resuspended in 50μl 2X Laemmli’s reducing buffer (Boston Bioproducts) or NuPAGE LDS sample buffer (Invitrogen), boiled, and analyzed by Western blot. For co-immunoprecipitations, SLP-76 was immunoprecipitated using a His-myc-tagged human Fab against amino acids 1-120 of murine SLP-76 generated by Antibodies by Design. This antibody was conjugated to magnetic Talon Dyna beads according to manufacture’s instructions (Invitrogen). Thymocyte lysates were incubated with conjugated beads for 2 h, washed four times and resuspended in 2x NuPage sample buffer. Samples were analyzed by Western blot using antibodies to Vav1 (Cell Signaling), 4G10 (Upstate), and SLP-76 (eBioscience). Itk was immunoprecipitated from thymocyte lysates using a rabbit polyclonal anti-Itk antibody (Upstate). Western blots were probed for Itk using a mouse anti-Itk antibody (Chemicon) and anti-SLP-76 antibody (eBioscience). Western blots were probed with the following antibodies: 4G10 anti-phospho-tyrosine (Upstate), phospho-PLCγ-1 (Tyr783), PLCγ-1, phospho-p44/42 MAPK (Thr202/tyr202), phospho-LAT (Tyr191), Vav1, Itk (all from Cell Signaling), ERK2 and Actin (Santa Cruz), phospho-SLP-76 (Tyr128) (BD biosciences), SLP-76 (e-biosciences).

Constructs and retroviral transduction of T cells

GFP-tagged hVav1 was a gift of Wojciech Swat (Washington University, St. Louis, MI) and GFP-tagged Itk KD was a gift of Pamela Schwartzberg (NIH, Bethesda, MD). GFP constructs were ligated into a MigR-1 retroviral vector (with GFP removed) a gift of Warren Pear (University of Pennsylvania, Philadelphia, PA). Peripheral T cells were induced to proliferate with 20 μg of phorbol myristate acetate (PMA)/ml, 200 μg of ionomycin/ml and 40 U of interleukin-2/ml overnight and then infected with retroviral supernatant and 0.8 μg Polybrene/ml (Sigma) by spinning at 2,000 rpm for 90 min on
two sequential days. They were then cultured in Iscove’s modification of Dulbecco’s medium (IMDM) with 10% fetal bovine serum, and analyzed 5 to 7 days later.

**Immunofluorescence and cell imaging**

Transfected T cells were washed and resuspended in serum-free IMDM and dropped onto pre-warmed coverslips coated with Poly-L-Lysine (unstimulated) or 10µg/ml anti-CD3 (2C11) and anti-CD28 (both from BD Pharmingen) and incubated at 37°C for 10 minutes. Excess media was aspirated and, at room temperature, cells were fixed in 2% paraformaldehyde (PFA; Electron Microscopy Sciences) in PBS for 20 minutes, quenched in 50mM NH₄Cl in PBS for 1 minute, permeabized with 0.3% Triton-100 (Sigma) in PBS, washed with PBS and incubated in blocking solution (0.01% Saponin, 0.25% Fish Skin Gelatin, 0.02% Sodium Azide (NaN₃) (all from Sigma) for 20 minutes. Cells were then stained with Phalloidin-Alexa Fluor 594 (Molecular Probes) for 1 hour, washed and mounted onto slides. Imaging was performed on a Perkin-Elmer UltraVIEW confocal spinning-disk scanner (Yokogawa) attached to a Nikon TE-300 inverted microscope equipped with a 1.4-numerical-aperture 100× objective and a z-axis controller (Physik Instruments). Samples were excited using 488-nm and 568-nm laser lines with a 488/568 red-green-blue dichroic mirror. A Hamamatsu Orca-ER camera was used to collect images. Image analysis was performed using IP Labs software (Biovision).

**T helper skewing assays**

Splenocytes were sorted for naïve (CD62L<sup>hi</sup>, CD44<sup>lo</sup>) or activated (CD62L<sup>lo</sup>, CD44<sup>hi</sup>) CD4<sup>+</sup> on a FACSaria (BD Biosciences). For conditional mice, populations were also sorted on YFP<sup>+</sup> expression. Cells were then cultured in 96 well plates pre-coated with 1
µg/ml anti-CD3 (2C11) and 5 µg/ml anti-CD28 (both from BD Pharmingen) and appropriate cytokines for 5, 6 and 3 days for Th1, Th2 and Th17 skewing conditions respectively. Skewing cytokines were as follows: 50U/ml rhuIL-12 (R&D Systems) and 10µg/ml anti-IL4 (Bioexpress) for Th1 skewing; 2000U/ml rmIL4 (R&D Systems), 10µg/ml anti-IL-12 and 10µg/ml anti-IFNγ (gift of Steve Reiner, University of Pennsylvania, Philadelphia) for Th2 skewing; 1ng/ml rhuTGFβ (ebiosciences), 10ng/ml rmIL6 (ebiosciences), 10ng/ml rmIL23 (ebioscineces), 10µg/ml anti-IL4 and 10µg/ml anti-IFNγ for Th17 skewing. Some experiments were performed with 40U/ml rhuIL-2 with no observable differences in outcome.

*T helper restimulation assays*

After culture T cells were stimulated in 96 well plates either pre-coated with 1µg/ml anti-CD3 (2C11) and 5µg/ml anti-CD28 (both from BD Pharmingen) or with soluble 500ng/ml ionomycin and 50ng/ml PMA in the presence of 1000µg/ml brefeldin A for 5 hours and then analyzed by intracellular cytokine staining.

*Infections*

Mice were infected intravenously with $2 \times 10^5$ CFU of LCMV Armstrong and sacrificed 8 days later for analysis of primary responses or >70 days later for memory responses. For rechallenge experiments, splenocytes from mice >70 days post-infection (p.i.) were pooled according to sex and genotype, and CD8+ T cells were purified using a MACs CD8+ T Cell Isolation Kit (Miltenyi Biotec). An aliquot of purified cells was analyzed by flow cytometry and the rest of the cells were equilibrated such that 7500 H2D^b: GP33 cells were transferred intravenously into each sex-matched congenic host. The following day, congenic hosts were infected with $4 \times 10^5$ CFU of LM: GP33 intravenously.
**LCMV peptide restimulation assays**

$1 \times 10^6$ splenocytes were left unstimulated or stimulated with 200ng/ml of GP33, GP276 or NP396 peptide (Genscript) or 500ng/ml ionomycin and 50ng/ml PMA in the presence of 1000ug/ml brefeldin A for 5 hours and then analyzed by intracellular cytokine staining.

**Statistical analyses**

Where indicated, $p$ values were determined by a two-tailed unpaired Student’s t test using Prism software (Graphpad). All graphs show average ±SEM. Slopes and corresponding $p$ values for CD62L and IL7rα expression were determined using nonlinear regression modeling using Prism.
Works Cited


SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. The Journal of biological chemistry 271, 19641-19644.


Hudrisier, D., Oldstone, M.B., and Gairin, J.E. (1997). The signal sequence of lymphocytic choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2D(b) and H-2K(b) molecules. Virology 234, 62-73.


122


