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Th2 Specific Immunity and Function of Peripheral T-Cells is Regulated by the p56Lck SH3 Domain

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TH2 SPECIFIC IMMUNITY AND FUNCTION OF PERIPHERAL T-CELLS IS REGULATED BY THE P56LCK SH3 DOMAIN

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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DEDICATION

I dedicate this dissertation work to my parents. It is not every child who can say of their parents that there will never be two more inspiring role models and lovers of life; who have taught me to see and love all the joy and beauty that God's great world has to offer.

Thank you for sharing your faith with me- that I may walk straight in the eyes of God, and for helping me learn that I am worthy of being held accountable to all that He has prescribed for me.

In my whole life there was never a moment when I did not know without absolute certainty that I was loved whole-heartedly; and that I was loved for nothing more than being myself.

It is a beauty not well known,
that breathes in darkest slumber and brightest day,
How magnificent is love's true sight
And for the dreamer, shall never stray.

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reach my dreams. With all my heart, Thank You. –I look forward to the next Elephant, and even the next Apostolina.

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LIST OF ABBREVIATIONS

-/-	Homozygous deletion of a gene
2C11	Anti-CD3 antibody
Ab	Antibody
Ag	Antigen
AP	Alkaline Phosphatase
APC	Antigen Presenting Cell
B220	mAb recognizing the murine CD45R; murine B cell marker
C57BL/6	Inbred mouse strain
CaMKII	Ca^{2+}/calmodulin dependent protein kinase
CD	Clusters of differentiation; cell surface marker identification
CD25	Clusters of differentiation 25; the IL-2 receptor alpha chain
CD28	Clusters of differentiation 28; a T-cell co-stimulatory molecule
CD3	Clusters of differentiation 3; part of the TCR complex
CD69	Clusters of differentiation 69; a T-cell activation marker
CD40L	CD40 Ligand
CFSE	Carboxyfluorescein diacetate succinimidyl ester

CPM	Counts per minute
CS	Circumsporozoite protein; a surface protein of sporozoites used for adhearance
DC	Dendritic cell
Dlgh1	Disks large homolog 1
DLN	Draining lymph node
DNP	Dinitrophenol
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal-rgulated kinase
ES cell	Embryonic Stem cell
F8	Eighth generation offspring
FACS	Fluorescent activated cell sorter
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
HEPES	Buffering agent
HepG2	Human hepatocellular carcinoma cell line
IFN	Interferon
IL	Interleukin
IL-2	Interleukin 2; a T-cell secreted cytokine
Ig	Immunoglobulin
i.p.	Intraperitoneal

ITAM	Immunoreceptor tyrosine-based activation motif
KIR	Killer-cell immunoglobulin like receptor
KLH	Keyhole limpet hemocyanin
L3	Life stage 3
LAT	Linker for activation of T-cells
Lck	Lymphocyte cell kinase
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHC-II	Major histocompatibility complex class II
OT-1	TCR transgenic CD8+ T-cell specific for SIINFEKL peptide
OT-2	TCR transgenic CD4+ T-cell specific for ova peptide
Ova	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PI	Phosphoinositol

PLC-γ1	Phospholipase C gamma 1
PMA	Phorbol myristate acetate
pNPP	p-nitropenyl phosphate
PTK	Protein tyrosine kinase
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute (as in the cell growth media)
RT-PCR	Reverse transcriptase polymerase chain reaction
SGP	Salivary gland pairs
SH2	Src homology 2
SH3	Src homology 3
SHP-2	Src homology 2 domain-containing phosphatase 2
SLP-76	Src homology 2 domain containing leukocyte-specific phosphoprotein of 76 kDa
SP	Single positive
Spl	Spleen
TCR	T-cell antigen receptor
Tg	Transgenic
Th1	T helper type 1
Th2	T helper type 2
W97A	The denotation for a pont mutation from tryptophan to alanine at position 97

WT	Wild-type
ZAP-70	Zeta-chain-associated protein kinase 70

ABSTRACT

"TH2 SPECIFIC IMMUNITY AND FUNCTION OF PERIPHERAL T-CELLS IS REGULATED BY THE P56LCK SH3 DOMAIN"

By Margaret Ellen M^{c}Coy

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Proper T-cell activation and effector function are essential for effective immunity. T-cell antigen receptor (TCR) signals are known to regulate the outcome of differentiation, but the mechanisms remain unclear. Recent work from our lab demonstrates that the Src family protein tyrosine kinase, p56Lck, is able to specifically link TCR signals to activation of the Mitogen Activated Protein Kinase (MAPK) pathway through the function of its SH3 domain. The MAPK pathway is known to be involved in T-cell activation downstream of TCR ligation and has previously been implicated in T-helper type 2 (Th2) effector function. We have utilized an Lck SH3 mutant knock-in mouse line (Lck W97A) to investigate the potential role of this regulatory signaling mechanism in determining T-lymphocyte activation and effector function. Our results demonstrate that the Lck SH3

domain function is required for normal activation of T-lymphocytes following TCR stimulation as indicated by significantly reduced proliferation, IL-2 production, and CD69 induction in Lck W97A T-cells. Biochemical studies confirm that activation of the MAPK pathway is selectively altered in Lck W97A T-cells as P-ERK1/2 induction is significantly reduced but phospho-PLCγ1 induction and calcium mobilization is unaffected. *In vivo* experiments demonstrate a specific and significantly impaired Th2 immunity in Lck W97A mice, with reduced serum levels of IgG1, IgE and IL-4 following immunization with DNP-KLH, or infection with the helminth *Nippostrongylus brasiliensis.* Th1 immunity does not appear differentially regulated in Lck W97A mice as serum levels of IgG3 and IgG2b are similar to WT following immunization with DNP-KLH, as well as serum levels of IFN-γ1 following immunization with heat-killed *Brucella abortus. In vitro* differentiation studies confirm that Lck W97A T-lymphocytes are able to be directed to the Th2 phenotype, as indicated by intracellular staining for IL-4, with significantly increased levels of IFN-γ under Th2 differentiating conditions compared to WT. These data indicate that the Lck SH3 domain regulates activation of T-lymphocytes by affecting MAPK pathway induction and demonstrate a novel and critical role for Lck in the regulation of Th2-type immunity.

The Lck SH3 domain has also been implicated in the pathogenesis of *Plasmodium*, the causative agent of malaria. The role of the mosquito vector on malaria pathogenesis is not well understood. Initial studies examining the role of vector salivary gland proteins on cells of the innate immune system indicate that *Anopheles stephensi* saliva is able to enhance macrophage activation and phagocytosis as well as enhance macrophage Ag-presentation to T-lymphocytes in an *in vitro* model.

INTRODUCTION

I. Innate and Adaptive Immunity.

The body's immune system is an intricate collaboration between highly adapted and specialized cells working together to mitigate and suppress invasion by pathogens. It is the exquisite partnership between these immune cells and responses of the innate and adaptive branches of the immune system that enable efficient and protective host immunity. The innate immune system is part of the host's first-line of defense against infection and includes antigen presenting cells (APCs) such as macrophages, dendritic cells and mast cells, which target infectious agents in a non-specific manner. These cells are able to process and present antigenic peptides derived from extra- and intracellular pathogens on the cell surface in the context of major histocompatibility complexes (MHC) in order to engage and prime cells of the adaptive immune system, such as T- and B-lymphocytes. The purpose of adaptive immunity is to provide specific and robust host protection from acute and recurring infections. T-cells are able to directly kill infected cells expressing foreign peptides on their surface by releasing cytotoxins, and function to assist the priming and activation of B-cells to produce antigen-specific antibodies. T-cells govern the cellular immune responses of adaptive immunity, whereas the humoral responses are mainly

controlled by B-cells and their secreted immunoglobulin (Ig) isotypes. B-lymphocytes also have membrane-bound Ig that can bind bacterial and viral antigenic epitopes on cell surfaces, further initiating B-cell activation and enhancing the immune responses to infection. Once activated, B-cells can differentiate into specialized cells termed plasma cells, which are able to secrete Ig that functions to tag foreign substances for neutralization or phagocytosis. Frequently, the Ig-targeted antigenic epitope is part of a functionally critical protein on the foreign agent required for invasion of the host, such as molecules required for adhesion to target cells (Rhoades). Binding to this type of epitope is termed neutralization.

A. B- and T-Cell Subsets. The tailored Ag-specific response of adaptive immunity relies, in part, on the proper development and activation of naïve lymphocytes. Although T-and B-cells both originate in the bone marrow as hematopoietic stem cells, they have divergent developmental programs, and distinct functions with regard to immunity. Whereas B-cells complete their development in the bone marrow, T-cells complete their development and maturation in the thymus. The designation of "B"- and "T"-lymphocytes is of note here, as T-cells are named as a result of the organ in which they mature, the Thymus. However, B-cells are named due to the organ in which they were originally discovered to develop, the avian-specific Bursa of Fabricius.

Following both naïve T-cell exodus from the thymus, and B-cell migration from the bone marrow, the naïve lymphocytes interact with cells of the innate immune system while circulating through the lymph organs in search of an antigenic match precursory to activation. T-cells are able to interact and engage APCs through surveillance of the

lymphatic system and secondary lymphoid organs, such as the spleen and lymph nodes. APC priming and activation of naïve T-lymphocytes is facilitated by ligation of the primary T-cell activating receptor, the T-cell antigen receptor (TCR), with MHC/peptide complexes on the APC surface. MHC class I and class II molecules, with cognate peptide, bind the class I binding protein CD8 or Class II binding protein CD4, respectively, on the surface of T-lymphocytes. This ligation induces and assists activation signals through the TCR, resulting in clonal expansion of the activated T-cell, cytokine secretion, and acquisition of effector function. The ability of T-cells to properly differentiate between self and foreign antigenic (Ag) peptides in the context of MHC is critical for understanding the processes leading to proper T-cell immunity and the improper response to self antigens leading to autoimmunity (Deng).

Once a T-cell has been activated and gained effector function, it has several possible fates. Either the cell can perform its primary immune function immediately, or it can segregate into a separate and long-lived population of “memory” cells. Memory cells are a reservoir of primed immune sentinel cells that are available to respond rapidly and with robust function upon secondary infection.

CD4+ and CD8+ T-cells comprise the two major subgroups within the T-lymphocyte population. In addition to their selective ability to bind MHC class II molecules, CD4+ T-helper (Th) cells are able to secrete various immune cytokines as well as bind and activate B-cells. Th-activated B-lymphocytes produce Ag-specific antibodies, the primary component of the humoral immune response. The other major group of T-lymphocytes, the CD8+ cytotoxic T-cells, recognize MHC class I molecules that have

bound cytosolic peptide (ie; viral), and, once primed, are able to cytolytically kill infected cells through the release of cytotoxins or through Fas-FasL interactions. This Ag-specific cellular immune response is important for the control and clearance of virally infected immune cells.

Another important T-lymphocyte subgroup is the regulatory T-cells (Treg). T-regs are a smaller but very important T-cell subgroup that acts to regulate T-cell mediated immunity as well as control auto-reactive T-cells. T-reg development in the thymus is regulated by the activity of the Foxp3 transcription factor, and express $CD4^{+}CD25^{+}$ on their cell surface. Although the mechanisms of T-reg suppression are the focus of intense study, it is known that they are able to secrete immunosuppressive TGF-β and IL-10 to control the scope of immune responses.

II. Immunity and the Activation and Function of T-Lymphocytes: A Role for the Src Family PTK, Lck.

In order to fully understand and manipulate the activity of T-lymphocytes, it is critical to examine the mechanisms that govern their development, activation, differentiation, and effector function. The Src family of non-receptor protein tyrosine kinases (PTKs) is of imminent value to this end, as they are involved in, and critical for, many of these processes (Thomas).

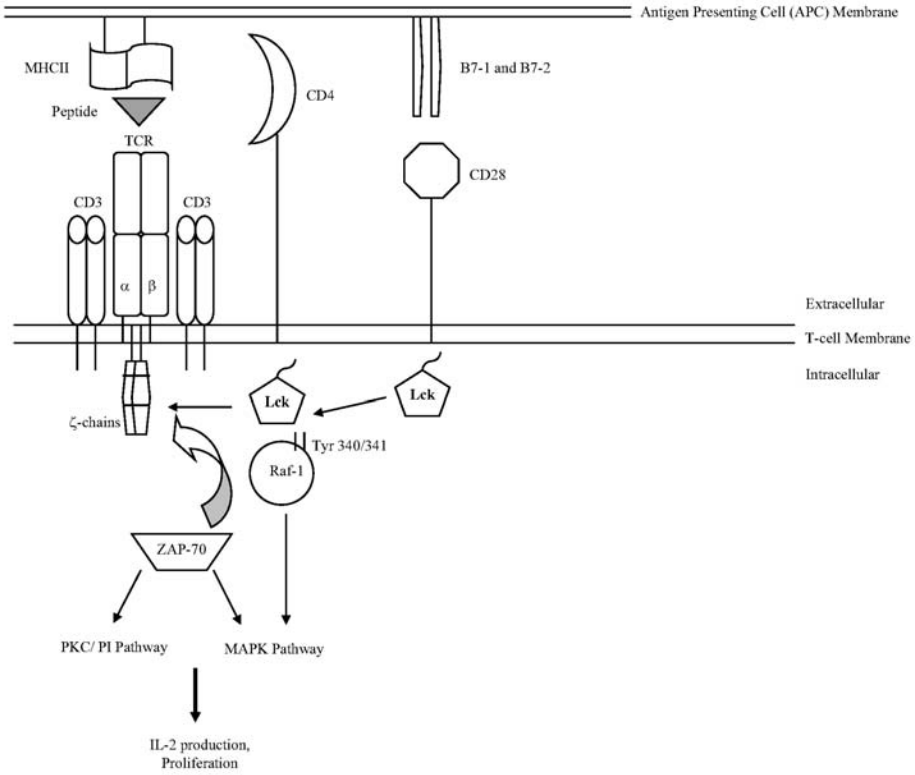
A. The Discovery of Src. A pioneer in the field of Src PTKs was Dr. Peyton Rous, who discovered the ability of the (now named) Rous Sarcoma Virus (RSV) to cause cancer in hens in 1911. Dr. Rous was later awarded the Nobel Prize in Physiology or Medicine in

1966 for his work, and became the first scientist to demonstrate that a virus is capable of inducing cancer (Nobel). Although Dr. Rous was unaware of the host cellular gene *src* in this infection model, his work was a major advancement for our understanding of the importance that this family of proteins plays in the immune system. It would not be until 1976, that Michael Bishop and Harold Varmus identified that it was, in fact, the cellular gene, "*sark*" (*src*), that was hijacked by the RSV to cause cancer (Stehelin). This body of work was the first to identify the Src PTKs, and classify them as proto-oncogenes. Bishop and Varmus were awarded the Nobel Prize in Physiology or Medicine in 1989 for their work with *src,* and an increased study of the nine Src PTK family members, which include: Src, Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes, and Ykr, would follow their discovery. Furthermore, one of the first properties of these kinases was discovered when mutations or over-expression lead to certain types of cancers (Potts, Stehelin). Of particular importance to the study of T-lymphocyte activation and function are the Src PTK family members, Lck and Fyn, as they are critical signaling components of the TCR.

B. The Src PTKs, P56Lck and p59Fyn. The lymphocyte cell kinase, Lck, was first discovered in 1985 by Jamey Marth (Marth), and subsequently, both Lck and Fyn have been shown to be critical for TCR signal transduction (Appleby, Goldsmith, Stein, Straus'92). In order to understand how Lck is involved in signaling through the TCR, it is necessary to understand the TCR structural components. The TCR is a hetero-oligomeric structure on the T-cell surface. Signaling via this receptor is required for T-lymphocyte activation. Ligand binding to this receptor is facilitated by heterodimeric immunoglobulin-like clonotypic chains ($\alpha\beta$, and/or $\gamma\delta$), which are noncovalently complexed with three pairs

of disulfide-linked molecules, termed the CD3 complex. The CD3 complex is comprised of γ-ε and δ-ε heterodimers and a disulfide-linked homodimer (ζζ, or ζ linked to its alternatively spliced form, the η chain). These CD3 subunits contain cytoplasmic tails essential for Ag-stimulated signaling downstream of ligand binding to the TCR (Frank). As the TCR has no intrinsic kinase activity, it is dependent on the recruitment and function of the Src family of PTKs, located within the cell, to propagate TCR-induced intracellular signals leading to T-cell activation, differentiation and function. (Fig. 1). Following TCR and co-receptor (CD4 or CD8) engagement with peptide/MHC complexes on the surface of an antigen presenting cell, Lck is able to facilitate signal transduction via binding to phospho-tyrosine residues located within immunoreceptor tyrosine-based activation motifs (ITAM) within the CD3-associated zeta chains (Straus '96). Once bound and activated, Lck is able to further phosphorylate tyrosine resides within the ITAM motifs, which promotes the binding of the Syk family member, ZAP-70, and subsequent propagation of activating signals. Lck activity is governed by several proteins, including the C-terminal c-Src kinase (csk) and CD45. Csk is localized at the T-cell membrane by association with the transmembrane scaffold protein, PAG1, and is thus able to continually phosphorylate Lck at its inhibitory tyrosine residue (Tyr^{505}). Following TCR ligation PAG1 is de-phosphorylated, releasing csk, and enabling CD45 de-phosphorylation of Lck at the inhibitory tyrosine residue. (DeFranco)

Fig. 1. Proximal TCR signaling events. The CD4- or CD28-associated Src family protein tyrosine kinase (PTK), p56Lck, is localized with Tyr residues within TCR zeta-chain ITAM motifs following TCR ligation. Lck also associates with the CD8 co-receptor (not shown). Lck phosphorylation of Tyr residues within these ITAM motifs provides a docking site for the Syk family member, ZAP-70. Subsequent phosphorylation and activation of ZAP-70 by Lck enables downstream activation of the PI and MAPK pathways, resulting in T-lymphocyte activation (including cytokine production and activation-induced cell proliferation). Furthermore, Raf-1 is able to associate with the Lck SH2 domain via Tyr residues 340/341 to induce MAPK pathway activation, independent of ZAP-70 activation.



Although Lck and Fyn expression is similar in both CD4+ and CD8+ thymocyte populations (Olszowy), Fyn is more highly expressed in peripheral T-cells (Denny'00). And though Lck and Fyn are both able to initiate tyrosine phosphorylation of the TCR, studies using the Lck deficient T-cell line, J.CaM1, have shown that the Fyn-specific phosphorylation pattern of TCR CD3 subunits and TCR zeta chains is distinct from that of Lck. Moreover, Fyn-specific phosphorylation of the Zeta-chain-associated protein kinase (ZAP-70), a Syk family PTK involved in proximal TCR signaling, is reduced compared to that induced by Lck. Subsequent activation of these J.CaM1 cells was also reduced, as indicated by reduced levels of IL-2 following TCR induced cell activation, resulting in a phenotype of partial T-cell activation compared with cells which rely solely on Lck activity. (Denny'00). Furthermore, Fyn null mice exhibit only partially dampened TCR signaling, whereas Lck null mice are almost completely lacking in peripheral T-cells due to severely arrested thymocyte development, with those cells that are found in the periphery having significantly impaired proliferation in response to TCR cross-linking (Stein, Molina). Examination of mice lacking both Lck and Fyn demonstrate a complete lack of αβT-cell development (Van Oers '96b). Therefore, the roles of these two Src PTKs are distinct with regard to T-cell development and proximal TCR signaling events and are not completely redundant, even though they perform similar roles in the processes that lead to T-lymphocyte activation.

C. The Roles of Lck and Fyn in T-Cell Signaling. As Lck is associated with both the CD4 and CD8 co-receptors on T-lymphocytes, ligation of the TCR and co-receptors with cognate MHC/peptide complexes on APCs localizes and couples the co-receptor-

associated Lck with the TCR enabling intracellular signaling cascade initiation. This concept is supported by work by Glaichenhaus et al, where the association between CD4 and Lck was found to be critical for T-cell activation. However, further examination of this model has indicated that while this interaction is necessary, it may not be the only kinase critical for this process (Veillette'89a-b, Luo, Sefton). (Fig. 1). While TCR ligation requires Lck for complete T-lymphocyte activation, it has been shown that tumors, such as Hodgkin's Lymphoma, are able to affect cellular immunity by inhibiting the function of this kinase. The mechanism of this suppression is believed to be a result of elevated secretion of prostaglandin E_2 (PGE_2) with the onset of this form of lymphoma, which has been shown to inhibit CD4+ activation by negatively regulating TCR and T-cell co-stimulatory (CD28) signaling via the inactivation of Lck (Chemnitz). Understanding the role that these signaling molecules play in the cellular response to co-stimulation is very important, not only as co-stimulation is responsible for lowering the threshold for signals received via the TCR to induce an intracellular signaling response and cytokine secretion (Viola), but for understanding the ability of specific receptor complexes to congregate and initiate various intracellular pathways leading to differential cell outcomes/fates. The ligation and association of particular co-receptors and co-stimulatory molecules permits the selective induction of distinct signal pathways which enables the regulation of various signaling cascades for divergent activation responses to different stimuli. For example, the ligation of CD28 with its ligands B7-1 or B7-2 (activating ligands on B-cells) has been shown to induce signaling required for immunogenicity and T-cell mediated rejection of weakly immunogenic tumors (Chen). As with many regulatory pathways *in vivo* which

have multiple responses, data has also shown that blocking CD28 engagement *in vivo* inhibits the development of autoimmune diseases (Cross, Lenschow, Perrin) and donor-specific tolerance to allogeneic organ transplants (Turka). The specific mechanisms by which these pathways are governed by signaling molecules are able to exquisitely direct the activation state and functional outcomes of the cells which they control. Moreover, Lck has been shown to associate with MHC class I–binding killer-cell immunoglobulin like receptors (KIR) on the surface of natural killer (NK) and T-cells (Moretta, Lanier). KIR receptors are type 1 membrane glycoproteins of the Ig superfamily. KIR receptor engagement acts to downregulate NK and T-cell cytotoxic effector function and cytokine production (Moretta). Lck has been shown to phosphorylate tyrosine residues of the KIR cytoplasmic tails. This phosphorylation promotes the association of PI3-K and its subsequent activation of the anti-apoptotic kinase AKT, resulting in a positive cell signal promoting cell survival, possibly as a consequence of insufficient viral or tumor MHC/peptide complex recognition. (Marti, Cannon). CD2 is found on the surface of NK and T-cells, and functions as a co-stimulatory molecule on the surface of the latter. This molecule is important for its ability to reverse anergy in T-lymphocytes following B7 blockage (Boussiotis), and has a dual function as an adhesion molecule (Wang). Moreover, this receptor is able to interact with the inhibitory receptor CD5 (Carmo, Castro) and modulate signaling at the cell surface via this interaction (Carmo, Teh). Lck has been shown to bind the cytoplasmic tail of CD2 via SH3 domain interactions (Bell), and has recently been shown to be important for the localization of CD2 to lipid raft microdomains in non-stimulated T-cells (J.CaM1) (Nunes). CD2 null mice do not appear to have any

significant alterations in their T-lymphocyte compartment or function (Killeen). Thus, Lck may act to regulate signaling cascades downstream of a variety of cell surface receptors in response to stimuli in order to enhance or inhibit cellular functions, even if these functions are not perhaps critical for their function.

Intracellular signaling following surface receptor engagement results in the association of various signaling proteins that interact to determine cell activation, as well as cell fate. Th2 T-lymphocytes (differentiated CD4+ T-cells) are critical mediators of allergic responses and provide essential functions for immunity to helminthic parasites such as the secretion of immune cytokines and assistance to B-cells to provide Ag-specific imunoglobulins. As IL-4 is a critical mediator of Th2 immunity, a deficit in this interleukin is of significant importance to the induction of a protective IgE response, and host immunity to parasite infection. Heyeck et al. have shown that Lck is able to phosphorylate a critical conserved tyrosine residue of Itk, and that this phosphorylation leads to specific activation of Itk and the induction of Itk kinase activity. Moreover, studies using a dominant-negative Lck transgenic mouse have shown that Th2 T-cell differentiation is selectively inhibited both *in vitro* and *in vivo* (Yamashita ’97) compared with that of Th1. Lck signaling has also been shown to influence the downstream MAPK pathway proteins Erk1/2, which have also been shown to be critical for controlling IL-4R function and Th2 cell differentiation (Skapenko, Yamashita ’99) by positively regulating STAT6 activity and IL-4 transcription (So). The importance of Lck, therefore, involving its role as a critical regulator of early TCR signaling events, its requirement for TCR and co-stimulatory signals necessary for T-cell activation, and intracellular protein associations during cell

signaling which are able to alter effector T-lymphocyte function and differentiation, is of great value to further understand and use these mechanisms to target and treat infection and immune dysregulation.

Lck is of particular interest to our lab, and is expressed in T-cells as well as NK cells, brain neurons, and B1 B-cells. Although I have touched on some of the aspects of Lck involvement in NK and T-cell activation and differentiation/function, its role in neurons and B1 B-cells is not as well understood.

Lck appears to be localized to the photoreceptor cell bodies in the outer nuclear layer and outer plexiform layer of the adult mouse retina. Neuronal dependence on Lck has been examined using mice lacking functional Lck (Lck KO). These mice present with partial retinal detachment with retinal infoldings and rosette formation similar to retinopathy of prematurity (ROP), a retinal injury frequent in human infants caused by oxygen-induced retinopathy (Omri). Furthermore, embryonic stem (ES) cells treated with retinoic acid (to enhance neuronal differentiation) in conjunction with inhibitors of Src family kinases blocks the terminal differentiation process of the excitatory neural phenotype (Theus). As ES cell differentiation into neuronal cells is a proposed treatment for various central nervous system (CNS) disorders, the regulation of Lck in this process is of great clinical importance.

B1 B-cells are a small subset of the B-lymphocyte population found in the spleen, lung, as well as peritoneal and pleural cavities, and are responsible for producing high levels of IgM. These cells have polyspecific receptors (low affinity for many different antigens) that have a preference for common bacterial polysaccharides. Splenic B1 B-cells

are devoid of Lck, but are induced to express Lck once in the peritoneum. Lck has recently been shown to be important for B-cell receptor (BCR) hypo-responsiveness, and, as such, Lck is believed to be important for the cell fate of B1 B-cells (Dal Porto).

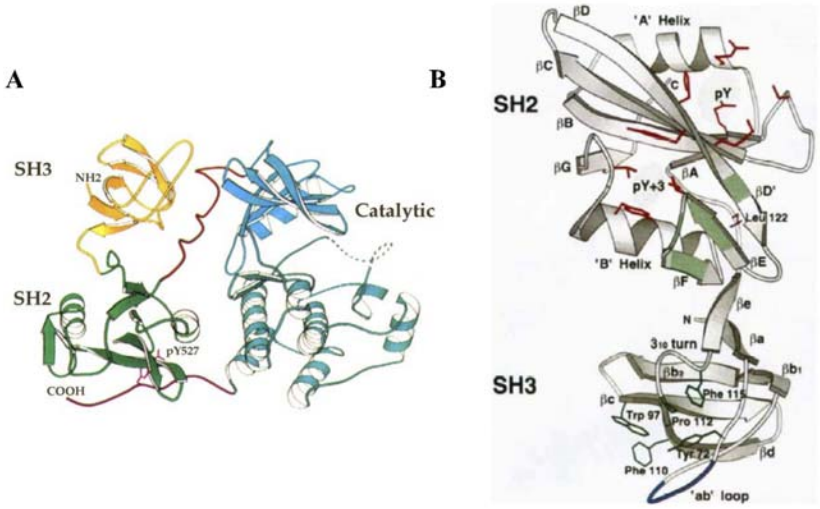
There are also studies reporting the importance of Lck in the ability of lymphocytes to maintain homeostatic balance within the periphery of a host animal. Homeostatic proliferation is a term used to describe the phenomenon by which lymphocytes are able to proliferate to fill a deficit in cell numbers in order to reach homeostatic balance within the host. Using a tetracycline-inducible system for regulating Lck levels in mice, Seddon et al. showed that in the absence of Lck, there was a defect in TCR-driven homeostatic proliferation of peripheral T-cells when in a lymphopoenic animal host- and that Lck was absolutely critical for this process. Lck was not, however, found to be essential for the long-term survival of peripheral T-cells, as Lck and Fyn had redundant roles in this process (also requiring IL-7) (Seddon). In order to better understand how Lck and its binding partners communicate with respect to T-cell development, activation and function, it is important to study the particular structural components of Lck, and understand how these domains interact to facilitate the specific roles and functions of Lck in these processes.

D. The Structure of Src. There is a conserved domain homology between Src PTK family members. The conserved domains of the Src PTKs are the SH3, SH2, catalytic (tyrosine kinase) domain (which includes the activation loop and an autophosphorylation site), and a short C-terminal tail (which contains a secondary site for auto-phosphorylation). As components of the TCR have no intrinsic kinase activity, the recruitment and activities of three classes of PTKs are essential for TCR function: Src

family members Lck and Fyn, Syk family members ZAP-70 and Syk, and Itk-Btk family member Itk) (Rawlings, Rudd ’94, Weiss ’94). A crystal structure for Src has been determined (Xu), but as of yet, there is no complete crystal structure for Lck. There are, however, isolated and compound crystal structures for the SH3 and SH2 domains of Lck (Boggon, Eck), (Fig. 2), and the kinase domain (Yamaguchi).

The N-terminal segment of SRC PTKs is attached to a variable unique domain and is important for the localization of the protein to the membrane as well as proximal association with various membrane-bound receptors. This occurs, in part, as the N-terminus can be modified by the covalent attachment of myristic and palmytic acid moieties. Myristic acid moieties can be cotranslationally added to glycine residues (position 2 in Lck) and Lck and Fyn are able to undergo further modification by palmitoylation at distinct cysteine residues antecedent to the unique domain. Palmitoylation of cysteine 3, and either cysteine 5 (Lck) or cysteine 6 (Fyn)) (Koegl, Paige), as well as cysteines 20 and 23, contributes to localization of Lck or Fyn within the cell (Shaw’90, Turner,). It is of note that Lck and Fyn maintain unique and distinct localizations within cells. This difference has been attributed to the function of additional protein-specific domain interactions unique to each of the Src PTKs (Ley, Lin). Lck is found at higher concentrations near the plasma membrane, whereas Fyn has greater affinity for the centrosomal and mitotic structures. The unique domain of Lck also plays a role in its reversible association with the T-cell co-receptors CD4 and CD8, both of which are important for T-cell activation (Abraham, Rudd‘88, Shaw, Veillette’88), via formation of a dicysteine motif in the unique domain with two cysteine residues (20 and 23)

Fig. 2 Crystal structures. A) c-Src ribbon diagram: Xu et al. 1997 B) Richardson diagram showing Lck SH2 and SH3 domain secondary structure: Eck et al. 1994.



(Rudd'88, Veillette'88) in each of the cytoplasmic domains of the co-receptors (Turner). Approximately 30-60% of total Lck is found associated with these receptors (Luo, Rudd'88, Veillette'88,'89). Further work has also demonstrated that GPI-anchored proteins can lead to the activation of T-cells (Robinson, Shenoy-Scaria'92, Su), and that this is made possible, in part, by the association of Lck with glycophosphatidylinositol (GPI)-anchored proteins via palmitylation and myristylation of the N-terminus of Lck (Shenoy-Scaria'93).

The SH2 domain consists of a central β-sheet flanked by single α-helixes on each side, and is able to engage ligands via binding with tyrosine residues. The ability of the Lck SH2 domain to bind to phosphotyrosines, and subsequently enable the phosphorylation of various tyrosine residues within ITAM motifs of the TCR zeta chains is critical for the propagation of activating signals upon TCR ligation. There are three ITAM motif repeats within each of the TCR zeta chains, as well as a single motif in each of the CD3 molecules (γ, δ, and ε) (Weiss '94). ITAM tyrosine phosphorylation provides, in turn, a docking site for the secondary signaling protein, ZAP-70 (Iwashima '94, Wange). Lck, being in proximity with ZAP-70, is able to bind and activate ZAP-70 via SH2 directed protein-protein interactions. (Chan, Duplay, Straus'92, '96). Activation of ZAP-70 leads to induction of the Ras/Raf pathway and downstream signaling cascades necessary for complete T-cell activation. The ability of the TCR to direct phosphoinositol (PI) pathway activation occurs rapidly upon TCR ligation. Phosphorylation of PLC-γ1 and the activation of PKC (serine/threonine kinase) in the PI pathway leading to the mobilization of intracellular calcium, have been shown to rely on Lck, and not Fyn, in T-cells (Lovatt), and

that this process requires the SH2 domain of Lck (Weber). Studies using mice lacking functional Lck show evidence that Lck is critical for T-cell development, and that TCR signaling is severely abrogated (Van Oers) with Zap-70 recruitment and downstream secondary signaling severely hampered. LAT, a downstream target of ZAP-70, is an adaptor molecule important for bringing signaling molecules together at the TCR including Grb2/SOS and resulting in downstream activation of Ras/Raf and the MAPK pathway protein, Erk. Another downstream molecule associated with ZAP-70 is SLP-76, an adaptor molecule involved in Itk activation and PI pathway induction. Therefore, defective ZAP-70 function significantly affects both MAPK and PI pathway signaling cascades following TCR ligation.

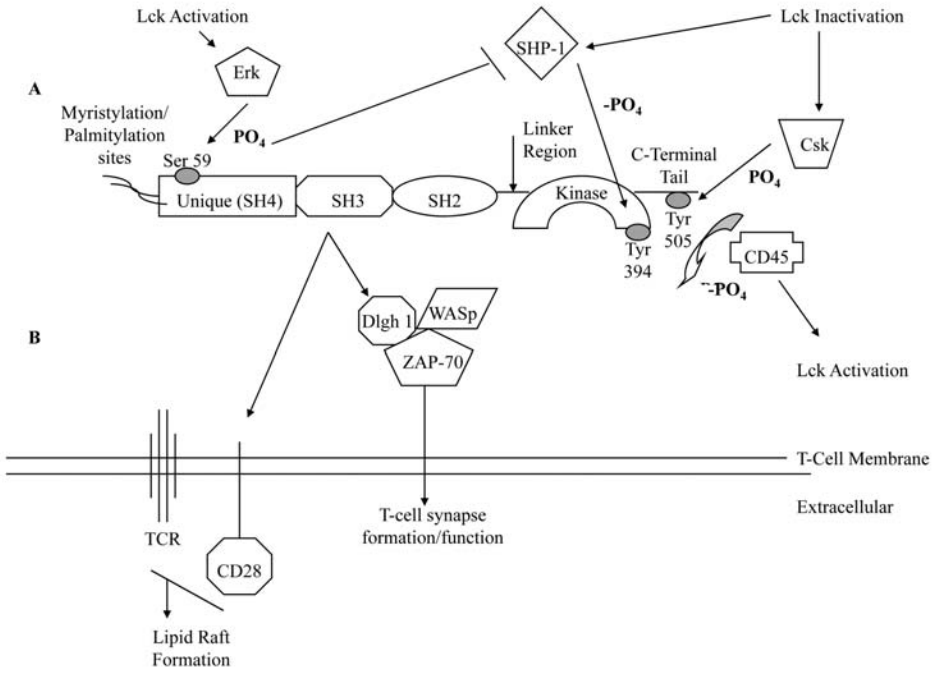
Data from Lck-null thymocytes has shown that Lck is critical for ITAM tyrosine phosphorylation, but that Fyn may be able to compensate to some degree in peripheral T-cells (Van Oers). Taken together, these data demonstrate the critical role for Lck in TCR signal transduction.

The C-terminal kinase domain of Src PTK family members functions to catalyze the phosphorylation of activating tyrosine residues on binding partners. The activating and inhibitory tyrosine residues responsible for the activation state of the Src protein itself are found in this domain (Abraham, Cooper). Lck further relies on low affinity intramolecular interactions between the C'-terminal phosphorylated inhibitory tyrosine (Tyr-505) and the SH2 domain, coupled with SH3 domain binding of the linker region, which results in a stabilized "closed" (or "autoinhibited") conformation of the protein (Erpel, Sicheri, Superti-Furga, Williams, Xu). This inactive form of Lck is due to the fact that the

activating tyrosine (Tyr-394) is structurally occluded, and the C-lobe of the kinase domain is displaced (which contains a catalytically important glutamine residue). High affinity ligand binding to either the SH3 or SH2 domain, or de-phosphorylation of the inhibitory tyrosine, releases the intramolecular constrictions in favor of an active conformation of the protein (Brown, Erpel).

E. Activation and Function of Lck. Several major modes of regulation are depicted in (Fig. 3) and are outlined in detail here. Direct activation of Lck is believed to occur by de-phosphorylation of the inhibitory tyrosine (Tyr-505) by CD45 (leukocyte common antigen), a transmembrane glycoprotein of the leukocyte-specific receptor-like protein tyrosine phosphatase (RPTP) family. Kinase activity involving CD45 *in vitro* has been shown to require Lck; and this association does not require the association of CD45/Lck with the TCR (Koretzky). Subsequent de-phosphorylation of Tyr-505 on Lck by CD45 renders the protein in a "primed" or "active" conformation (Hermiston, Mustelin'89, '90). Conversely, inhibition of Src PTK activity occurs as a result of phosphorylation of the inhibitory tyrosine residue (Tyr-505-on Lck) by the protein C-terminal Src kinase (Csk) (Hermiston, Okada, Sondhi). Furthermore, the ubiquitin ligase, Cbl, is able to bind Lck via its SH3 domain and target it for lysosomal degredation as part of a negative feedback mechanism following TCR ligation and CD4 co-receptor engagement (Rao). In another negative feedback-type mechanism of regulation, activated Lck is able to phosphorylate and activate the tyrosine phosphatase, SHP-1, which is able, in turn, to mediate Lck inactivation. This is facilitated by T-lymphocyte ligands that induce activating signals through the TCR, and subsequently activate Lck and the MAPK pathway

Fig. 3. Lck activation and secondary signaling. A) Schematic of Lck structure and its regulation. Major modes of direct activation and inactivation of Lck by the signaling proteins, Erk, SHP-1, Csk, and CD45. B) CD28-associated Lck is able to assist lipid raft aggregation and cytoskeletal reorganization following TCR ligation by SH3 domain binding to the scaffold protein Dlgh1 and subsequent recruitment of WASp and ZAP-70, which are required for complete synapse formation and function during an Ag-dependent co-stimulatory-associated T-cell/APC engagement.



signaling protein, Erk. Erk is able to phosphorylate Lck at serine 59, which prevents the association of Lck with SHP-1, and thus allows the activation of T-cells by activating ligands. (Germain). Therefore, viable activation signals received via the TCR, and the duration of that activation, compared with that of antagonizing signals, are able to be controlled by the interaction between Lck and SHP-1 (Stefanova '02, '03). Lck also contains an activating tyrosine located within the activation loop of the protein (Y394), which is able to be autophosphorylated following de-phosphorylation of Y505 (Brown, Williams). Moreover, Lck has been shown to be able autophosphorylate at this site upon the induction of the CD4 and CD28 accessory molecule stimulation in the absence of CD45 activity. This occurs following Lck recruitment by CD4 or CD28 to the T-cell/APC interface and CD28-induced anti-PY394 reactivity. (Holdorf '02, Tavano). Furthermore, CD45 has been shown to negatively regulate CD44 mediated adhesion in T-cells, also requiring Src PTK activity (Li '01), and has recently been implicated as able to de-phosphorylate the Lck activating tyrosine (Y394) following CD44 ligation (Wong). CD44 ligation has been shown to activate Lck (Taher), so this new finding indicates that CD45 inhibition of CD44 induced Lck activation is able to result in the inhibition of sustained Lck activation, and reduced actin rearrangement resulting in elongated T-cell spreading required during T-cell/APC engagements (Wong).

F. The Role of Adaptor Protein Interactions. Adaptor proteins play a critical role in the organization of signaling clusters and molecules required for T-cell signaling through receptors, and function at many different levels to ensure proper

associations downstream of receptor ligation. The T-cell-specific adaptor protein (TSAd) is a critical adaptor molecule required for proper regulation of cytokine gene transcription. Cells lacking TSAd produce significantly reduced levels of IL-2, IL-4, and IFN-γ following TCR engagement (Drappa, Rajagopal). Marti et al. have shown that TSAd is able to activate Lck in peripheral T-cells. Although TSAd contributes to Lck activation, it is not sufficient for full Lck activation, and requires binding via both the SH3 and SH2 domains of Lck (potentially by acting to release Lck from its autoinhibitory conformation). With the ability to affect the activation of Lck, Rlk, Itk, and MEKK2 (Rajagopal, Sun), as well as the ability to stimulate calcium mobilization, TSAd is able to affect multiple pathways leading to the transcription of cytokine genes. The Lck-interacting molecule (LIME) is an adaptor molecule involved in the regulation of T-cell activation following co-receptor ligation with either CD4 or CD8. CD4 or CD8 ligation is able to activate LIME, whose function enables the proximal association of Csk and Lck (via binding to the Lck SH2 domain (Hur)), so that Csk is able to negatively regulate the activity of Lck following receptor engagement (Brdickova). The Lck SH3 domain has also been shown to recognize and bind scaffold proteins, such as SLP-76 (Sanzenbacher), which complexes and localizes various signaling proteins to enable the efficient transmission of activating signals downstream of receptor ligation. The linker for activation of T-cells (LAT, a transmembrane scaffolding protein) is able to recruit SLP-76 to the membrane, where the Syk family member, ZAP-70, is able to bind and phosphorylate these adaptor proteins (Wardenburg). Scaffolding molecules rely on the activity of tyrosine kinases, as they lack intrinsic kinase activity. SLP-76 and LAT interactions are important for T-cell activation,

as they recruit phospholipase Cγ1 (PLCγ1), the guanine nucleotide exchange factor VAV, the Tec family kinase interleukin2-inducible T-cell kinase (Itk), and the Src family PTK, Lck (Jordan). With these proteins localized together, they are able to rapidly interact to release intracellular calcium stores and facilitate downstream activating signals required for complete T-cell activation.

G. SH3 Domain Interactions. SH3 domains are important protein-protein binding domains, and are utilized by many proteins to facilitate functional interactions. To understand the specific requirements for the Src kinase, Lck, with regard to its SH3 domain, it is important to further examine the structure of Lck and how these domains interact. Following the conserved SH2 domain, there is a two amino acid polypeptide linker region that connects the SH2 and SH3 domains in all nine of the Src PTKs (Eck), which is important for the aforementioned intramolecular interactions. Src SH3 domains are formed by five β-strands and contain two protruding loops (RT and n-Src loops) that border the hydrophobic and aromatic residues of the proline recognition site (Musacchio, Noble, Yu). This structure confers a binding orientation for class I ligands of the RxxPxxP motif (the class II ligands are named due to their opposing binding orientation to class I ligands) (Feng). This orientation is particularly sensitive to the position of a basic residue (usually arginine) relative to the core proline residues (Feng). Polyproline SH3 domain ligand binding occurs at the hydrophobic patch formed by the loops and at a point of contact between the β_4 and β_5 strands (Larson). Although the specific mechanisms and roles for interactions with the Lck SH3 domain have not yet been fully elucidated, several proteins known to be important for TCR signaling have been identified as binding partners

for the Lck SH3 domain, including: LckBP1 (identical to HS1; important for B- and T-cell selection) (Takemoto, Taniuchi), phosphatidylinositol-3-OH kinase (PI-3-kinase) (Prasad, Vogel), c-Cbl (adaptor molecule, ubiquitin ligase, lipid raft localization) (Hawash), and the T-cell co-stimulatory molecules, CD28 (Holdorf) and CD2 (Bell).

In vitro studies have investigated the importance of the Lck SH3 domain with regard to T-cell activation using a J.CaM1 cell line containing an Lck SH3 domain mutant. This body of work has indicated that the Lck SH3 domain is critical for activation of the mitogen activated protein kinase (MAPK) pathway, as indicated by reduced Erk induction, but not for early TCR signaling events (including TCR zeta chain phosphorylation and ZAP-70 recruitment and activation) (Denny'00, Patel), which rely more heavily on the function of the Lck SH2 and kinase domains.

In order to further investigate the observed defect in Erk induction following TCR ligation in Lck W97A T-cells *in vitro*, Li et al. examined potential downstream signaling mediators leading to MAPK pathway activation. TCR/CD4 engagement leads to the association of Lck and Raf-1, and the subsequent activation of Raf-1 (Popik). Tyrosines 340 and 341 are required for activation of Raf-1, and have been shown to be activated by Src kinases (Fabian). Li et al. have shown that defective Erk induction by TCR ligation of Lck W97A T-cells *in vitro*, occurs as a result of defective Lck activation of Tyrosines 340/341 on Raf-1.

Aside from a role for Lck in proximal TCR signaling events, it has also been implicated downstream of TCR signaling as a regulator of TCR/co-stimulation-induced lipid raft formation/recruitment to the TCR contact "cap". Lipid rafts are considered to be

microdomains rich in glycolipids, cholesterol, and saturated phospholipids, and are embedded with various cellular proteins (Simons). Previous work has indicated that T-cell co-stimulation is able to induce lipid raft recruitment to the TCR, enabling the association of various receptors and signaling molecules with the TCR (Moran, Tavano, Viola '99). CD28 co-stimulation in T-cells has been shown to rely on the Lck SH3 domain *in vitro* (Holdorf). Moreover, the Lck SH3 domain has been implicated as an important mediator of TCR/CD28 co-stimulation-induced lipid raft aggregation as well as sustained TCR/CD28 co-stimulation-induced protein tyrosine phosphorylation and IL-2 production (Patel). In order to study the role of the Lck SH3 domain in lipid-raft/immunological synapse formation, cells of the BI-141 T-cell hybridoma cell line were modified to contain a mutation in the Lck SH3 domain, ablating the function of this domain. Patel et al. found that the Lck SH3 domain was not only important for the recruitment of the TCR into lipid rafts in a co-stimulation-dependant fashion using this cell line model, but prolonged TCR engagement and signaling for this process is defective in T-cells lacking Lck SH3 domain function.

Once lipid rafts aggregate in response to co-stimulation, a more compact synapse structure forms between immune cells. The lymphocyte function-associated antigen-1 (LFA-1), a leukocyte integrin, binds to the inter-cellular adhesion molecule-1 (ICAM-1), to promote conjugate formation between T-cells and APCs in an Ag-dependent fashion. This interaction results from "inside-out" signaling downstream of surface receptor stimulation that alters the adhesive state of integrins. Actin accumulation and cytoskeletal reorganization occur following receptor ligation, to assist the interactions that occur at the

T-cell/APC binding site. A role for the Lck SH3 domain in this process has been shown by Morgan et al., who demonstrated that the Lck SH3 domain is required for conjugate formation, without relying on the activity/function of ZAP-70, implying a role for Lck to effect LFA-1-dependant conjugate formation via SH3 domain interactions independent of ZAP-70. Lck-deficient T-lymphocytes, and those having a mutation in the Lck SH3 domain, both have significant defects in actin-remodeling and LFA-1 recruitment during T-/B-cell conjugate formation, irrespective of the function of the MAPK protein, Mek1 (Morgan). In order to fully understand how Lck could be modulating conjugation, the effects of Lck on cytoskeletal reorganization have also been examined. The guanine nucleotide exchange factor (GEF), Vav-1, is an important regulator of Rho family GTPases, which are important for the process of actin remodeling. Defective Lck activity downstream of CD28 signaling has been implicated as having significantly negative effects on Vav-1-dependent Rac-1 activity and NF-AT activation, and thus, defective cytoskeletal remodeling and conjugate formation (Carey, Michel). Moreover, interference with Lck-dependant activation of the Rho protein, CDC42, at the APC interface interrupts conjugate formation (Cannon'01). CDC42 is required for the activation of the Wiskott-Aldrich Synrome protein (WASp). Activation of WASp allows it to activate the Arp1/2 complex resulting in nucleation of F-actin. Dlgh1, a member of the membrane-associated guanylate kinases (MAGUK) family of molecular scaffolds, acts to bring together Lck, Zap-70 and WASp downstream of TCR/CD28 ligation to assist T-cell synapse formation and function. The SH3 domain of Lck has been shown to bind Dlgh1 and assist its localization at the membrane so that it can act as a scaffold for the secondary signaling molecules required

for antigen-induced actin polymerization and raft clustering (Round). Lck has therefore been proposed to play a role at two stages during TCR/CD28 signaling for synapse formation by positively affecting CDC42 function and Dlgh1 localization resulting in actin accumulation and synapse formation.

While Lck has been implicated in signaling affecting various signaling cascades and protein-interactions, it has also been hypothesized as a critical mediator of pathogen invasion of immune cells. In order to examine the potential role that Lck may play in the pathogenesis of *Plasmodium*, it will be necessary for me to provide some background on malaria and what is known so far about infection by this protist.

III. Immunity to *Plasmodium.*

Malaria is considered to be an infection with the eukaryotic microorganism, *Plasmodium*, a genus of parasitic protozoa of the family Plasmodiidae. The genome of this organism consists of 14 chromosomes, one mitochondrion, and a plastid. Four species are known to infect humans, and more than 200-300 million people present with acute malaria infections each year. Infection by *plasmodium* is a leading cause of death in the tropics, claiming the lives of more than 3 million persons annually (Bjorkman), with the majority of these being children in sub-Saharan Africa. 9 out of 10 deaths from malaria are due to the highly fatal strain *Plasmodium falciparum*.

A. In the Beginning: The Foundation of Discovery. There is a long history that has lent itself to the study of malaria. Several major advances had their beginnings after Dr. Ronald Ross was awarded the Nobel Prize in 1902 for his work in 1898, demonstrating

the presence of *Plasmodium* in the midgut and salivary glands of the *Culex* mosquito. This work, combined with that of Dr. Giovanni Battista Grassi, who determined that only the *Anopheles* mosquito was able to transmit the human-infective *plasmodium*, and is also credited with the characterization of the complex *plasmodium* life-cycle, have provided critical insight for the life-and-death struggle against one of the world's deadliest diseases.

B. *Plasmodium* Life-Cycle and Pathology. There are two main stages of the life cycle of the parasite which requires two hosts: a female mosquito vector and a vertebrate host. Only female mosquitos are able to transmit the parasite, as only female mosquitos bite. This is believed to be due to the increased requirement for protein by the females, which rely on blood as well as the common nectar meals, in order to lay eggs. Following host infection, sporozoites are able to travel to the liver and rapidly infect hepatocytes. During the course of about a week, the hypnozoites (latent stage sporozoites) differentiate into the morphologically different merozoite stage, and rapidly replicate to such an extent (one sporozoite is believed to develop 30,000-40,000 merozoites) that they burst the hepatocytes (Nardin). Hypnozoite reactivation has been observed for *P. malaria*, *P. ovale*, and *P. vivax*, and can occur up to thirty years after initial infection of the host. Merozoites target and infect erythrocytes, where they take on a trophozoite form (ring form), and asexually divide during the schizont stage to such an extent that the red blood cells (RBC) swell and lyse, allowing renewed erythrocyte infection by the new merozoite population every 2-3 days (depending on the species of *plasmodium*), and result in the characteristic "cycles" of "malaria fever" (resulting form merozoite secreted molecules which stimulate the host inflammatory immune response) every 4-5 days. Splenomegaly is observed, as the

reticuloendothelial system tries to clear the infected RBCs, and the host presents with anemia, as the patient is deprived of their red blood cell population due to parasite infection. Some asexual parasites are able to take on gametocyte form (both male and female) that can be ingested by mosquitoes during a blood meal; and gametes can fertilize each other in the mosquito midgut to create motile zygotes termed ookinetes, that mature into sporozoites in the gut membrane and migrate to the salivary glands for infection of a new vertebrate host. Symptoms from an initial host infection can take approximately 1-2 weeks to appear.

Of the hundreds of species of plasmodium, only four are known to infect humans, and of these, only *P. falciparum*, and *P. vivax* are known to lead to fatality. The major cause of death is due to occlusion of brain microvessels by infected RBCs following *P. falciparum* infection (Miller). Specific immunity is acquired slowly, and is rarely long-lived. Immunity to *plasmodium* is partly mediated by cytotoxic T-cell killing of infected hepatocytes and the subsequent induction of more specific Ag-induced T-cell proliferation and Ab-induced IFNγ production during the blood stage infection (Good ‘98, Miller). Several key antigens have been discovered to induce effector CD4+ and CD8+ T-cell function, including the sporozoite circumsporozoite protein (CS) (Plebanski ’97, ’99), the sporozoite thrombospondin related adhesive protein (TRAP) (Flanagan), and the *P. falciparum*-specific merozoite protein 1 (PfMEP1) (Newbold). These antigens are being intensively studied for their ability to induce immunity and protection against these stages of infection in the human host. No vaccine has successfully induced strong immunity using single antigens alone, even including those of CS (Good ‘98, Herrington, Nardin).

Although pre-erthyrocytic Ag-immunization can confer host protection from hepatocyte infection by plasmodium, it is only able to protect if there is sufficient induction of the CD8+ T-cell dependant IFN-γ response (Gilbert, Good ’99, Plebanski ’98, Schneider, Sedegah). The most effective vaccine candidate available is one that uses irradiated sporozoites to induce a strong CD8+ T-cell-dependant IFN-γ response (Good ’99). Aside from Ag-vaccines, there are plasmid DNA vaccines (Wang), non-replicating vector vaccines: Ty-particles/modified vaccinia virus *Ankara* (MVA) (Gilbert, Plebanski), and recombinant hepatitis B fusion protein particles in conjunction with MPL and QS-21 adjuvants (Herrington) which all show great promise in Phase I and Phase II vaccine trials. Antigenic polymorphisms, poor antigen immunogenicity, parasite-induced immunosuppression, and the complex parasite life-cycle all contribute to the difficulty of effective vaccine development, and encourage the use of multi-factoral vaccine approaches.

More recent work has indicated a critical interaction between the Plasmodium falciparum sporozoite TRAP protein (type 1 membrane protein important for motility and liver cell invasion) and the Src family tyrosine kinase, Lck, for proper localization and infection of sporozoites within hepatocytes (Akhouri). Furthermore, this association has been shown in the HepG2 liver cell line, and has illustrated the requirement for the SH3 domain binding for this interaction (Akhouri). Experiments using TRAP null sporozoites have shown that these sporozoites are unable to infect mosquito salivary glands and the liver of the vertebrate host (Sultan). If an antagonizing agent were used to disrupt the Lck SH3 domain interaction with the sporozoite TRAP protein, it is possible that the

sporozoites would not be able to properly infect hepatocytes in a vertebrate host, and there would be no subsequent malaria infection following sporozoite entry into the host. Furthermore, previous work has shown that the *Ixodes scapularis* tick salivary protein, Salp15, is able to reduce T-cell activation by inhibiting Lck activity downstream of CD4-assisted TCR signaling (Juncadella). Therefore, it is possible that salivary proteins are able to directly act on Lck activity, and may affect its ability to interact with the sporozoite TRAP protein.

C. Vector Contributions to *Plasmodium* Pathology. Sporozoite infection models have, until recently, not included the role of the vector in the infection process. Extended use of malaria prophylactics such as Doxycycline, Tetracycline, and Larium, has resulted in the emergence of skin rashes that are subsequently treated with anti-histamines, such as Benadryl. A notable finding was that use of Benadryl, while it cleared the rashes on the patients, was, more importantly, able to induce some level of protection of the host against malaria. This led to comparisons between *i.v.* injection and bite-administered sporozoite delivery, where sporozoite infection via mosquito bite was found to be more infectious (Vaughan). Partially as a result of this research, there has been increased investigation into the role of mosquito saliva and cells of the innate immune system, such as mast cells, which secrete histamine (vasoactive amine), and how these interactions play a role in host immunity to malaria (Beghdadi, Demeure, Depinay, Furuta). Increased pathogen infectivity has been shown with many arthropod vectors (Titus), and so the role of salivary components and their effect on immune regulation and parasite infection are not novel undertakings. These modulating effects include mast-cell TNF production (Furuta), saliva-

induced histamine release (Demeure, Depinay), and histamine binding to H1R and H2R to increase *plasmodium* susceptibility (Beghadadi).

Several proteins present in mosquito saliva have been identified as critical inducers of inflammation at the site of mosquito bites aside from histamine, including the induction of neutrophil chemotactic factor (NCF) in *Anopheles stephensi* (Owhashi). Furthermore, *Aedes aegypti* mosquito saliva has been shown to inhibit T- and B-cell responses (Wanasen, Wasserman), which has potential for hampering immune responses to infection with *plasmodium*. Contrary evidence has also been proposed by Donovan et al., indicating that pre-exposure *in vivo* to mosquito saliva skews the immune response to a Th1-type immunity, resulting in elevated and protective levels of IL-12, IFN-γ, and inducible nitric oxide synthase in a murine model of *Plasmodium yoelii* infection. Earlier studies using pre-exposure of mice to *Ixodes sacpularis* tick saliva conferred resistance to *Borrelia bergdorferi* (Wikel). This was also found to be true with repeated exposure to sand fly bites and subsequent *Leishmanis spp.* (Kamhawi).

The quest for a vaccine against malaria is evolving to include both factors of the vector and host, as well as combined immunization strategies against multiple stages of the *Plasmodium* life-cycle.

IV. Dissertation Objective.

In order to fully understand the role of the Lck SH3 domain in T-cell development, activation and function *in vivo,* our laboratory created a novel animal model by designing a construct with a knock-in mutation of the *lck* allele with a critical amino acid change in the

Lck SH3 domain (W97A). This point mutation has previously been shown to ablate ligand binding (and function) of the SH3 domain (Denny'99, Patel). Lck is known to be involved in thymocyte signaling via the pre-TCR and TCR (Hashimoto, Levin, van Oers), but it was not until recently that a role for the Lck SH3 domain has been shown *in vivo* for this process. Data from our lab using thymocytes from Lck W97A mice has shown that ablation of Lck SH3 domain function *in vivo* results in defective development of CD4+ and CD8+ thymocytes, as well as reduced activation of the MAPK pathway (Erk activation), while early TCR signaling (including TCR zeta chain phosphorylation and ZAP-70 recruitment and activation) appear unaltered (Rudd'06).

The work of this thesis has expanded the investigation of the role of the Lck SH3 domain *in vivo* with regards to peripheral T-lymphocytes in order to examine its relevance in T-cell activation and function, including *in vivo* immune responses to antigen and parasite infections.

Moreover, novel reports have raised questions as to the relevance of Lck SH3 domain function in the pathology of malaria. As there is currently no vaccine for malaria, it was the purpose of a brief internship during the course of this dissertation research to investigate malara and the role that the vector plays in host immunity, as well as to attempt to translate cell line data indicating a role for Lck SH3 domain in hepatocyte infection by sporozoites into *ex vivo* analysis of this hypothesis.

MATERIALS AND METHODS

I. Animals and Media Reagents

A. C57BL/6 mice. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Lck W97A mice, generated by our laboratory (described in Rudd '06), were backcrossed to the C57BL/6 strain for 8 generations and maintained in specific pathogen free housing until use. Experimental mice were age and sex-matched. Mice were used between 6 and 12 weeks of age. All animal studies were reviewed and approved by the IACUC of Virginia Commonwealth University, Richmond, VA (Protocol #0508-3117).

B. OT-1 mice. OT-1 Tg mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Lck W97A mice were backcrossed onto the OT-1 background for three generations and maintained in specific pathogen free housing until use. Experimental mice were age and sex-matched. Mice were used between 6 and 12 weeks of age. All animal studies were reviewed and approved by the IACUC of Virginia Commonwealth University, Richmond, VA (Protocol #0508-3117).

C. OT-2 mice. OT-2 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Lck W97A mice were backcrossed to the OT-2 line for 5 generations and maintained in specific pathogen free housing until use. Experimental mice were age and

sex-matched. Mice were used between 6 and 12 weeks of age. All animal studies were reviewed and approved by the IACUC of Virginia Commonwealth University, Richmond, VA (Protocol #0508-3117).

D. Mice for *Plasmodium* Studies. C57BL/6, OT-1 and OT-2 mice were obtained from Charles River Laboratories International, Inc. (L'Arbresle Cedex, France) and maintained in aseptic housing until use. Experimental mice were age and sex-matched. Mice were used between 6 and 12 weeks of age. All animal studies reviewed and approved by the IACUC of the Institut Pasteur, 28 Rue Dr. Roux, Paris, France.

E. Media Reagents. Dulbecco's modified Eagle's medium, 1x (DMEM) (Mediatech, Inc., Herndon, VA, Cat# 15-013-CM) was used throughout. Media was supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, Cat# SH30071.03), 5ml. of nonessential amino acid solution for MEM 100x (cellgro, Mediatech, Herndon, VA, Cat# 25-025-CI), 5ml. of penicillin, streptocmycin, L-Glutamine 100x (Invitrogen, San Diego, CA, Cat# 10378-016), and 50μM β-mercaptoethanol (Calbiochem, San Diego, CA, Cat# 444203). Stored at 4°C. *NB*: New β-mercaptoethanol was added every 6 weeks if entire solution was not used by this point.

II. Analysis of Cell Surface Phenotype.

Single cell suspensions were made from lymph nodes (inguinal, brachial, cervical, axillary) and spleen. Lymph node single cell suspensions were made by physical disruption of nodes through a nitex nylon mesh (Sefar America Inc., Kansas City, MO, Cat# 03-100/32) in 2ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

fetal bovine serum, L-glutamine, nonessential amino acids, penicillin, streptomycin, and 50μM β-mercaptoethanol in 35mmx10mm cell culture dishes (Corning, Corning, NY, Cat#430165). Splenic preparations were used following RBC lysis (Ack Lysing Buffer, Quality Biological, Inc., Gaithersburg, MD, Cat#118-156-101). The surface antigens of primary murine lymphocytes were evaluated by flow cytometry following cell surface staining with 20μg/ml fluorochrome-conjugated antibodies recognizing CD4, CD8, TCRβ, CD90.2, CD69, CD25, CD44, and/or CD62L. All antibodies were obtained from BD Biosciences (San Diego, CA, **CD4-PE** L3T4, GK1.5: Cat#553730, **CD8-FITC** 53-6.7: Cat# 553031, **Biotin anti-mouse CD8α** (Ly-2, 53-6.7) Cat# 553028, **TCRβ -FIT**C H57-597: Cat#553171, **CD90.2-PE** 53-2.1: Cat# 553005, **CD69-FITC** H1.2F3: Cat# 557392, **CD25-FITC** (IL-2R α xhain, p55, 7D4) Cat# 553071, **Biotin anti-mouse CD44** (Pgp-1, Ly-24, 1M7) Cat# 553132, **CD62L-FITC** (L-selectin, LECAM-1, Ly-22, MEL-14) Cat# 553150, **Streptavidin-PE-Cy7** (Sav-PE-Cy7) Cat# 557598. Cells were analyzed by FACS.

III. *In vitro* Cell Stimulations for Proliferation and Cytokine Production.

A. C57BL/6 mice. Single cell suspensions of primary murine lymphocytes were labeled with 2.5μM CFSE (Sigma, St. Louis, MO, Cat# 21888, 100mM stock dissolved in DMSO, store in light-proof vial). Briefly, cells were washed and resuspended in 1x PBS at a concentration of $5x10^6$ cells/ml. An equal volume of CFSE in 1xPBS was added and slowly rotated every minute for five minutes (it is critical to never vortex the CFSE at any stage). Equal volume of ice cold FBS and ~10ml ice cold DMEM complete was added to

stop the reaction. Cells were spun at 309.66xg for 7 minutes, washed 1-2 times with ice cold DMEM complete and resuspended in room temperature DMEM complete and plated at $1.25x10^6$ cells/ml with a total volume of 1 ml in 24 well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ. Cat# 35-3047). In experiments where supernatants were harvested, T-cells were purified by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA. Cat# 130-090-861, used per manufacturer's recommendations) before plating. Cells were stimulated with 0.1-10μg/ml bound or soluble anti-CD3 (145-2C11, BD Pharmingen, San Diego, CA, Cat#553058) and/or 1μg/ml anti-CD28 (BD Pharmingen, San Diego, 37.51, Cat# 557393) antibodies (with or without 10ng/ml phorbol-12-myristate-13-acetate (PMA, Calbiochem, San Diego, CA, Cat# 524400). Cells cultured in DMEM complete were incubated at 37°C, 7.5%CO_2 for 48 hours. For CFSE analysis, cells were stained with anti-CD90.2 for T-cell gating, and intensity of CFSE staining was analyzed by FACS. For IL-2 analysis, supernatants were harvested after 24 hours and analyzed using the OptEIA mouse IL-2 ELISA kit (BD Pharmingen, San Diego, CA, Cat#555148) per manufacturer's protocol. Briefly, 96 well flat-well MaxiSorp plates (NUNC via Fisher Scientific, Pittsburgh, PA, Cat# (F96) 12-565-135) were coated with mIL-2 capture Ab O/N at 4°C. Wells were washed and blocked at RT for one hour. Culture supernatants were incubated at RT for two hours following which the working detector was added for 1 hour at RT and detected with substrate detection and ELISA reading. All substrates purchased with the kit or made following manufacturer's recipe protocols.

*handy reference for conversion of RPM to xg:

https://www.msu.edu/~venkata1.gforce.htm. Otherwise, formula used is $g=(1.118x10^{-5})RS^2$, where g is the gravitational force, R is radius of the rotor, and S is the RPM value.

B. OT-1 mice. Single cell suspensions of primary murine lymphocytes from both Lck W97A and WT OT-1 Tg mice were labeled with 2.5μM CFSE, as described above. Splenocytes were harvested form WT OT-1 Tg mice and used following RBC lysis and irradiation by 2,000 RADS. Cells were plated in 24 well tissue culture plates at a concentration of $2x10^7$cells/ml in 1ml of culture media with a LN:Spl ratio of 1:2. Plates were briefly centrifuged at 150xg for 5sec. before incubation at 37°C with 7.5%CO_2. SIINFEKL peptide was used at doses of 0.003, 0.003, 0.03, 1, and 3μg/ml (Sigma Genosys, The Woodlands, TX, Peptide Cat#99058-1). Cells were harvested at 48 hours and analyzed by FACS as described above.

IV. Activation Marker Induction.

Single cell suspensions of primary murine lymphocytes were plated at $0.5x10^6$ cells/ml in 24 well tissue culture plates and incubated in DMEM complete with various doses of soluble anti-CD3 (2C11) for 4 hours at 37°C, 7.5%CO_2. Cells were then stained with anti-CD90.2 and anti-CD69 conjugated fluorochromes (see reference above for product information) and analyzed by flow cytometry.

V. TCR Signaling Analysis- Western Blot Protocol.

Single-cell suspensions of primary murine T-lymphocytes were purified by negative selection (Miltenyi Biotec, Auburn, CA) and used at a concentration of 2-4 x 10^7

cells/ml in HEPES-buffered saline (25mM HEPES, 125mM NaCl, 5mM KCl, 1mM Na_2HPO_4, 1mM $CaCl_2$, 0.5mM $MgCl_2$, pH 7.4). 50µl cell suspension (~$0.64x10^6$ cell equivalents) was warmed at 37°C and stimulated with 3-5 µg/ml anti-CD3 antibody (2C11) for three minutes. Cells were lysed with 15µl 1%NP-40 with phosphatase and protease inhibitors (*) and insoluble material was removed by centrifugation at 300xg for 10 minutes at 4ºC. Lysates were denatured by the addition of equal volume sodium dodecyl sulfate (SDS) sample buffer with β-Mercaptoethanol (1ml 2x SDS-sample buffer, 50µl 0.2% Bromophenol Blue) and incubation at 100°C for 3-4 minutes. Samples were analyzed by western blotting following SDS-gel electrophoresis and transfer to PVDF membrane Millipore Corporation, Bedford, MA, Immobilon-P, Cat#IPVH00010). Briefly, samples were run on an 8% polyacrylamide gel and run on a Bio Rad electrophoretic apparatus at constant current (12mAmp) and transferred onto an equilibrated PVDF membrane (equilibrated with MeOH and rinsed with 1xTBST) on a Bio Rad Trans-Blot-SD, semi dry transfer machine at 24Volts for 35 minutes. The membrane was then blocked with either a filtered (Fisher Scientific, Pittsburgh, PA, 0.2µm syringe filter. Cat# 190-2520) 3%BSA/1xTBST solution O/N at 4ºC for total mAbs or in a 2-5%milk/1xTBST solution for 15min.-1hr. RT and probed with phospho-mAbs O/N at 4ºC. Primary antibodies recognizing **Lck** (BD Biosciences, San Jose, CA, Cat#610098), **PLC-γ1** (Cell Signaling, Danvers, MA, Cat#2822), **P- PLC-γ1** (Biosource, Carlsbad, CA, P-PLCγ1 Cat#44-696G), **Erk** (Cell Signaling Technology, Inc., Danvers, MA, p44/42 MAP Cat#9102)**, P-Erk,** (Cell Signaling Technology, Inc., Danvers, MA, P-p44/42 MAP

Cat#9101S), **4G10** (Upstate, Charlottesville, VA, Cat#05-321), **ZAP-70** (Cell Signaling Technology, Inc., Danvers, MA, Cat# 2705), **P-ZAP-70** (Cell Signaling Technology, Inc., Danvers, MA, Tyr493, Cat# 2704), **P38** (Cell Signaling Technology, Inc., Danvers, MA, Cat# 9212), **P-P38** (Cell Signaling Technology, Inc., Danvers, MA, Thr180/Tyr182, Cat# 9211S), **P-LAT** (Biosource, Camarillo, CA, Cat# 44-228), **SLP-76** (Cell Signaling Technology, Inc., Danvers, MA, Cat# 4958), and **CamKII** (kind gift of Dr. Tombe's Laboratory, VCU) were detected by incubation with primary antibodies and HRP-conjugated secondary antibodies (Southern Biotech, Birmingham, AL, Cat# 1010-05 HRP-Goat anti-mouse Ig (H+L) human adsorbed, Invitrogen, Carlsbad, CA, Cat# 65-6120 HRP-Goat anti-rabbit IgG (H+L)) and detected using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, Cat# 34080). *for 1 ml lysis buffer: 10µl 1M NAF, 10µl 40M Na_3VO_4, 10µl 100mM PMSF, 10µl 40mM EDTA, 10µl 1mg/ml leupeptin, 959µl 1% NP-40.

VI. Calcium Mobilization.

Single-cell suspensions of primary murine T-lymphocytes were washed and resuspended in warm HEPES-buffered saline (25mM HEPES, 125mM NaCl, 5mM KCl, 1mM Na_2HPO_4, 1mM $CaCl_2$, 0.5mM $MgCl_2$, pH 7.4) at a concentration of $3x10^6$ cells/1.5ml. 1.5µl Fura 2 (Fura-2, AM, Invitrogen, Eugene, OR, Cat# F1221) was added to cell suspension and placed in a 37°C incubator for 30min. 1.5ml HEPES was then added to the cell suspension and cells were spun at 269.75xg for 7min. Cells were washed again with 1.5 ml. HEPES, resuspended in a total volume of 1.35ml HEPES, and incubated at

37°C for 30min. 90µl of cell suspension was added to designated wells of a glass bottom plate (glass bottom 96-well plates No. 1.5, Uncoated, γ-irradiated, MatTek Corporation, Ashland, MA, Part No. P96G-1.5-5-F). 2µl stock biotin-anti-CD3α (2C11) was added to the negative control and stimulation wells. A separate 96-well reagent plate (Becton Dickinson, Franklin Lakes, NJ. Cat# 35-3047) with a base volume of 200µ HEPES was used to load the stimulations. Added to the reagent plate was: 1) warm HEPES alone for negative control, 2) 20µl stock Peroxidase labeled anti-biotin (Vector Laboratories, Burlingame, CA, Cat#SP-3010) to cross-link anti-CD3 mAb in stimulated samples, and 2µl of a 1mM Ionomycin was added for the positive control. Automatic injections of 10µl from the reagent plate were added at a designated injection point and kinetic time points were taken using a NOVO Star plate reader (NOVO Star S/N 700-0122, BMG Labtech, Offenburg, Germany) at 10 flashes per time point. Ratiometric analysis was determined from the ratio of fluorescence at 340- and 380-nm excitation λ (emission 510-520 λ).

VII. *In vitro* Conjugation.

In order to examine conjugate formation between T-and B-cells, OT-2 LN T-cells were harvested from WT and W97A C57BL/6 mice. Splenocytes were harvested and lysed (previously described) from the spleens of OT-2 WT mice and placed at a concentration of $3x10^6$ cells/tube in 15ml Falcon polypropylene culture tubes (Fisher Scientific, Pittsburgh, PA, Cat#352059) (approx. volume 150ul/tube). 500µl 1xPBS was added to each tube and then spun at 309.66xg for 7min. Supernatants were aspirated and cells resuspended in

500µl 1xPBS. 25µl of a 10mg/ml ova stock (Sigma, St. Louis, MO, Chicken ovalbumin, Cat#A5503) solution was added per tube (500µl volume) into 4 of the 5 tubes (the 5th is a negative control). The tubes were then incubated at 37ºC for 1 hr (7.5%CO_2). Following the incubation, the volume of each tube was split into two separate conical tubes A and B (250µl each): so that one half of the cell content is designated A and the other half designated B. The cells were then washed with 1ml 1xPBS. Cells were resuspended in 200µl 1xPBS.

1x10^6 LN from WT mice were added to the A series of tubes, and 1x10^6 cells from W97A C57BL/6 mice were added to the B series of tubes. This equals a T:APC ration of 1:1.5. The tubes were pulse spun up to 500RPM and incubated at 37ºC for 5', 10', 15', and 30' (7.5%CO_2). When one set of tubes (A and B) were removed at the designated time interval, a 1:1 solution of 2% PAF was added and allowed to incubate at RT for 15'. The cells were subsequently washed with 1ml of a 0.5%BSA/PBS (BSA, Sigma, St. Louis, MO, Cat#A-3059) solution and spun at 1200RPM for 5'. Cells were resuspended in 0.5ml of the 0.5%BSA/PBS solution and stored at 4°C O/N.

The following day, the cells were stained by adding 230µl of the cells from each tube into Falcon FACS tubes (Fisher Scientific, Pittsburgh, PA, Cat#352008) and washed with 0.5ml 0.5%BSA/PBS solution. Following centrifugation at 1200RPM, cells were aspirated and resuspended in 30µl staining volume with the addition of 3µl B220-PE and 3µl TCRβ-FITC flurorescent conjugated Abs for 20' on ice in the dark (BD Biosciences Pharmingen, San Jose, CA, B220 (CD45R) RA3-6B2, Cat#553089, TCRβ, H57-597,

Cat#553171). Cells were then washed with 1ml 0.5%BSA/PBS and resuspended in 300µl 1xPBS and analyzed by FACS for conjugate formation (double-staining for TCRβ and B220).

VIII. Ag Immunization.

A. DNP-KLH Ag Immunization. p56Lck W97A mice 4-6 weeks of age, sex- and age-matched with Jackson C57BL/6 wild type (WT) mice, were injected sub-cutaneously (s.c.) at the base of the neck with a 22G1" needle (BD, Franklin Lakes, NJ, Cat#305155) containing 100µg di-nitrophenol-keyhole limpet hemocyanin (DNP-KLH, Calbiochem, San Diego, CA, Cat# 324121) reconstituted in sterile de-ionized H_2O in a 50/50 solution with Titer Max Gold adjuvant (Sigma, St. Louis, MO. T2684-127K1534, CAS 145380-33-2), mixed using glass syringes, on day 0. A boost was performed on day 14 by injecting 50µg DNP-KLH s.c. without adjuvant. Blood was drawn via tail vein nick on days 0 (pre-immunization), 14 (pre-boost), and day 21 at time of sacrifice. Serum was collected by placing blood samples on ice for 1hr. and then spinning at 660.8xg for 5 min. and removing serum to new tube for storage at –20ºC until use.

B. Heat-Killed *Brucella Abortus* Immunization.

Heat-killed *Brucella abortus* was a generous gift of Dr. Fred Finkelman. Stock solution (10% wet weight) was diluted to a 1:25. 200µl of the 1:25 dilution was injected i.p. into groups of either WT C57BL/6 or Lck W97A C57BL/6 mice. Blood was taken at days 4, 7, and 10 following immunization via tail vein nick (cardiac stick on day 10).

Blood was allowed to coagulate 30min-1 hour on ice and spun at 660.8xg for 5 minutes. Serum was collected and stored at –20°C until analysis by ELISA.

C. *In vitro* Antigen Re-Stimulation.

LN cells were isolated from DNP-KLH immunized animals following sacrifice on day 21 (see above). $200x10^5$ lymphocytes were resuspended in 0.1ml media complete in a 96 well flat-bottom plate (Falcon 96 well plate, BD Labware, Franklin Lakes, NJ, Cat#35-3072). Various doses of DNP-KLH were added (0, 1µg, 10µg, 100µg/ml) and incubated for 48 hours at 37ºC, 7.5%CO_2. After 48 hours incubation, 1µCi of [H^3]-thymidine (Perkin Elmer, Waltham, MA) in complete media was added/well and allowed to incorporate for 18 hours. Plates were then harvested using a Filtermate cell harvester onto GFC plates. Assays were read using a Topcount Plate Counter (Perkin Elmer, Waltham, MA).

IX. Helminth Infection.

A. *Nippostringylus brasiliensis* Infection.

Lck W97A mice 4-8 weeks of age, sex- and age-matched with Jackson laboratories C57BL/6 WT mice were injected with 500-700 *Nippostrongylus brasiliensis* L3 larvae s.c. on day 0. Blood was drawn via tail vein nick on days 0 (pre-infection), 8, 11, 14, 18, and 21 at time of sacrifice. *Nippostrongylus brasiliensis* L3 larvae were generously provided by Dr. Dan Conrad and Dr. Joe Urban.

B. *In vivo* Cytokine Capture Assay.

In vivo cytokine capture assay (IVCCA*)* was performed on days 5, 7, 10 and 12 of *Nippostrongylus brasiliensis* infection. To capture IL-4 for serum analysis, 10µg biotin-

BVD4-1D11 anti-IL-4 antibody was prepared in a 2% autologous serum and injected i.p. Mice were bled 3-4 hours post injection and IL-4-biotin-anti-IL4 complexes were detected by coating microtiter ELISA plates with BVD6-24G2.3 and analyzed by luminescent ELISA as previously described (Urban'98). The IVCCA, and analysis of the same was generously provided and performed by Dr. Fred Finkelman's laboratory.

X. ELISAs.

A. Ig ELISAs. For Ig analysis, mouse serum was collected on days 0, 14, and 21 following DNP-KLH immunization or at days, 0, 8, 11, 14, 18, 21 following *Nippostrongylus brasiliensis* infection.

1. Total IgE ELISA. For total IgE ELISAs, 96 well microtiter plates were coated with 10µg/ml BIE3 (Generous gift of Dr. Dan Conrad) in BBS (to 350ml deionized H_2O add: 41.2g Boric Acid (H_2BO_4), 29.2g NaCl, q.s 4L and pH 8.5) O/N at 4°C (50µl/well). Plates were washed twice with deionized water and blocked (1/100 dilution of 10mM Hepes, 2%FCS, in 1xPBS) for 1hr. at 37degC (70µl/well). Plates were washed twice in deionized water and standards (starting at 1,000ng/ml) and serum (at various dilutions) were added for 2 hrs. at 37ºC (50µl/well). Plates were washed twice with water, twice with 1xPBS/0/02% Tween-20, and then twice with water and biot-R1E4 (Generous gift of Dr. Dan Conrad) was allowed to bind for 1 hr. at 37ºC (50µl/well) when plates were washed as previously described. A 1/400 dilution of StreptAvidin –AP (Southern Biotech, Birmingham, AL, Cat#7100-04) was allowed to bind for 1 hr. at 37ºC (50µl/well). Plates

were washed as above, and developed using 100µl/well substrate solution (into 300ml H_2O add: 0.1g $MgCl_2 \bullet 6H_2O$, 0.2g NaN_3, 50ml Diethanolamine, q.s. to 500ml and pH to 9.8. Add 1 P-Nitrophenyl Phosphate Disodium Salt substrate tablets (Sigma, St. Louis, MO, Cat#S0942-200Tab) Tablet/5ml solution for a final concentration of 1mg/ml. Plates were read at OD 405/650 on an ELISA reader.

2. Ag-Specific ELISA. For Ag-specific Ig ELISAs, microtiter plates were coated with 5µg/ml DNP in BBS, or 5µg/ml unlabeled isotype specific goat anti-mouse IgG1, IgG3, or IgG2b O/N at 4degC (Southern Biotech, Birmingham, AL, Cat #1070-01, 1100-01, 1090-01, respectively, 50µl/well). Plates were washed twice with deionized water and blocked for 1 hr. RT. Plates were washed as before and standards and samples were diluted in block buffer and plated for 1hr. at 37ºC (50µl/well). Plates were washed 2x water, 2x PBS/Tween-20, 2x water and goat anti-mouse isotype specific Ap-tagged Abs (Southern Biotech, Birmingham, AL, Cat# 1070-04, 1100-04, 1090-04, respectively) were used at a 1/400 dilution for 45min. at 37ºC (50µl/well). Plates were washed as before and developed using 50µl/well substrate (as described previously). Plates were then read at OD 405/650 on an ELISA reader.

B. IFN-γ ELISA. Blood was collected from experimental mice via tail vein nick on days 4, 7, and 10 following immunization with heat-killed *Brucella abortus*. Serum was collected and analyzed using BD Biosciences BD OptEIA Mouse IFN-γ (AN-18) kit (BD Biosciences-Pharmingen, San Diego, CA, Cat# 551866) per manufacturer's recommended protocol.

XI. *In vitro* T-Cell Differentiation.

In vitro studies were performed by Jane Hu-Li of the Bill Paul Laboratory, NIAID. Briefly, primary murine CD4+ T-cells were negatively purified using a selection of biot-fluorochromes (BD PharMingen) and biot-microbeads using a MACS column. Specifically, FITC-conjugated fluorochromes were used for: **anti-CD8** (53-6.7; Cat#553031), **anti-CD45R/B220** (RA3-6B2; Cat#553088), **anti-I-A^b** (AF6-120.1; Cat#553551- for C56BL/6), **anti-CD16/32** (2.4G2; Cat#553144), **anti-CD24** (M1/69; Cat#553261), **anti-NK1.1** (PK136; Cat#553164-not on BalbC), **anti-CD11b** (M1/70; Cat#553310), **anti-CD49b/Pan NK cells** (DX5; Cat#553857), and **anti-Ly-6G (Gr-1) and Ly-6C** (RB6-8C5; Cat#553127). Cells were then cultured in 24-well plates/1ml volume media with or without 5ng/ml IL-2, and cytokines were added at final concentrations as follows:

For Th1 Differentiation: anti-IL-4 (10μg/ml), Mouse IL-12 (10ng/ml), anti-CD3 (1μg/ml), and/or anti-CD28 (1μg/ml).

For Th2 Differentiation: IL-4 (2ng/ml), anti-IFN-γ1 (10μg/ml), Anti-IL-12 (10μg/ml), anti-CD3 (1μg/ml), and/or anti-CD28 (1μg/ml).

If the cells grew quickly, new reagents should be added (minus the anti-CD3 and anti-CD28 stimulations). Cells were cultured until days 4 or 5 and washed with HBSS. Cells were then resuspended in fresh medium containing 5ng/ml human IL-2 for 2-3 days, re-stimulated with 10ng/ml PMA and 1μM Ionomycin 4-6 hours, and treated with

Monensin (golgi block) before being analyzed for intracellular IL-4 or IFN-γ by FACS analysis.

XII. Mosquito Salivary Gland Preparation.

Harvest mosquitos by placing in 70%ETOH. Wash by performing two subsequent washes in 1xPBS solution. Use micro-wire tweezers to dissect mosquito in a drop of 1xPBS on a glass slide under a microscope. Quickly dislocate head from thorax and tweeze salivary glands from body cavity. Place glands in small microcentrifuge tube containing 1xPBS. When desired glands have been collected, freeze at –20ºC. Thaw at later time and sonicate by 100% 2sec.ON/2sec.OFF pulsing for 20min. Keep on ice. Spin at 1116.75xg for 15min. at 4ºC. Remove and store supernatant at-20ºC until use.

XIII. Ozaki Method for Harvesting Sporozoites from Mosquitos.

Place mosquitos in 70%ETOH. Line mosquitos on the lid of a Petri dish and cut off heads with scalpel. Using tweezers, place heads in a double-barrel centrifuge tube (small porous inner tube contained within a 1.5ml centrifuge tube) containing wool. Cover with parafilm and add 300µl complete media to the top and spin at 951.55xg for 10min. at 4ºC. A pellet will form in the bottom of the tube. Remove the media from the flow through and place back into the top of the tube. Spin at 1295.17xg for 10min. at 4ºC and resuspend the pellet and transfer to a clean tube. Add to Petri counting grid and allow sporozoites to settle 15-30min. before counting.

XIV. Dendritic Cell Differentiation.

Harvest bone marrow from both femurs of a mouse in media complete (flush bone with media three times). Culture marrow in a large Petri dish, undisturbed, at a concentration of $1x10^6$ cells/3ml media supplemented with GMCSF. *Alternatively,* culture in a 24 well plate in 1.5ml media at a concentration of $0.5x10^6$ cells/well. Change the media every 2 days or as necessary by removing/replacing 1ml media. Cells should "cluster". Use cells on day 7 for O/N or same day experiments. Harvest by flushing the wells, do not use a scraper or ice to detach cells.

XV. Harvesting of Peritoneal Macrophages.

Intraperitoneal injection of 1.5ml thioglycolate with Resazurin (BioRad, Cat# 6D0384) to enhance infusion of peritoneal macrophages. Harvest via peritoneal lavage on day 5 by injecting 3-4 ml of complete media into euthanized mouse, agitating belly and inverting mouse, and removal via p1000pipette. Add one more ml of media into mouse using pipette and mixing with the pipette *in vivo* to ensure efficient harvest. Cells will be slightly activated, so it may be necessary to rest cells at 37ºC for 24 hours to get cells back to a baseline level of activation.

XVI. Microsphere Uptake and Activation of Dendritic Cells and Macrophages.

Peritoneal cells were plated at $1x10^6$cells/well in a 24 well tissue culture plate in a volume of ~100-200µl of media complete for 2-3 hours. The media was then removed and 100-200µl media complete was re-administered. 5-10 salivary gland pairs were added

concomitant with 0.1-1μg/ml LPS (positive control for macrophage activation) O/N at 37ºC. Cultures of macrophages were also used without the addition of LPS with the addition of 4ul of a 1:10 dilution in media of FITC-fluoresbrite carbocylate microspheres (Biovalley Polysciences, Cat#15700, 0.5μYG, diameter 0.496μ) for 1hr. Culture plates were placed on ice for exactly 10min. to loosen macrophage attachment to plates. Plates were scraped gently with plate scrapers and stained with **M5** for MHCII (anti-mouse I-A/I/E M5/114.15.2, BD for C57BL/6 mice, rat IgG2bk), **CDllb** (Mac-1, M1/70, cat#557396), **CD80** (activation marker), **CD86** (B7-2, activation marker), and **CD40** (activation marker). Cells were analyzed by FACS for presence and intensity of fluorescence or by fluorescent microscope for visual analysis of microsphere uptake.

XVII. Circumsporozoite (CS) Staining.

Co-culture macrophages and sporozoites on a culture microscope tray and incubate for desired amount of time (10min.-3hrs.) in complete media at 37ºC. Add a 4% PAF solution (Heat 450ml 0.1M Phosphate Buffer, after reaching 45ºC, add 20g paraformaldehyde and stir. When solution clears, remove from heat. Do not let solution go above 60ºC. Cool and filter. Add buffer to make 500ml. Store in refridgerator and use cold. Use within 48 hours or freeze.) for 20min. Remove and wash 2 times with 1xPBS, resuspend in 1xPBS and store O/N at 4ºC (if desired). Permeabilize cells by adding 0.5%Triton at RT for 5min. Wash 3x with 1xPBS. *Alternatively*, cells were not permeabilized and were washed 3times with 1xPBS. Then, 1xpBS/2%BSA was added for 15min. RT to block and washed 3 times with 1xPBS. Anti-CS-PE Ab was added to the

culture slide for 1 hr. at RT and then washed 3x with 1xPBS to detect the levels of CS on the surface of the macrophages using fluorescent microscopy.

XVIII. HepG2 Cell Line and Primary Murine Hepatocyte Analysis for the Presence of Lck.

In order to determine if Lck was present in the HepG2 liver cell line as well as in primary murine hepatocytes, western blot analsysis was performed.

HepG2 cells were cultured and harvested and used at a concentration of $10x10^6$cells/0.5ml. Cells were spun at 7.86xg for 5' and gently aspirated. Cells were resuspended in 1ml 1% NP-40 lysis buffer with inhibitors (see biochemistry protocol above) and incubated for one hour on ice with intermittent vortexing. HepG2 cells were generously provided by the laboratory of Dr. Phil Hylemon, VCU. Bradford analysis of protein content indicated an average of 6,380μg/ml protein.

Primary murine hepatocytes were used following mouse perfusion with saline solution. Primary hepatocyte lysates were made as per HepG2 lysates above, but with a total volume of lysis buffer being 1ml (not 0.5ml). Mouse perfusion performed by the laboratory of Dr. Phil Hylemon, VCU. Bradford analysis of protein content indicated an average of 8,400μg/ml protein.

Both HepG2 and primary murine hepatocyte lysates were run on a 10.5% polyacrylamide gel (see Western blotting protocol above). Membranes were blocked in 3%BSA/PBS solution and probed with anti-Lck COOH polyclonal Ab (see Western blotting protocol for Ab information).

RESULTS

I. Lck W97A Murine Phenotype.

The T-lymphocyte subset of immune effector cells is essential for the formation of a functional adaptive immune response. The development of thymocytes and subsequent activation of naive T-cells relies on signals received via the T-cell antigen receptor (TCR) (Kane, Love, Werlen). A role for the proximal TCR signaling protein, p56Lck, in T-cell development has been implicated (Hernandez-Hoyos, Legname, Molina, van Oers) as well as for T-lymphocyte differentiation and activation (Zamoyska).

Thymocyte development is known to be regulated by the nature of signaling through the TCR, however, much less is known about how TCR signals influence the differentiation and effector function of mature T-lymphocytes. Independent work has implicated the MAP kinase pathway as a critical regulatory pathway for T-cell differentiation (Jorritsma, Yamashita‘99), although it is unclear how modifying TCR signals can specifically regulate MAPK pathway activity to determine the outcome of T-lymphocyte differentiation.

In order to examine the particular roles that Lck has in T-cell development and function, it was necessary to isolate specific interactions of Lck and determine how these

relationships were involved in the processes that lead to thymic development and, ultimately, T-cell activation and function in the periphery. The reliance of Lck on its unique domain for localization within the cell, the role of the SH2 domain for initial T-cell signaling (via binding to the TCR and ZAP-70), as well as the critical importance of the kinase domain for the phospho-tyrosine function of the protein are relatively well-studied. However, the specific binding partners for the Lck SH3 domain, as well as their role and function in T-cell development and activation, are not well understood.

To further examine the role of the Lck SH3 domain *in vivo*, a murine line was created by our laboratory. This was accomplished by using a targeting construct with genomic *lck* sequences of the 129 mouse line. The genomic sequence encompassed 3kb upstream of the *lck* gene into exon 8. A neomycin resistance cassette was introduced at exon 3 and flanked by lox sites. PCR was used to create a critical tryptophan to alanine point mutation in exon three. This particular mutation has previously been shown in cell line data to ablate ligand binding to the SH3 domain of Lck, and creates an Lck SH3 domain null phenotype (Denny'99, Patel). Neomycin-resistant embryonic stem (ES) cell clones were selected and correctly targeted clones were detected by Southern blotting with a probe recognizing a region of exon 3, 3'to the targeted/modified sequence. Blastocysts were injected, and animals containing the mutation were backcrossed onto the C57BL/6 murine line. Mating with Jackson Laboratory Tek-Cre mice subsequently removed the neomycin resistance cassette. Reverse-transcription (RT)-PCR sequencing of the entire Lck coding region of thymocyte RNA confirmed the *lckW97A* allele mutation, as well as assured that no other mutations were introduced into the construct (Rudd'06).

Mice containing the Lck W97A mutation were backcrossed to the F8 generation with the Jackson Laboratory C57BL/6 line. Initial investigation of thymic development in these mice identified reduced CD4+ and CD8+ thymocyte population development and maturation. There was also a noticeable reduction in MAPK pathway induction (Erk) independent of initial TCR signaling events, which were found to be unaltered in Lck W97A thymocytes compared to WT. (Rudd'06). This work showed for the first time that Lck had the potential to play a role in downstream pathways critical for T-cell activation, irrespective of the role for Lck in initial TCR signaling events, and that this process was mediated by the action(s) of the Lck SH3 domain.

Rudd et al. further showed that there were approximately half the total thymocyte numbers in Lck W97A mice compared to WT, and that these mice had two-fold reductions in double-positive thymocytes, possibly as a result of a block in development involving the β-selection step. There was also a two- to three-fold reduction in the number of CD4+ and CD8+ thymocytes, indicating defective positive selection in Lck W97A mice. Single-positive T-cells were found in the periphery of Lck W97A mice.

p56Lck is essential for the activation and differentiation of T-lymphocytes, however, it is unclear if Lck is able to act via specific mechanisms in order to regulate this process (Palacois, Molina, Mosmann). It is also unknown to what extent this mutation affects T-cell activation and function in the periphery following thymic exodus. An investigation into the phenotype and characteristics of the peripheral T-cell populations in Lck W97A mice is investigated here.

A. Phenotypic Characterization of Primary Lck W97A T-lymphocytes.

As with all new animal models, it is important to note general appearances and behavioral effects that may arise due to the knocking-in or –out of a gene of interest. With regards to the Lck W97A mice, no gross physiological abnormalities have been observed. The mice are fertile and healthy.

1. Similar Lck W97A Total Cell Counts Compared to WT. In order to examine more subtle phenotypic differences in Lck W97A mice, total cell counts from two major secondary lymphoid structures, the lymph node and spleen, were each analyzed in Lck W97A mice. Although thymic data from these mice suggested there might be differences in total numbers of T-cells in the periphery as a result of reduced thymic numbers (Rudd '06), analysis of secondary lymph structures did not indicate any substantial differences in total numbers of cells compared to WT (Fig. 4). It is possible that homeostatic proliferation of thymic SP T-cells occurs following thymic exodus to elevate the number of Lck W97A T-cells to a more "normal" physiological level in the periphery. If this were the case, one would expect these T-lymphocytes to have slightly elevated levels of activation, compared to WT, as a result of their heightened proliferation.

2. Lck W97A Animals Have a Reduced TCRβ Population. A closer look at the T-cell population in Lck W97A animals does, however, demonstrate a significantly reduced percentage (5-15% reduction) of TCRβ+ cells, with the total number of TCRβ+ being only slightly reduced compared to WT (Fig. 5). As the cumulative data shown is comprised of only five individual mice, is possible that with further examination of the total number of TCRβ+ cells in the Lck W97A murine population, this value may in fact be significant, reflecting the observed reductions in the percent TCRβ+ population of total

Fig 4. Similar total cell counts in secondary lymph organs between Lck W97A and WT mice. Single cell suspensions from lymph node and spleen were washed and counted. Cumulative data from 5 experiments is shown. Statistical analysis was performed using Student's T-test.

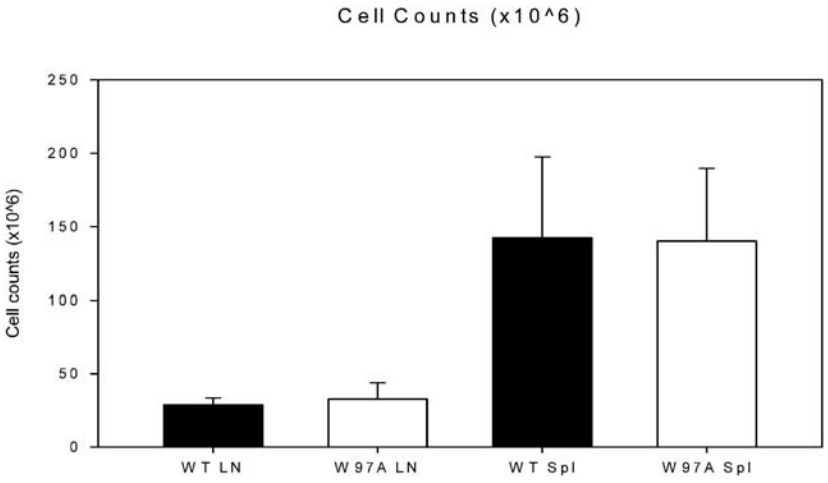
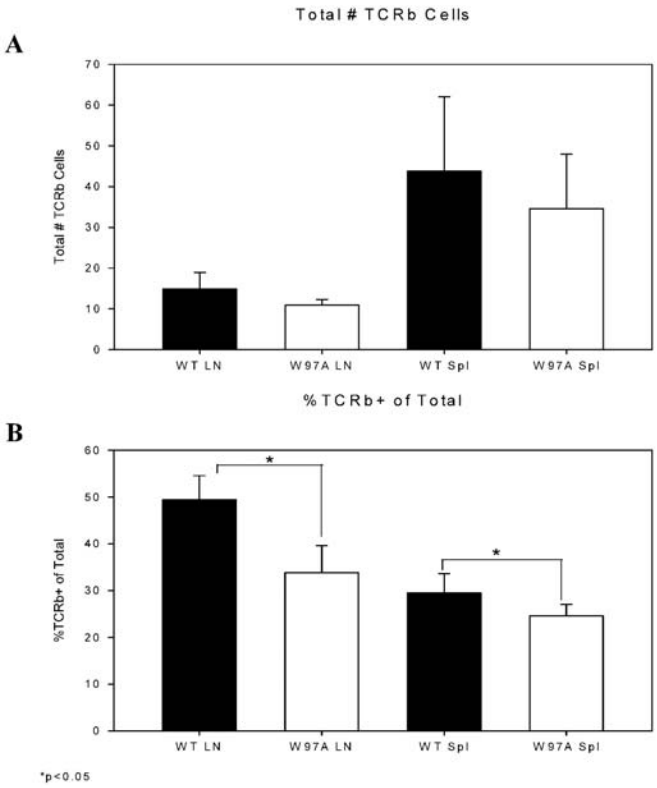


Fig 5. Significantly reduced percentage of TCRβ$^+$ Cells in Lck W97A mice. Single cell suspensions from lymph node and spleen were counted and stained with fluorochrome conjugated TCRβ antibody and analyzed by flow cytometry. A) Total numbers of TCRβ$^+$ cells in lymph node and spleen from WT and Lck W97A mice. B) TCRβ$^+$ cells shown as a percentage of total lymph node or spleen cell counts. Cumulative data from 5 experiments is shown. Statistical analysis was performed using Student's T-test.



cell counts from the lymph node and spleen. Alternatively, there could be another population of cells that have expanded in these secondary lymphoid organs to compensate for reduced T-cell numbers, and analysis of different cell population numbers should be examined. This finding led us to examine the specific T-cell compartments within the lymph node and spleen in Lck W97A mice in order to determine if there were differential requirements for the Lck SH3 domain in T-cell differentiation and/or survival in the periphery.

3. Reduced Numbers of Lck W97A CD8+ T-Cells. Analysis of the different T-cell compartments within the lymph node and spleen indicates significantly reduced numbers of CD8+ T-cells in Lck W97A animals, with the CD4+ T-cell compartment being only slightly reduced (Fig. 6). This significant reduction in numbers of CD8+ T-cells helps to explain reductions in the percent TCRβ population in Lck W97A mice, and also indicates that the Lck SH3 domain may play a differential role in T-cell subset development and/or survival in the periphery.

Aside from total numbers of cells, and understanding what percentage of the T-cell population expresses the unique co-receptor markers, it is also important to examine co-receptor expression differences between WT and Lck W97A T-cells.

4. Reduced CD4 Co-Receptor Expression in Lck W97A Peripheral T-Cells. Co-receptor and TCRβ surface expression on peripheral T-cells from Lck W97A and C57BL/6 WT mice was examined from both LN and spleen. CD8 and TCRβ cell surface expression levels were normal in Lck W97A mice with CD4 expression being modestly reduced (Fig. 7). These data, taken together with the distribution data previously described,

Fig 6. Modest reduction in peripheral T-cell numbers in Lck W97A mice.

Single cell suspensions from lymph node and spleen were counted and stained with fluorochrome conjugated antibodies and analyzed by flow cytometry. CD4 and CD8 data are shown for total cell populations in lymph node and spleen. Cumulative data from 4-5 experiments is shown. Statistical analysis was performed using Student's T-test.

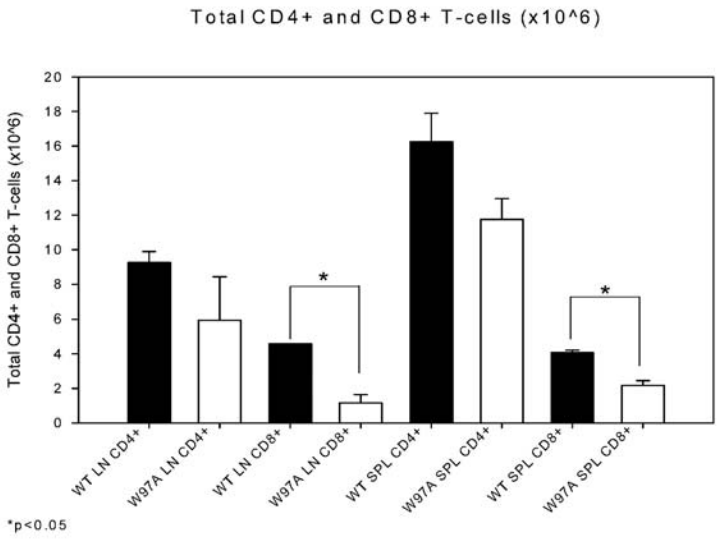
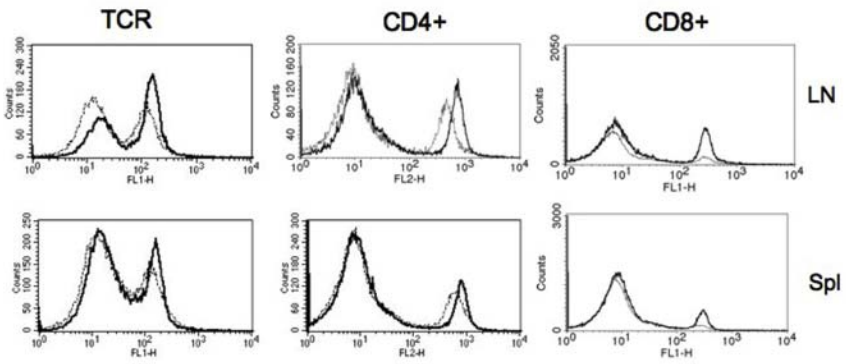


Fig 7. Modest reduction in CD4 expression in Lck W97A mice. Single cell suspensions from lymph node and spleen were counted and stained with fluorochrome conjugated antibodies and analyzed by flow cytometry. Representation of $TCR\beta^+$, $CD4^+$ and $CD8^+$ T-cells from lymph node and spleen of WT and LckW97A mice. CD4 and CD8 data are shown gated on $TCR\beta^+$ populations. Data is representative of 5 experiments.



Bold= WT
Thin= W97A

suggest that the SH3 domain of Lck is important for the establishment of proper T-cell population numbers as well as normal CD4 expression in primary T-lymphocytes.

5. Basal Levels of the Activation Markers CD44 and CD62L in Lck W97A T-Cells are Significantly Different. The concept that Lck W97A T-cells may be proliferating in an attempt to regain homeostatic balance as a result of reduced thymic output, it was important to look at basal expression of markers known to be involved in the activation-induced proliferation that accompanies homeostatic proliferation, specifically $CD44^{hi}$ and $CD62^{low}$ expression. This ability of T-cells to fill a lymphopoenic "void" is not well understood. It is believed that T-lymphocytes are able to sense "space", potentially as a result of decreased competition for cytokines, such as IL-7, IL-12 and IL-15, as well as the more controversial MHC/self-peptide interactions (Cho, Goldrath). For this study, we examined the total T-cell compartment, as well as the unique CD4+ and CD8+ compartments, in order to determine if there were inherent differences between these two subsets. We found that there are significant 10-15% increases in the percentage of Lck W97A lymphocyte populations expressing CD44, a marker of activation and memory, and those of WT (Fig. 8a). There is also a significant 10-20% increase in the expression of CD44 between WT and Lck W97A T-cells. Lck W97A T-lymphocytes have a significantly higher MFI expression of CD44 on both CD4+ and CD8+ cells in the lymph node and splenic compartments compared to WT (Fig. 8b). This elevated level of CD44 expression on Lck W97A T-cells could indicate a higher level of activation, or be indicative of a memory T-cell phenotype.

Fig 8a. $CD44^+$ populations are significantly increased in Lck W97A mice. Single cell suspensions from lymph node and spleen were counted and stained with fluorochrome conjugated antibodies and analyzed by flow cytometry. A) Total $CD44^+$ T-lymphocytes, B) $CD4^+$ gated $CD44^+$ T-lymphocytes, C) $CD8^+$ gated $CD44^+$ T-lymphocytes. Cumulative data from lymph node and spleen of WT and Lck W97A mice. n=4. Statistical analysis performed using Student's T-test.

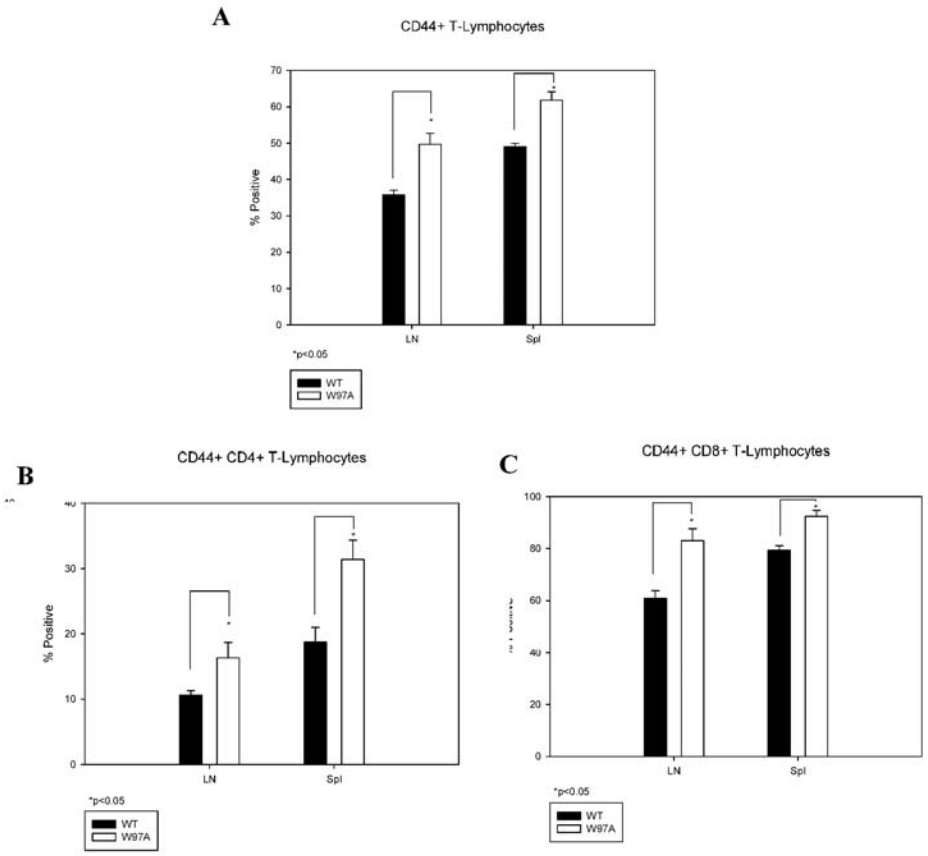
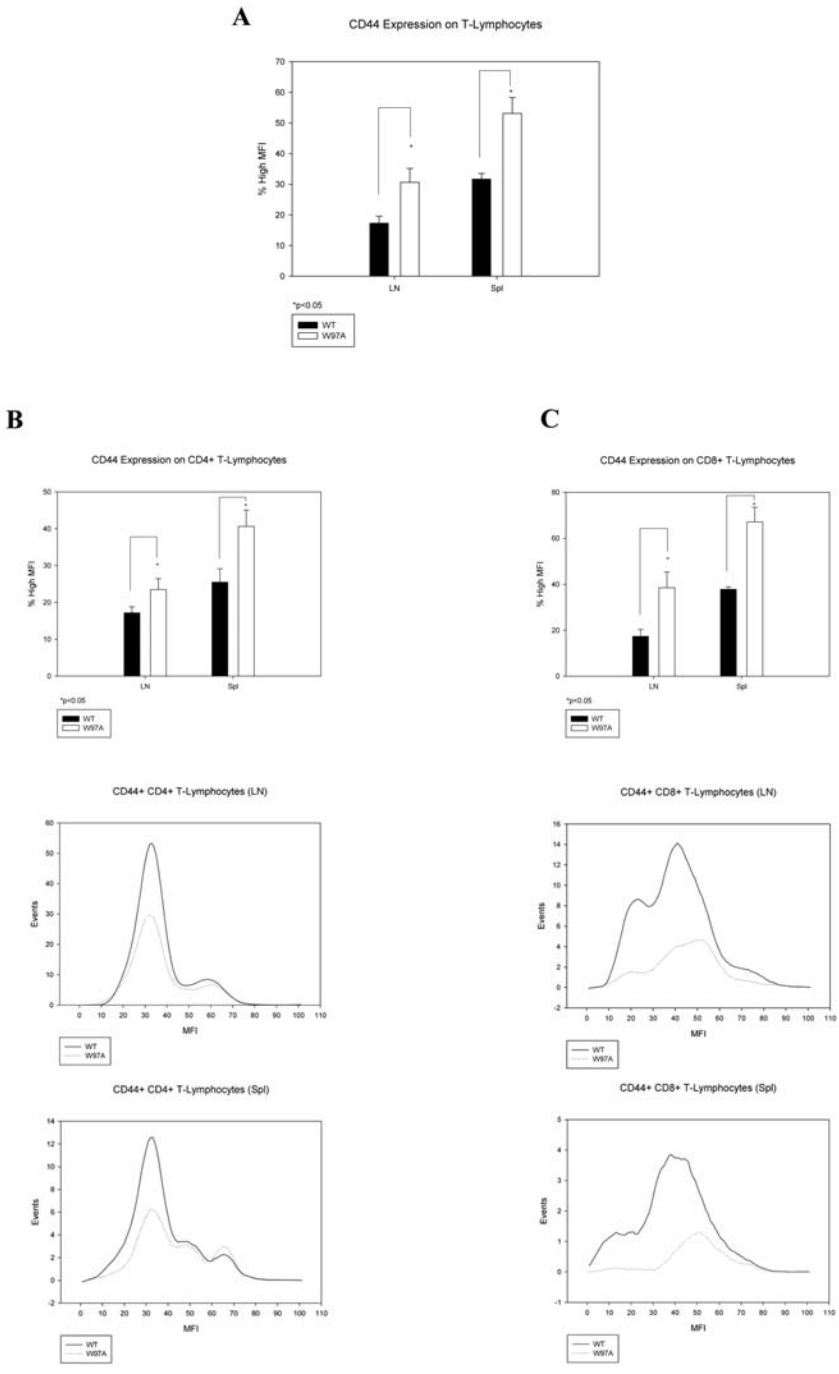


Fig 8b. CD44 expression is significantly increased on Lck W97A T-lymphocytes. Single cell suspensions from lymph node and spleen were counted and stained with fluorochrome conjugated antibodies and analyzed by flow cytometry. A) CD44 expression on total T-lymphocyte population. B) CD44 expression on $CD4^+$ gated T-lymphocytes from lymph node and spleen. C) CD44 expression on $CD8^+$ gated T-lymphocytes from lymph node and spleen. Cumulative data from lymph node and spleen of WT and Lck W97A mice. n=4. Histogram data are representative of 4 independent experiments. Statistical analysis performed using Student's T-test.



We also notice significantly reduced numbers of the Lck W97A CD4+ lymphocyte population expressing CD62L (L-selectin) (~10%) (Fig. 9a). It is possible that there are significant reductions in the percent CD62L+ CD8+ T-cell population in Lck W97A mice, however, the standard deviation and error are too great from these experiments to be able to draw firm conclusions. Although there was no significant reduction in the MFI expression of CD62L in Lck W97A T-lymphocytes, it is also possible with larger sample size, the trend observed that the Lck W97A mice have slightly reduced CD62L expression could become significant (Fig. 9b). This data, taken together, could indicate that these cells are indeed slightly more activated in response to homeostatic proliferation in order to fill a T-cell "void" in the periphery- as they would classically have elevated levels of CD44 expression and reduced levels of CD62L. Furthermore, this heightened state of activation could also negatively affect the degree to which Lck W97A T-cells can be activated in response to stimuli.

II. T-Cell Activation in Lck W97A T-Lymphocytes.

A. Ab-Induced Proliferation and IL-2 Production. T-lymphocyte activation is the result of TCR ligation and subsequent induction of intracellular signaling pathways, such as the MAPK and PI pathways. TCR signaling relies on non-receptor tyrosine kinases to link receptor engagement with activation of intracellular signaling pathways (Mosmann). The Src-family of protein tyrosine kinases (PTKs), in particular, Lck, are important for TCR and ZAP-70 phosphorylation which leads to the regulation and

Fig 9a. $CD4^+CD62L^+$ populations are reduced in Lck W97A mice. Single cell suspensions from Lymph node and spleen were counted and stained with fluorochrome conjugated antibodies and analyzed by flow cytometry. A) Total $CD62L^+$ T-lymphocytes. B) $CD4^+$ gated $CD62L^+$ T-lymphocytes. C) $CD8^+$ gated $CD62L^+$ T-lymphocytes. Cumulative data from lymph node and spleen of WT and Lck W97A mice. n=4.Statistical analysis performed using Student's T-test.

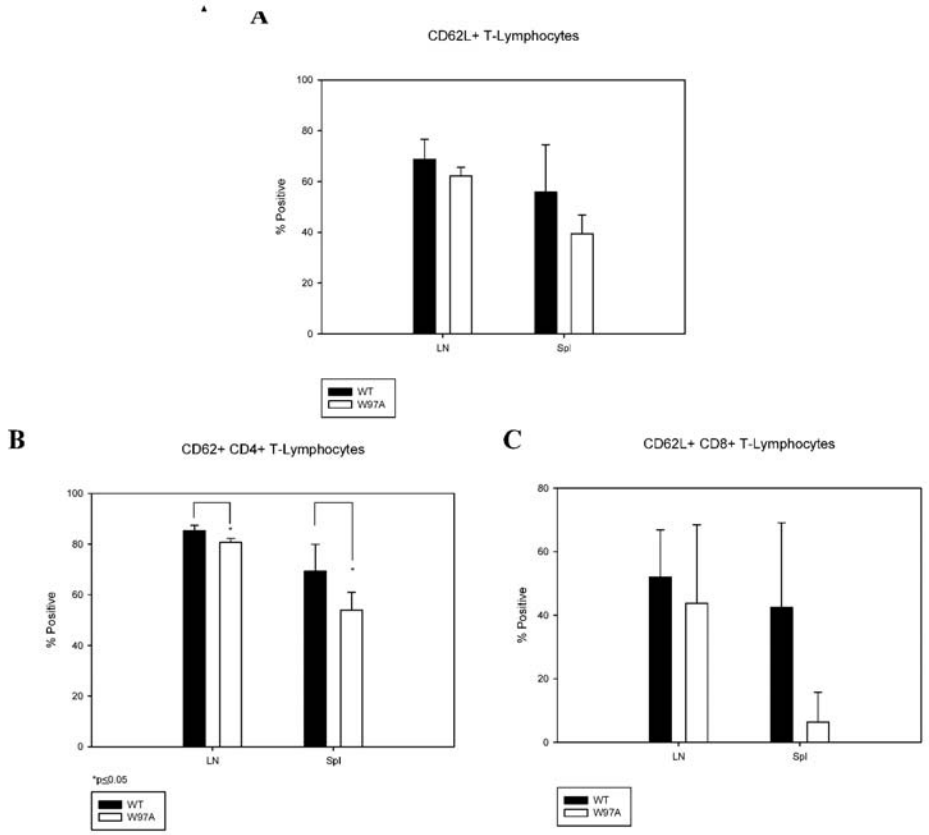
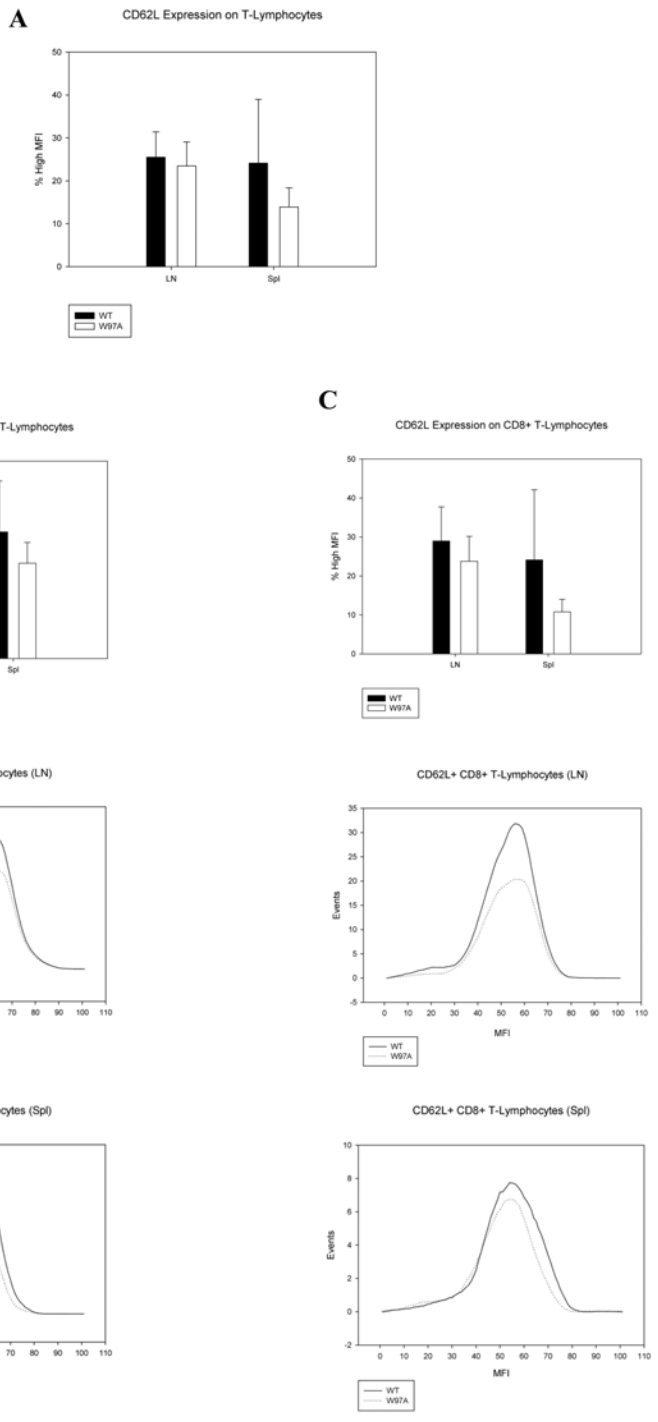


Fig 9b. CD62L expression is similar between Lck W97A and WT T-lymphocytes. Single cell suspensions from lymph node and spleen were counted and stained with fluorochrome conjugated antibodies and analyzed by flow cytometry. A) CD62L expression on total T-lymphocytes. B) CD62L expression on $CD4^+$ gated T-lymphocytes. C) CD62L expression on $CD8^+$ gated T-lymphocytes. Cumulative data from lymph node and spleen of WT and Lck W97A mice. n=4. Histogram data are representative of 4 independent experiments. Statistical analysis performed using Student's T-test.



recruitment of PI and MAPK pathway activators in T-lymphocytes (Chan, Iwashima, Lovatt, Palacios, Straus'92, Zamoyska).

As p56Lck is known to be important for T-cell activation, and differences in the expression of activation markers were observed in Lck W97A peripheral T-cells (Figs. 8-9), potential defects due to loss of function of the Lck SH3 domain in Lck W97A mice were examined by observing outcomes of T-lymphocyte activation. We assessed proliferation, cytokine production and early activation marker expression in T-cells from Lck W97A mice using anti-CD3 to stimulate Lck W97A T-cells *ex vivo* in order to examine potential differences in Lck W97A T-cell activation compared to WT.

Proliferation studies were performed by staining LN cells with CFSE and stimulating with the anti-CD3 mAb, 2C11. Lck W97A T-lymphocytes show a consistent 3-4.5 fold reduction in the number of T-cells that are able to respond to TCR ligation compared to WT. This difference is observed with either high or low doses of anti-CD3, although higher levels of anti-CD3 stimulates greater proliferation of Lck W97A T-cells, and cells that do respond undergo the same number of divisions as WT (Fig. 10). To confirm our studies on the activation induced proliferation defect observed in Lck W97A T-cells, we examined the levels of secreted IL-2 in stimulation cultures. IL-2 is commonly used as readout of T-cell activation (Irving). Analysis by ELISA indicated a 10-fold reduction in the level of IL-2 in Lck W97A culture supernatants compared to WT at 24 hours following 1μg/ml anti-TCR stimulation (Fig. 11). These findings indicate that Lck W97A T-cells have significantly impaired activational responses compared to WT, and

Fig 10. Lck W97A T-cell proliferation is significantly reduced following anti-TCR stimulation compared to WT. T-cell proliferation following *in vitro* stimulation. CSFE labeled peripheral lymph node cells were stimulated with indicated doses of anti-CD3 antibody. Data show fluorescence histograms of CFSE labeled T-cells following 48hrs. of stimulation. Representative of 4 independent experiments. Statistical analysis performed using Student's paired T-test.

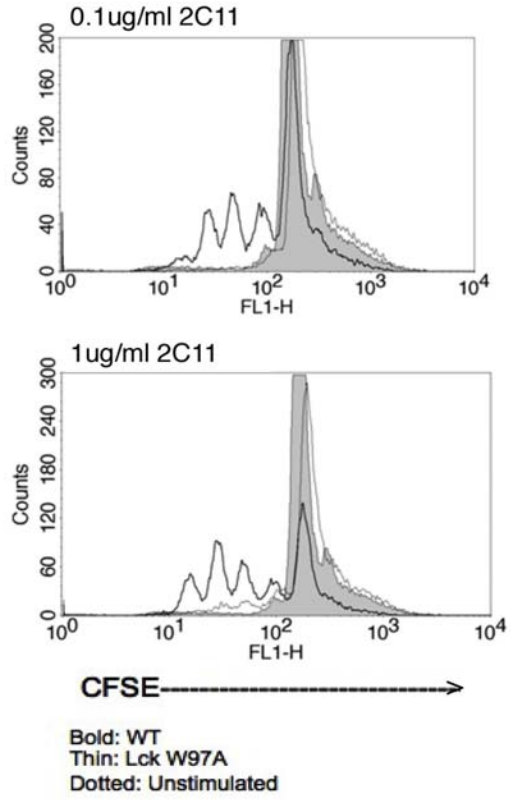
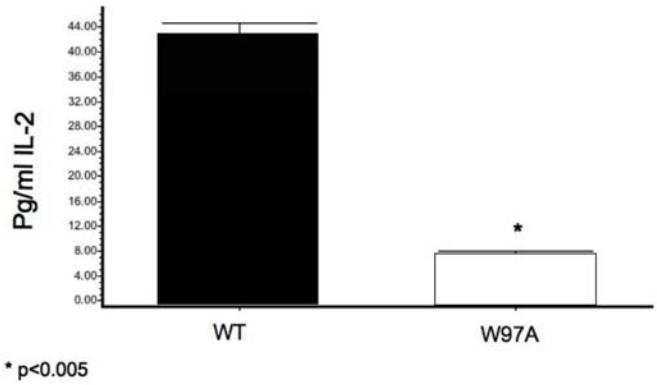


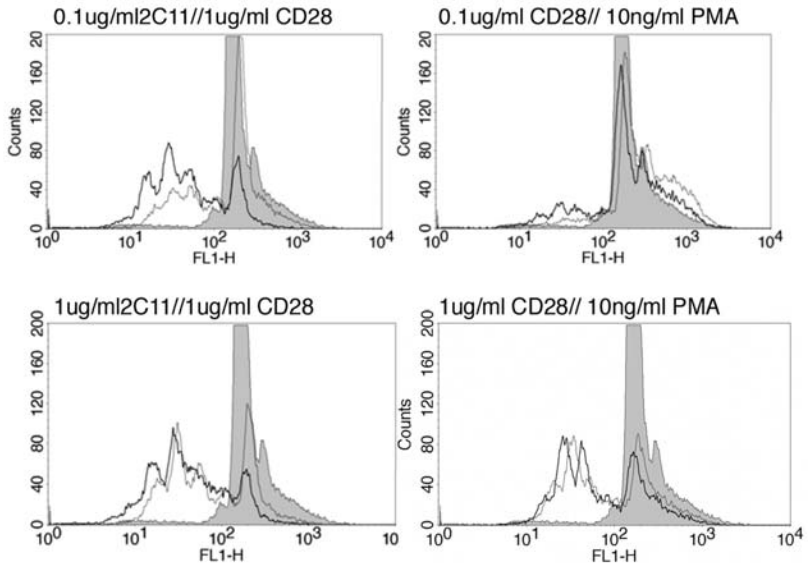
Fig 11. Lck W97A IL-2 production is significantly reduced following anti-TCR stimulation compared to WT. Peripheral lymph node cells were purified by negative selection and stimulated with 1μg/ml anti-CD3 antibody. Culture supernatant levels of IL-2 were measured by ELISA. Cumulative data shown. n=4. Statistics analyzed using Student's T-test.



though higher Ab stimulations appear to improve Lck W97A T-cell proliferation, there remain significant defects in proliferation, as well as in the production of IL-2.

B. Co-Stimulation With CD28 Modestly Improves Lck W97A T-Cell Proliferation. T-cell activation, as well as T-dependant B-cell responses, is assisted by signaling via co-stimulatory molecules such as CD28 (Acuto). It has been shown that Lck binds to CD28 via the SH3 domain of Lck, and that this interaction may assist TCR signaling for complete T-cell activation (Holdorf). We wished to examine the potential effects on activation of Lck W97A T-cells with co-stimulation of CD28 in order to determine the role that Lck, and, in particular, the SH3 domain of Lck, facilitates the intracellular signaling cascades involved downstream of proximal TCR signaling leading to T-cell activation. The CD28 co-receptor is known to bind the SH3 domain of p56Lck, suggesting that mutation of the p56Lck SH3 domain might lead to a reduction in co-stimulatory signal transduction efficacy (Mosmann). Surprisingly, we found that CD28 receptor engagement concomitant with TCR ligation in Lck W97A T-lymphocytes strongly stimulated proliferation so that there were approximately 1-1.5 fold more T-cells able to respond to stimulation (Fig. 12). As with activation-induced proliferation following anti-CD3 stimulation (Fig. 10), higher doses of anti-CD3 appear to improve the ability of Lck W97A T-cells to respond to stimulation with the addition of anti-CD28 co-ligation (Fig. 12). However, the population of undivided cells still remains increased roughly 3-fold in Lck W97A T-cells (Fig. 12) notwithstanding CD28 co-receptor engagement when compared to WT. In order to examine the potential effect of the Lck W97A mutation on CD28 signaling in isolation from that of the T-cell receptor, the cellular response to CD28

Fig 12. Lck W97A T-cell proliferation is mitigated but reduced following anti-TCR/anti-CD28 stimulation compared to WT. T-cell proliferation following *in vitro* stimulation. CSFE labeled peripheral lymph node cells were stimulated with indicated doses of anti-CD3 antibody with, or without 1μg/ml CD28 antibody, or CD28 antibody with 10ng/ml PMA. Data show fluorescence histograms of CFSE labeled T-cells following 48hrs of stimulation. Representative of 4 independent experiments.



and PMA was examined. The p56Lck SH3 domain makes little contribution to CD28 signals that are independent of pathways stimulated by PMA (Fig. 12). These studies indicate that Lck SH3 domain function is required for proper T-lymphocyte activation, and that the Lck SH3 domain appears to be primarily involved in mediating signaling through the TCR rather than CD28.

C. Activation Marker Induction Following Ab-Induced Activation is Reduced in Lck W97A Peripheral T-cells. Following our observations that Lck W97A T-cells appear to have significant defects in activational responses, we wanted to further examine parameters of cell activation, including investigation of induction of the early activation markers CD69 and CD25 (IL-2Rα). CD69 induction following 2C11 stimulation is significantly reduced by 10-30 percent in Lck W97A mice using various doses of anti-CD3 (Fig. 13a). Although the level of expression on responding cells is comparable with that on WT T-lymphocytes, the percentage of T-cells able to induce CD69 is significantly reduced (Fig. 13a). These data indicate a defect in the activation of Lck W97A T-cells following anti-TCR specific stimulation resulting from a loss of Lck SH3 domain function. Preliminary analysis of CD25 expression on Lck W97A T-cells, another marker of early activation, indicates that this marker is elevated to a greater degree basally, and remains modestly elevated over a period of 4-31 hours following stimulation with anti-CD3 antibody, compared to WT (Fig. 13b). Further investigation into this phenomenon will be required to get a clear picture of these differences in CD25 expression. However, it appears that Lck W97A T-cells do have defective CD69 induction, which correlates to the

Fig 13. Lck W97A induction of CD69 is significantly reduced following anti-TCR stimulation compared to WT. Induction of early activation marker expression following *in vitro* stimulation. A) Peripheral T cells were stimulated with indicated doses of anti-CD3 for 4 hrs and stained for CD69 expression. Data show the percent of T cells expressing elevated levels of CD69 (MFI >20). Statistical analysis performed using Student's paired T-test using data from 3 independent experiments. B) Peripheral T-cells were stimulated with 3μg/ml anti-CD3 for 0-31 hours and stained for CD25 expression. Data show the percentage of T-cells expressing CD25. n=1.

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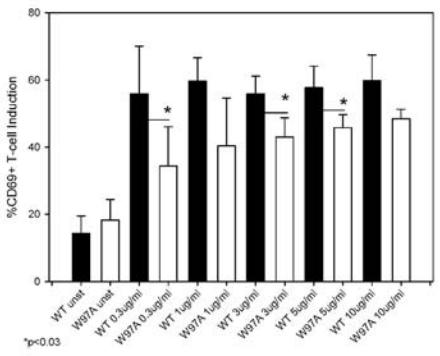

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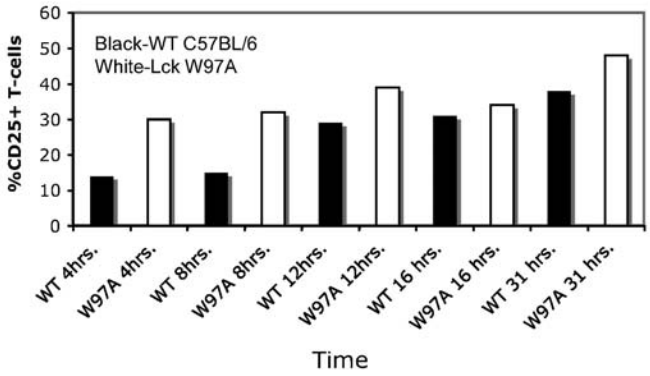
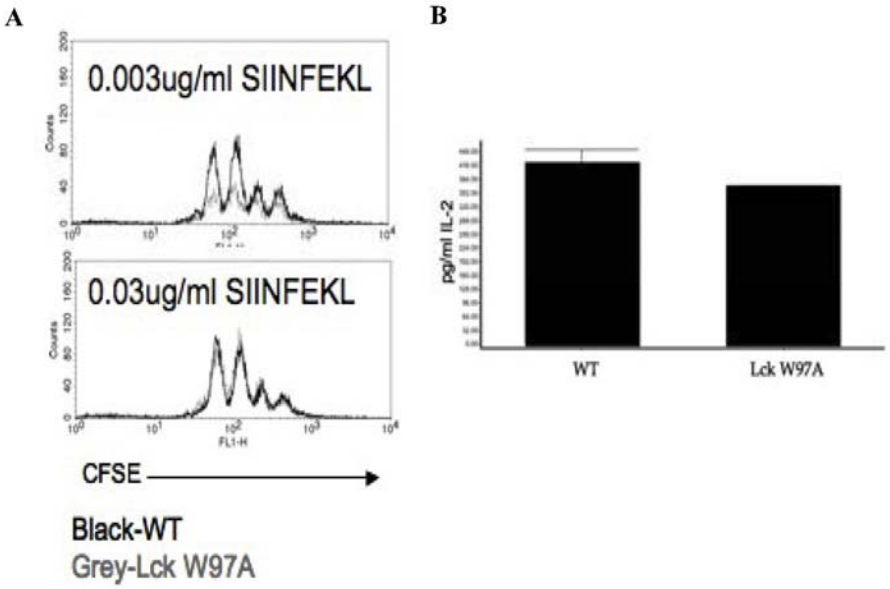


observed reductions activational responses as examined by both proliferation and IL-2 production following Ab-induced activation.

D. Ag-Induced Activation is Modestly Reduced in Lck W97A Peripheral T-Cells. Observing the importance of the Lck SH3 domain in MAPK pathway induction and T-cell activation following TCR-specific ligation, as indicated by reduced activation-induced expression of CD69 (Fig. 13), proliferation (Fig. 10) and IL-2 production (Fig. 11), even with the assistance of the co-stimulatory molecule CD28 (Fig. 12), we wanted to investigate the importance of multiple co-stimulatory signals on the activation state of Lck W97A T-cells to determine if multiple signals could assist in overcoming these observed activational defects. In order to examine complex co-stimulation, *in vitro*, p56Lck W97A mice were backcrossed onto mice expressing the OT-I transgenic (Tg) T-cell receptor (restricted for K^b/SIINFEKL peptide) for three generations. Peripheral T-cells were stimulated in an antigen-dependent fashion to determine differences in proliferation and cytokine production.

Various doses of SIINFEKL peptide were used to examine potential differences in the responses of Lck W97A T-cells to antigen presentation compared to WT. Ag-induced proliferation of Lck W97A OT-I T-cells was modestly reduced (approx. 2-fold) at lower concentrations of SIINFEKL peptide, compared to WT, with higher peptide doses mitigating proliferation and IL-2 production to a similar level with that of WT (Fig. 14). The Lck W97A T-cells appear to be able to have similar rounds of division when compared with that of WT, but the percentage of cells that undergo this proliferation is severely reduced, especially at later rounds of division (Fig. 14). This is only a preliminary

Fig 14. Lck W97A OT-1 T-cell Ag-induced activation is modestly reduced compared to WT A) OT-1 T-cell proliferation following *in vitro* stimulation. CSFE labeled peripheral lymph node cells were stimulated with indicated doses of SIINFEKL peptide. Data show fluorescence histograms of CFSE labeled T-cells following 48hrs of stimulation with peptide and irradiated WT splenocytes. Representative of 1 independent experiment. B) IL-2 levels secreted by T-cells stimulated with 20μg/ml peptide. Culture supernatants were measured for levels of IL-2 using ELISA. Cumulative data from 2 independent experiments. Statistics analyzed using Student's T-test.



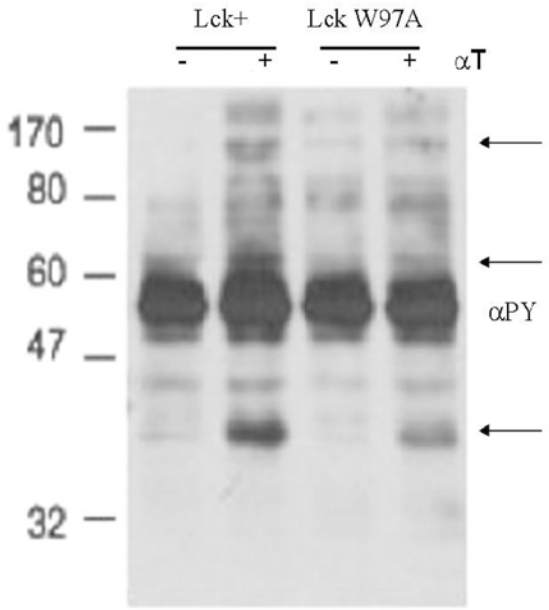
investigation into the ability of Lck W97A T-cells to respond to Ag-specific stimulation and should be repeated with various doses of SIINFEKL as well as kinetic analysis performed to determine whether the Lck W97A T-lymphocytes peak at different times following stimulation compared to WT. The observed decrease in reliance of Lck SH3 domain for T-cell activation with Ag *in vitro,* especially with stronger Ag stimulation, led us to examine if there were any potential differences in T-effector function and immunity *in vivo*.

III. T-Cell Signaling in Lck W97A T-Lymphocytes.

To understand the basis for defective activation of T-lymphocytes from Lck W97A mice, regulatory pathways critical for cellular activation downstream of TCR signaling were examined. In particular, we wished to examine the MAP kinase pathway, which we have previously found to be regulated by Lck SH3 domain function in thymocytes (Rudd'06).

A. Reduced Phospho-Tyrosine Induction in Lck W97A Peripheral T-Cells. To analyze signaling, we purified peripheral LN T-cells from Lck W97A and WT mice using negative selection (to eliminate any basal levels of activation that positive sorting might incur) and stimulated with anti-CD3. Western analysis of lysates probed with anti-phosphotyrosine (PY) antibodies did not indicate substantial differences in the levels of tyrosine phosphorylation in anti-CD3 stimulated Lck W97A peripheral T-cells (Fig. 15). Although the pattern of PY induction appears similar between groups and treatments, several proteins have slightly reduced levels, as indicated by lower phospho-tyrosine

Fig 15. Similar levels of phosphotyrosine induction in Lck W97A T-cells compared to WT. Purified lymph node T–cells were stimulated with 3-5μg/ml anti-CD3 antibody (2C11) for 3 min and 1% NP-40 lysates were analyzed by western blotting with the indicated antibodies. Representative protein tyrosine phosphorylation induced by TCR stimulation. n=6.

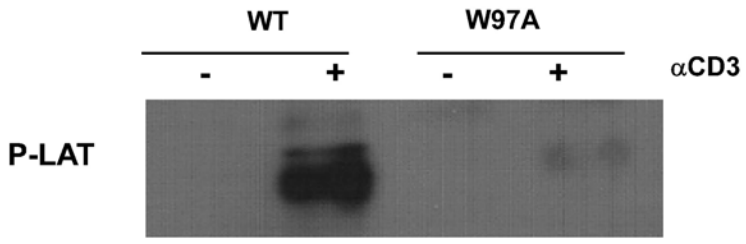


induction, following 2C11 stimulation in Lck W97A peripheral T-cells (Fig. 15). It is possible that several of these proteins may represent activation defects of signaling proteins known to bind the Lck SH3 domain and be activated by Lck, or are downstream targets of Lck SH3 domain function.

B. Adaptor Molecules in Lck W97A T-Cells Appear Differentially Regulated.

Our interest in various bands that appear differentially regulated in PY immunoblot analysis of Lck W97A T-cells following anti-CD3 stimulation (Fig. 15) led us to examine several proteins known to interact with Lck or Lck binding partners that are about the observed sizes noted in the anti-PY immunoblot analysis. Preliminary data, as analyzed by Western immunoblot, suggests that activation of the adaptor molecule, LAT, is significantly reduced in peripheral Lck W97A T-cells following anti-CD3 Ab-induced activation (Fig. 16). This protein runs about 38kDa, and correlates to a major band that appears reduced on the PY immunoblot (Fig. 15). LAT is an important adaptor molecule, acting as a scaffolding protein for various signaling proteins near the TCR. Moreover, data from our lab in Lck W97A thymocytes has also indicated that there is reduced phosphorylation of LAT following anti-TCR stimulation (Rudd ‘06), indicating that the Lck SH3 domain may play a role in the activity and function of LAT following TCR ligation. This result will need to be further investigated in order to discern if this is a representative finding. A control blot recording total LAT levels will also be required to confirm that this finding is truly reflective of a difference in activation of this molecule, and not a result of different levels of total LAT in Lck W97A T-cells.

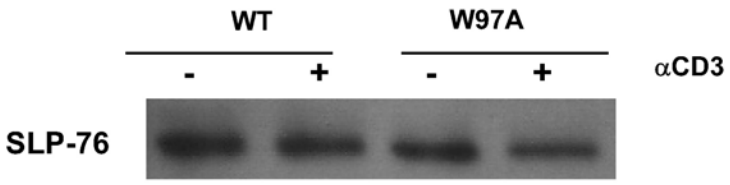
Fig 16. Impaired LAT induction following TCR stimulation in Lck W97A T-cells. Purified lymph node T–cells were stimulated with 3-5μg/ml anti-CD3 antibody (2C11) for 3 min and 1% NP-40 lysates were analyzed by western blotting with anti-P-LAT. Total levels of protein were examined by coomassie staining. n=1.



Another molecule of interest is SLP-76. This adaptor molecule is believed to be a part of the LAT complex proximal to the TCR following TCR ligation and, thus, has been implicated in MAPK/Erk pathway induction. Furthermore, SLP-76 has been shown to bind the Lck SH3 domain *in vitro* (Sanzenbacher), and was of interest to us as we have previously shown defects in MAPK/Erk pathway induction in thymocytes (Rudd '06). Preliminary immunoblot analysis indicates only modest reduction in total SLP-76 levels (Fig. 17). Further examination of total SLP-76, as well as potential differences in phosphorylated levels of SLP-76 will be of critical interest with regards to differential functionality in Lck W97A T-cells and SLP-76 activity in peripheral T-cells. Of ultimate importance is the effect on binding between SLP-76 and Lck having a mutated SH3 domain (W97A). Future work, including immunoprecipitation of SLP-76 and Lck (W97A), will determine if there is reduced binding between these proteins due to the ablation of Lck SH3 domain binding capability.

C. Normal ZAP-70 Induction Following Ab-Induced Activation of Lck W97A T-Cells Compared with WT. In order to rule out differences in early activation events following TCR ligation that may lead to the observed activational differences observed in Lck W97A T-cells, immunoblot analysis of purified T-cells from both WT and Lck W97A mice was examined for differences in ZAP-70 activation. ZAP-70 is an early mediator of TCR signaling and is directly activated by Lck for downstream cascade activation of the MAPK pathway. Preliminary immunoblot analysis indicates higher basal levels of P-ZAP-70 and similar levels of induction of the early signaling molecule, ZAP-70, following anti-

Fig 17. Similar levels of total SLP-76 in Lck W97A T-cells. Purified lymph node T–cells were stimulated with 3-5μg/ml anti-CD3 antibody (2C11) for 3 min and 1% NP-40 lysates were analyzed by western blotting with anti-SLP-76. n=1.



TCR ligation in Lck W97A T-cells (Fig. 18). This data corroborates previous analysis in Lck W97A thymocytes from our lab (Rudd '06).

D. Reduced MAPK Pathway Induction in Lck W97A Peripheral T-Cells. Next, we wanted to investigate proteins of the MAPK pathway, as this pathway has previously been shown to be affected by the Lck W97A mutation. Recent work has shown Lck is also directly involved in regulation of the MAPK pathway downstream and independent of initial phosphorylation of the TCR and ZAP-70, and that this role in MAPK pathway induction selectively requires the p56Lck SH3 domain (Rudd'06, Denny'00, Li).

Activation of the MAPK pathway proteins Erk1/2, was significantly reduced in Lck W97A peripheral T-cells following anti-CD3 stimulation (Fig. 19a). P-Erk 2 appears moredramatically reduced in Lck W97A T lymphocytes (4 fold reduction) when compared to P-Erk 1 (2 fold) between Lck W97A and WT mice using densitometric analysis to determine fold inductions from basal levels of Erk following anti-CD3 stimualtion. Total levels of Erk1/2 are comparable between Lck W97A T-cell and WT cells (Fig. 19b). Analysis of total Lck was examined to confirm that lysates from both WT and Lck W97A mice had equal levels of Lck, ensuring that differences in Erk activation were not due to differences in the levels of Lck, but rather, its activity (Fig. 19). Furthermore, the defect in Erk induction in Lck W97A T-cells does not appear to be due to an issue of kinetics, as Erk induction is never restored in Lck W97A peripheral T-cells even after fifteen minutes following initial anti-TCR-induced activation (Fig. 20).

Fig 18. Similar levels of P-ZAP-70 induced following anti-TCR stimulation in Lck W97A T-cells. Purified lymph node T–cells were stimulated with 3-5μg/ml anti-CD3 antibody (2C11) for 3 min and 1% NP-40 lysates were analyzed by western blotting with anti-P-ZAP-70. Control blots showing levels of total ZAP-70 were run in parallel using the same lysates. n=2.

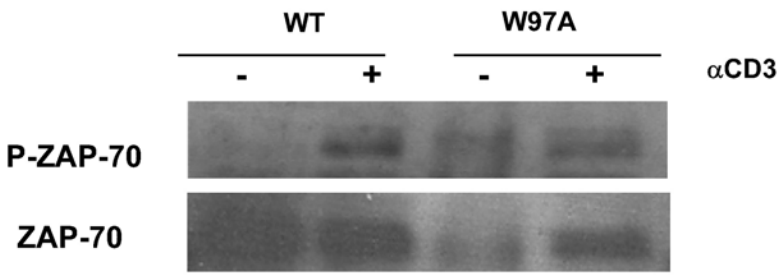
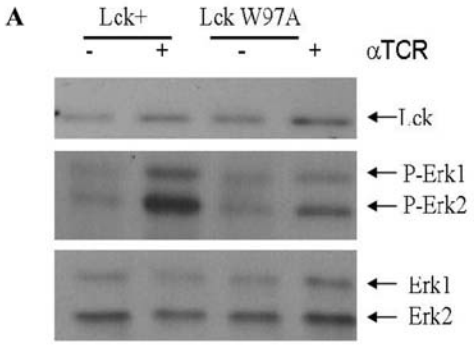


Fig 19. Reduced phospho-ERK induction in Lck W97A peripheral T-cells. ERK activation induced by TCR stimulation. A) Purified lymph node T–cells were stimulated with 3-5μg/ml anti-CD3 antibody (2C11) for 3 min and 1% NP-40 lysates were analyzed by western blotting with anti-phospho-ERK1/2. Control blots showing levels of ERK and Lck were run in parallel using the same lysates. n=6. B) Quantitative analysis of ERK activation. Densitometric data for phospho-ERK1 and phospho-ERK2 is shown relative to unstimulated phospho-ERK levels in wildtype lysates. Statistical analysis was performed using Student's T-test. n=4.



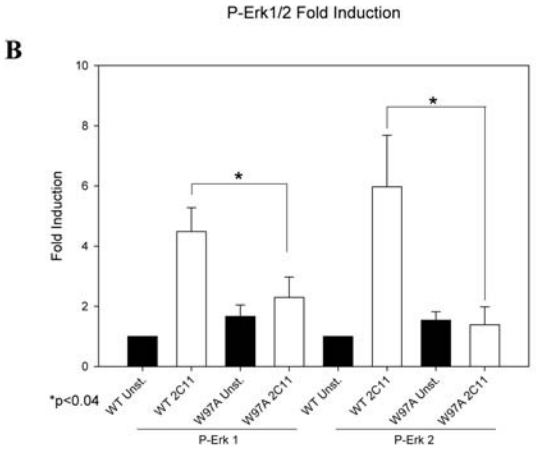
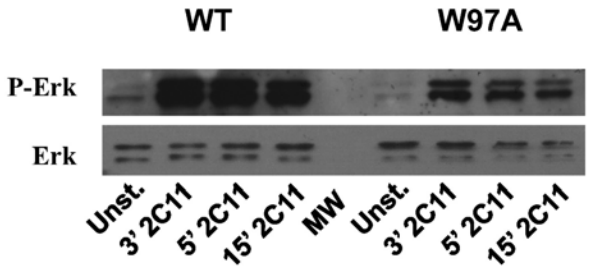


Fig 20. Reduced phospho-ERK induction in Lck W97A peripheral T-cells is not recovered over time. Purified lymph node T–cells were stimulated with 3-5μg/ml anti-CD3 antibody (2C11) at various kinetics and 1% NP-40 lysates were analyzed by western blotting with the indicated antibodies. Representative P-ERK activation induced by TCR stimulation. n=3. Lysates were blotted with anti-phospho-ERK1/2. Control blots showing levels of ERK were run in parallel using the same lysates. n=2-3.

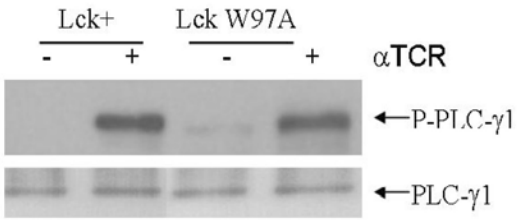


E. Similar Levels of PLC-γ1 and Calcium Mobilization in Lck W97A T-cells Compared to WT. The Lck SH3 domain has been shown to interact with Tec kinases, which are known to play a role in normal phosphorylation of PLC gamma and activation of the PI pathway. Therefore, we wanted to examine PI pathway function in Lck W97A peripheral T-cells.

Immunoblot analysis of lysates with anti-phospho-PLC-γ1 antibody revealed that P-PLCγ1 induction was similar in T-lymphocytes purified from Lck W97A and WT mice (Fig. 21). These results appear to suggest that induction of the phosphoinositol (PI) pathway does not require the Lck SH3 domain. With little change in overall phosphorylation or PLC-γ1 activation following TCR stimulation, the Lck SH3 domain appears to be selectively required for MAPK pathway activation, which may help explain the role of the Lck SH3 domain in the activation of T-lymphocytes.

As Lck, and not Fyn, has been shown to regulate the activation of PLCγ and PKC to mobilize intracellular calcium (Lovatt), we wanted to further confirm that the Lck W97A mutation does not affect the PI pathway and calcium mobilization. Lck W97A and WT T-lymphocytes were stained with the ratiometric dye Fura-2 in order to examine potential differences in calcium mobilization following anti-CD3 cross-linking. Ionomycin, a calcium ionophore was used as a positive control to ensure that the Fura-2 stain was able to measure calcium mobilization, and that both WT and Lck W97A T-cells were able to mobilize calcium in response to such a strong stimuli. Examination of calcium flux reveals that although the ability of Lck W97A peripheral T-cells to mobilize calcium is not differentially regulated compared with WT following anti-CD3 ligation, there do

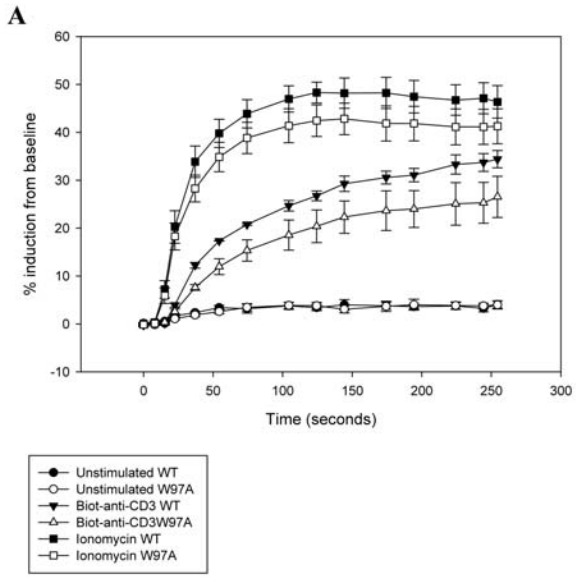
Fig 21. Similar levels of P-PLCγ1 induced following anti-TCR stimulation in Lck W97A T-cells. Purified lymph node T–cells were stimulated with 3-5μg/ml anti-CD3 antibody (2C11) for 3 min and 1% NP-40 lysates were analyzed by western blotting with anti-phospho-PLCγ1. Control blots showing levels of total PLCγ1 were run in parallel using the same lysates. n=3.



appear to be reduced calcium stores in Lck W97A T-lymphocytes as indicated by the reduced maximum mobilization with Ionomycin compared with WT (Fig. 22).

There are signaling molecules which are affected by the release of calcium, including the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII is activated by released stores of calcium and acts to antagonize TCR signaling via SHP-2. It has also been shown that CaMKII can act to impair positive selection of thymocytes, and is involved in the activation of Erk/2. Therefore, it was of interest for us to determine if activity of this protein might be regulated by the Lck SH3 domain with regards to both PI and MAPK pathway function, or that alternative regulation of CAMKII might potentially lead to altered Lck activity and downstream Erk induction. Furthermore, calcium-induced activation of Erk has been shown to require activation of CaMKII and that this is followed by activation of Lck (Franklin), though the mechanisms behind the relationship between CamKII and Lck has not been elucidated. Preliminary examination of CaMKII appears to indicate a slightly elevated (3-5 fold) induction of CaMKII in Lck W97A T-cells (Fig. 23). This study requires further investigation into reproducibility of this finding. Potentially, increased levels of CaMKII could mean that the activity of the Src homology 2 domain-containing phosphatase 2 (SHP-2) is altered, and may thus be an alternative pathway (aside from Ras/Raf activation) regulated by the Lck SH3 domain, further acting to dampen Erk/2 activation.

Fig 22. Similar levels of calcium mobilization following anti-TCR cross-linking in Lck W97A T-cells. Lymphocytes were harvested and loaded with Fura2 and labeled with biot-anti-CD3. Cells were either stimulated with the addition of biot-Streptavidin, or dosed with ionomycin. Cells were analyzed by ratiometric analysis for calcium mobilization. A) Negative control (biot-CD3 alone), stimulated (cross-linked with streptavidin), or positive control (ionomycin) results are plotted as percent induction from baseline. B) Data has been normalized to the positive control. n=3. Statistical analysis performed using Student's T-test.



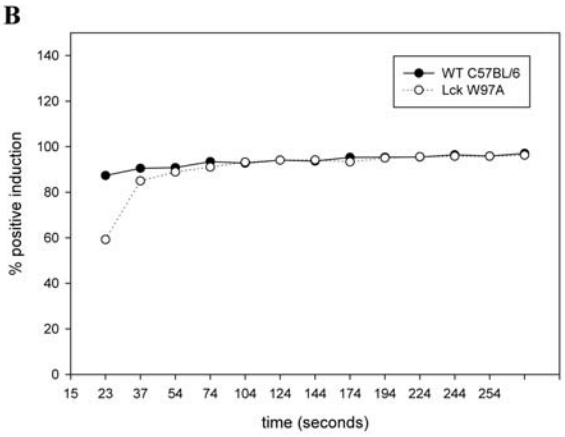
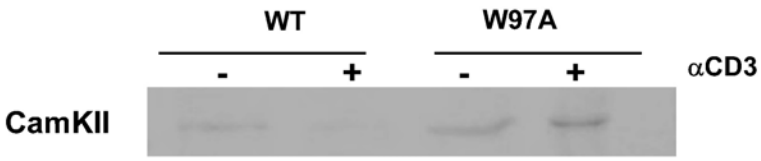
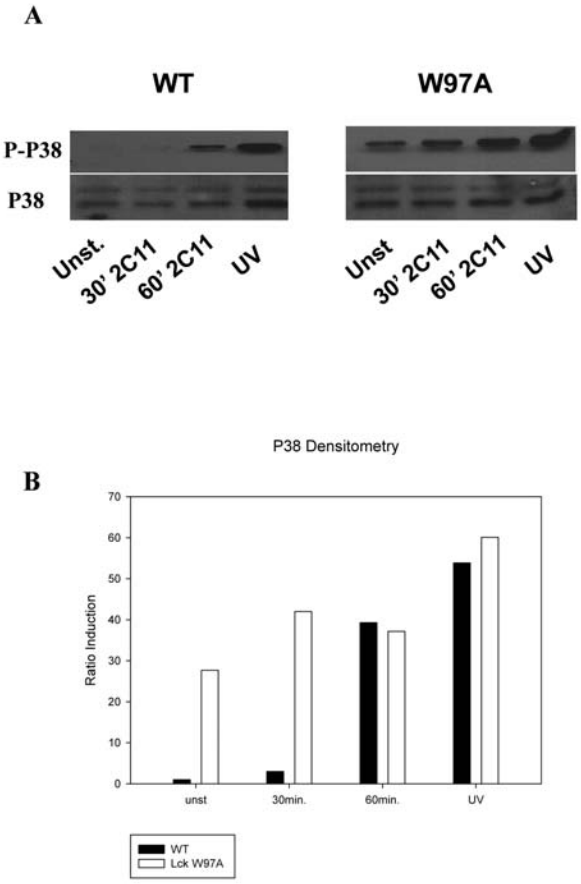


Fig 23. Slightly elevated levels of CaMKII following anti-TCR stimulation in Lck W97A T-cells. Purified lymph node T–cells were stimulated with 3μg/ml anti-CD3 antibody (2C11) for 3 min and 1% NP-40 lysates were analyzed by western blotting with anti-CaMKII. n=1.



F. P38 Activation is Potentially Induced Earlier But Not to a Greater Degree at Later Time-Points Following Ab-Induced Activation in Lck W97A T-Cells. To further examine how alternative signals at the TCR could result in defective cell activation and induction of the MAPK/Erk pathway, we examined the induction of p38. It has been shown that p38 activity can be regulated by the membrane-associated guanylate kinase (MAGUK) family member, Dlgh1. Dlgh1 has also been shown to interact with the SH3 domain of Lck, enabling proper membrane targeting and function of Dlgh1. Therefore, potential differences in Lck SH3 domain interactions with Dlgh1 could ultimately affect p38 activation and function for proper NFAT activity. Immunoblot analysis indicates that P-p38 induction appears to be significantly elevated basally, and earlier (30min.), in Lck W97A T-cells, but does not surpass the level of activation in WT T-cells at one hour post stimulation with anti-CD3 (Fig. 24). This finding indicates that while there may be differences early on in p38 activation as a result of the Lck W97A mutation, the effects on this pathway are not significantly impacted at later points during activation. This enhanced early p38 induction could result from heightened basal Lck activity, as p38 has been shown to be activated downstream of Lck activation of ZAP-70 (Salvador), and may not be a result of stress-induced responses. Dlgh1 has also been shown to be involved in TCR/CD28 signal transduction. As TCR/CD28 co-stimulation appears to somewhat recover activation-induced defects in TCR-specific activation (Fig. 12), it is unlikely that there are significant defects in Lck SH3/Dlgh1 interactions that would result in defective p38 activation.

Fig 24. Similar levels of Phospho-P38 induction following 60' anti-TCR stimulation in Lck W97A T-cells. A) Purified lymph node T–cells were stimulated with 3μg/ml anti-CD3 antibody (2C11) at various kinetics and 1% NP-40 lysates were analyzed by western blotting with anti-phospho-P38. Control blots showing levels of P38 were run in parallel using the same lysates. B) Quantitative analysis of phospho-P38 activation. Densitometric data for phospho-P38 is shown relative to unstimulated phospho-P38 levels in wildtype lysates. Statistical analysis was performed using Student's T-test. n=2-3.



IV. Lck SH3 Domain and T-Cell Function.

In order to determine if the differences between Lck W97A and WT T-cell activation might affect T-lymphocyte effector function and immunity, we examined several different immunization and infection protocols to observe if and how these immune processes were differently affected between these groups.

A. Conjugation Between Lck W97A T-Cells and B-Lymphocytes is Similar to that of WT. Proper differentiation and effector function of T-lymphocytes, particularly the T-helper subset, is critical for host survival and regulation of immune responses to pathogens (Mosmann'86, Syrbe). Two major components of T-cell subset function are cytotoxic killing (CD8+ T-cells) and B-cell help (CD4+ T-cells). Primed effector CD8+ T-cells that recognize antigenic peptides concomitant with MHC molecules on a target/infected cell, are able to release the cytotoxins perforin and granulysin via exquisite specificity to target and destroy infected cells. CD28, a co-stimulatory molecule on T-lymphocytes, binds the CD80 or CD86 receptors on antigen presenting cells (APCs) to assist the interaction of the cytotoxic T-cell and infected cell. Lck is believed to play a role in CD28 signaling, and, more specifically, this interaction has been proposed be facilitated by the Lck SH3 domain (Holdorf). Potential defects in the ability of CD28 to assist the engagement of CD8+ effector T-cells could be expected as a result of the Lck W97A mutation in peripheral T-cells, and was investigated here. Ag-dependant contact between B-cells and CD4+ T-cells relies on the interaction between Ag/ MHC II and CD4 receptors on T-cells as well as via the interaction of CD40 on T-cells and CD40 ligand (CD40L) on B-cells. Cd40/CD40L interactions facilitate the transduction of activation signals enabling

B-cell growth, and differentiation leading to the production and secretion of IgE (Noelle, Rousset, Jabara).

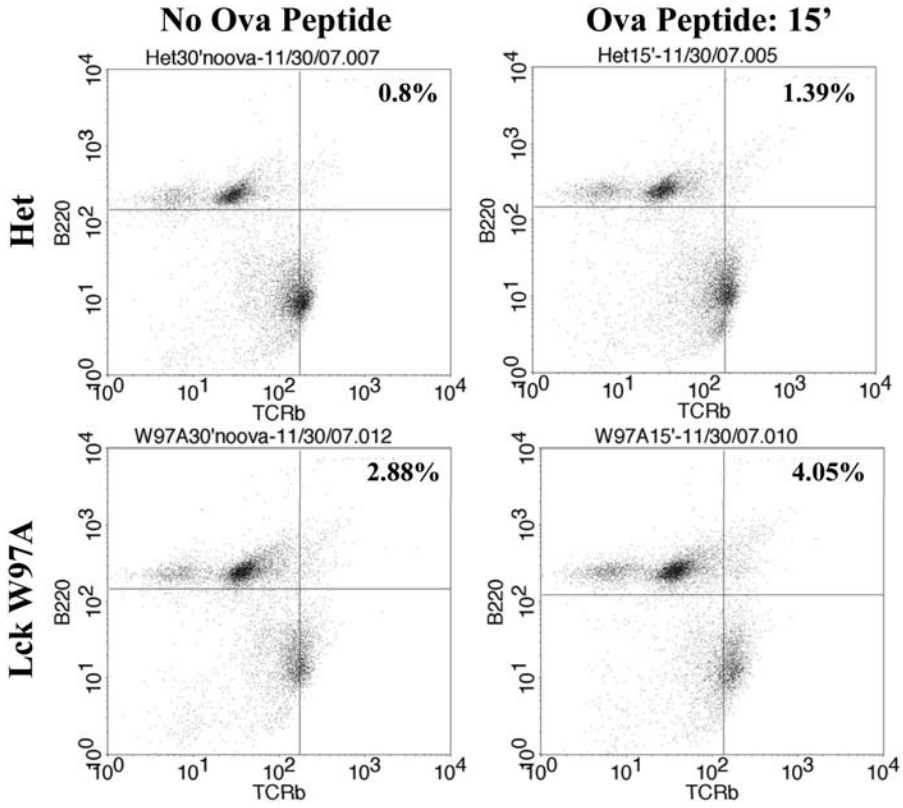
In order to examine potential differences in peripheral Lck W97A T-cell function, we examined the role of the Lck SH3 domain on the ability of T-lymphocytes to carry out their effector function both *in vitro* and *in vivo*. For *ex vivo* investigation of T-effector function, we examined the ability of Lck W97A T-cells to properly form conjugates with B-cells following Ag-induced activation. This cell-to-cell interaction is critical for the ability of T-cells to activate the B-lymphocyte group of the immune system during host infection or immunization and provide a productive adaptive immune response.

Preliminary evidence indicates that Lck W97A T-cells are able to form proper conjugates with B-lymphocytes (Fig. 25). Whereas the induction of conjugate formation is modestly increased with WT T-cells following the addition of the OT-II-specific Ag ligand, ovalbumin (ova), to cell cultures, Lck W97A OT-II T-cells appear to have a generally higher basal level of conjugate formation, which is also increased to a greater degree following Ag-specific activation (Fig. 25). As with OT-1 Ag-induced activation of Lck W97A T-cells, it would be interesting to see if conjugate formation was also effected/altered by varying the dose of ova peptide.

B. Lck W97A Animals Have a Significant Defect in Th2 Ag-Specific Immunity. In order to examine potential differences in the effector CD4+ T-cell population, we examined several immunization and infection strategies.

CD4+ T-cells are further subdivided into Th0, Th1, Th2, Th17, and T-regulatory T-cells, which have specific roles in immunity. Of particular interest are Th1 T-cell clones,

Fig 25. Conjugation of Lck W97A T-cells to B-lymphocytes is similar to that of WT OT-2 T-cell conjugation to B-lymphocytes following *in vitro* stimulation. Data show dot plot analysis of OT-2 T-cells co-cultured with 0.5mg/ml ova Ag-pulsed splenocytes from WT mice for 0-30 minutes. Cells were fixed and stained with anti-B220 and anti-TCRβ and analyzed by FACS following co-culture. Plots are gated on total T-lymphocyte population, and quadrants set to determine conjugate population induction following ovalbumin stimulation. Representative of 1 independent experiment.



which are characterized by the production of IL-2, IFN-γ and TNF-β, and Th2 T-cells, which secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Mosmann'86). Moreover, Lck has been implicated in Th2 differentiation, as T-cells from dominant negative Lck transgenic mice have selective defects in Th2 T-cell differentiation both *in vitro* and *in vivo* (Yamashita'98).

Thus, understanding the outcomes of alterations in components of TCR signaling pathways and how they regulate this immune process is of clinical interest for the control of immune responses to antigen immunity and pathogen infections. It is known that altering the strength of TCR signaling can regulate differentiation of T-lymphocytes (Brogdon). Initial studies showed that T-cell differentiation was influenced by altering antigen concentration (Constant, Reudl). More recent studies have shown that mutations in early TCR signaling components also modify differentiation (Yamashita'98, Schaeffer, Marsland, Sommers). It was the purpose of these experiments to determine the initial effects of the Lck W97A mutation on basic T-cell functions such as conjugation and the ultimate effect that the Lck W97A mutation had on the ability of Lck W97A animals to mount immune responses to antigen immunization and parasite infections.

Given the defects in MAPK pathway induction in Lck W97A T-cells, and evidence supporting an essential role for this pathway in Th2 immunity (So,Yamashita'99), we examined the ability of Lck W97A mice to respond to immunization with DNP-KLH antigen/adjuvant.

1. Ig Isotype Analysis. Immune responses were determined by monitoring Ag-specific (anti-DNP) Ig serum levels in Lck W97A and WT mice. Baseline serum analysis

did not reveal any difference in the overall levels of Ig isotypes between Lck W97A and WT mice (Fig. 26a,c). However, we found significantly reduced levels of DNP specific IgG1 in Lck W97A compared to WT mice following immunization (Fig. 26a). In contrast Ag-specific IgG2b and IgG3 isotypes were found to be comparable between both groups throughout the immunization (Fig. 26b-c). The particular deficit in IgG1 suggests defective Th2 immunity in Lck W97A mice.

2. Ag Re-Stimulation. In order to rule out the possibility that Lck W97A T-cells are simply unable to proliferate in response to activating Ag-stimulation, re-stimulation of T-lymphocytes from Lck W97A and WT animals following immunization with DNP-KLH was examined. Re-stimulation data does not indicate defects in the ability of T-cells from immunized animals to proliferate in response to Ag re-exposure (Fig. 27). On the contrary, preliminary investigations into the ability of T-cells from Lck W97A mice immunized with DNP-KLH appear to be slightly better responders to re-stimulation than their WT counterparts (Fig. 27). Though this type of experiment has only been performed several times, it does indicate that the lack of Lck W97A T-cell responsiveness *in vivo* is not a result of their inability to proliferate, or that they are not initially primed by exposure to the immunogen. It is also of note that the levels of proliferation as cpm in the figure shown are somewhat low (expected cpm for ag re-stim are anywhere from 5-20,000 cpm) compared to similar antigen re-stimulation experiments. This could be due to several parameter variables, including the strength of Ag re-stimulation, the time at which the tritium was added to the stimulated culture, and/or the length of time the tritium was

Fig 26. Lck W97A mice have a significant defect in Ag-specific IgG1 production. Age and sex-matched animals were immunized with 100μg DNP-KLH in TiterMax Gold adjuvant. Sera was collected on days 0 and 14 (pre-boost) and day 21 following boosting with 50μg of DNP-KLH on day 14. Serum levels of DNP-specific IgG1 (A), IgG2b (B), and IgG3 (C) were analyzed by ELISA. Representative data are shown as the log of the concentrations in μg/ml. Statistical analysis was performed using Student's T-test. n=4.

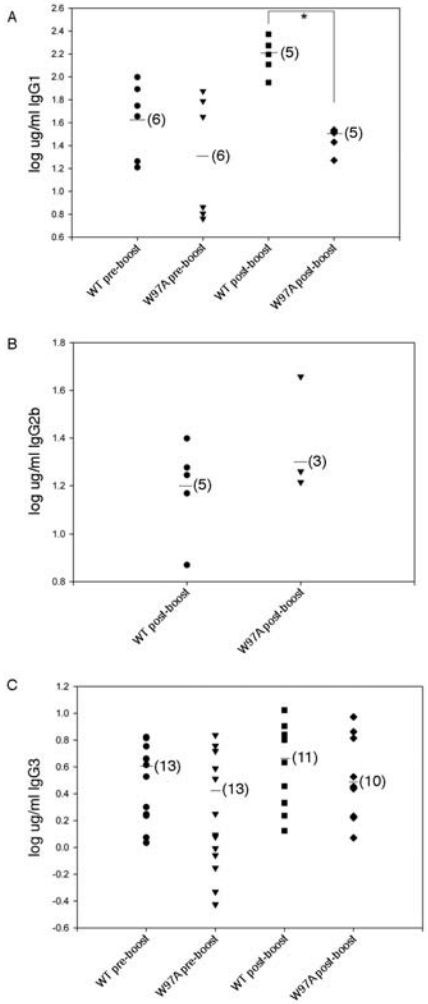
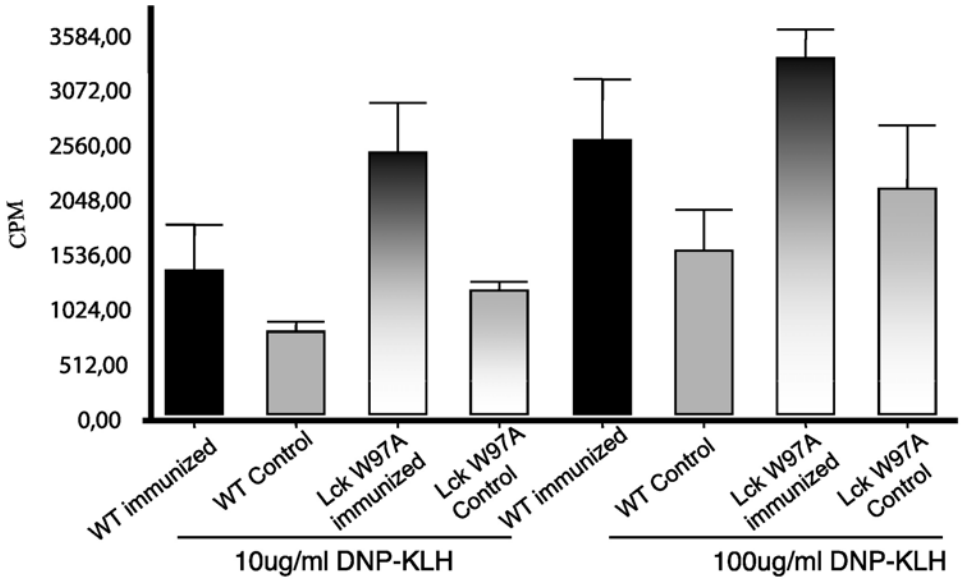


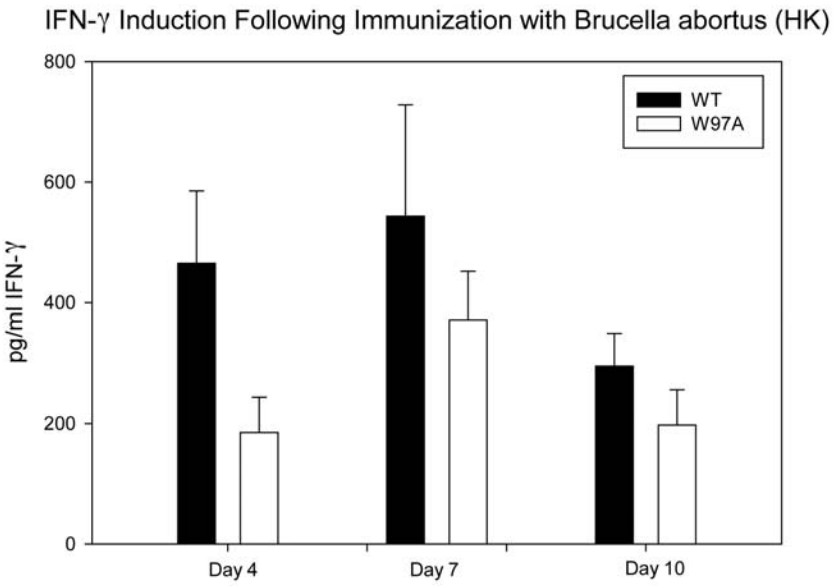
Fig 27. Similar levels of proliferation following re-stimulation of T-lymphocytes between DNP-KLH immunized Lck W97A and WT mice. Age and sex-matched mice were immunized with 100μg DNP-KLH in TiterMax Gold adjuvant. Sera was collected on days 0 and 14 (pre-boost) and day 21 following boosting with 50μg of DNP-KLH on day 14. Lymphocytes were harvested from the draining lymph nodes on day 21 and either re-stimulated or not with various doses of DNP-KLH. n=2. Statistical analysis performed using Student's T-test.



allowed to incorporate. Further optimization will need to be performed in order to examine re-stimulation of Lck W97A T-lymphocytes more effectively

3. Th1 Immunity is not Impaired in Lck W97A Mice. In order to further determine if the defect in Lck W97A immunity was limited to alterations in the IgG1 isotype specific response to immunization, or was indicative of a more general defect in the Th2 immune responses in these mice, a parasite infection model was utilized. In order to further confirm that there is not a defect in Th1 immunity, and that the observed IgG1 deficit in Lck W97A mice following Ag- immunization truly reflects a defect in Th2-specific immunity, a heat-killed *Brucella abortus* was utilized as an immunogen to induce a robust Th1 response. IFN-γ, an inflammatory cytokine associated with robust Th1 responses, was used as a readout for Th1 immunity following immunization with the heat-killed *Brucella abortus.* Serum was analyzed at days 4, 7, and 10 during infection, and IFN-γ was measured by ELISA. Analysis does not indicate significant defects in IFN-γ production by Lck W97A mice at days 7 and 10 during infection, which would indicate defective T-cell responsiveness in Lck W97A mice (Fig. 28). However, there does appear to be a large deficit in IFN-γ production in Lck W97A mice around day 4, indicating a potential defect in the innate immune responsiveness to the immunogen (Fig. 28). This data suggests that Lck W97A mice are indeed able to mount a productive Th1 immune response. To further examine the parameters of the defective Th2 immune response in Lck W97A mice, a helminth model of infection was utilized, which should induce a robust Th2 immune response in mice.

Fig 28. Similar levels of serum IFN-γ following immunization with heat-killed *Brucella abortus* in Lck W97A mice compared to WT. Age and sex-matched mice were immunized with heat-killed *Brucella abortus* i.p. Sera was collected on days 0, 4, 7, and 10 following immunization and analysed by ELISA for levels of IFN-γ. Cumulative data shown for 8 mice per group. Statistical analysis performed using Student's T-test.



4. Lck W97A Animals Have a Significantly Reduced Th2 Immunity to Infection.

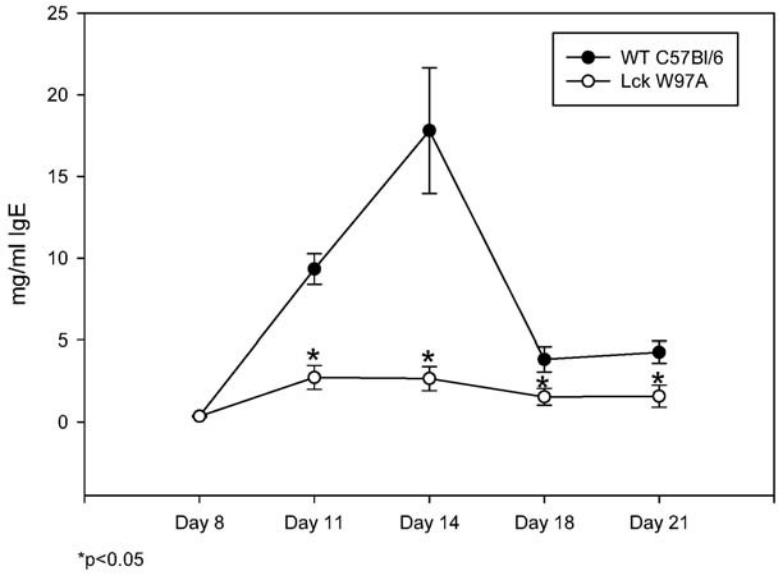
Nippostrongylus brasiliensis, a well-studied model of helminth infection, and a strong inducer of Th2 immunity, was used to further examine potential differences in Th immunity in Lck W97A mice. Mice were infected with L3 stage parasites and total serum IgE and IL-4 levels were examined during the course of infection.

i. IgE Serum Levels Are Significantly Reduced Following Helminth Infection in Lck W97A Mice. IgE, a robust immunoglobulin characteristic of a Th2 immune response, was examined during the course of infection as a readout of Th2 immunity in Lck W97A and WT mice. Lck W97A mice have significant reductions in serum levels of IgE compared to C57BL/6 WT mice after day 8 of infection and continuing throughout the course of the study (day 21) (Fig. 29). There is a maximum defect in serum IgE on day 14 in Lck W97A mice, amounting to a roughly 6 fold reduction in levels compared to WT mice (Fig. 29), with only nominal inductions noted for IgE in Lck W97A mice. These results indicate that the SH3 domain of Lck acts in a critical fashion in peripheral T-lymphocytes to determine the outcome of Th2 specific immune responses in C57BL/6 mice following both Ag immunization and helminth infection.

ii. IL-4 Serum Levels Are Significantly Reduced Following Helminth Infection in Lck W97A Mice. IL-4 is a critical cytokine for maintaining Th2 immunity and regulating IgE levels, as well as the propagation of functional T-helper type 2 T-cells during parasite infection in mice (Finkelman'91, Gros, Jorritsma, Kopf, Mora, Urban'98). To investigate whether deficient IL-4 production could explain the observed deficit in IgE levels in Lck W97A mice following *Nippostrongylus brasiliensis* infection, serum levels of

Fig 29. Lck W97A mice have significantly reduced serum IgE following infection with *Nippostrongylus brasiliensis* compared to WT mice.

Nippostrongylus brasiliensis induced serum IgE levels. Age and sex-matched mice were infected with 600-700 L3 *Nippostrongylus brasiliensis* larvae. Sera was collected on days 0, 11, 14, 16, 18 and following sacrifice on day 21. Total serum IgE levels were determined by ELISA. Statistical analysis performed using Students T-test on cumulative data. n=4.



IL-4 were examined on days 5-12 during the course of infection (Fig. 30). To assess IL-4, we used an *in vivo* cytokine capture assay (IVCCA), which enabled retention of IL-4 in the serum for improved isolation and characterization by fluorometric analysis. Lck W97A mice appear to have roughly half the available serum IL-4 during the height of the IL-4 response *in vivo* compared to WT (Fig. 30). This data demonstrates a significant defect in the ability of Lck W97A mice to maintain Th2 immunity and cytokine production during an immune response to parasite infection.

Lck W97A animals, therefore, have a specific and significant reduction in Th2 immunity as demonstrated by reduced levels of IgG1, IgE, and serum levels of IL-4 following either immunization (DNP-KLH) or infection with the helminth *Nippostrongylus brasiliensis.* Thus, the SH3 domain of Lck plays a critical role in the function of Lck as regards the Th2-specific immune response *in vivo.*

iii. Th1/Th2 *in vitro* Differentiation Indicates Slightly Elevated Levels of IFN-γ. In order to determine if Lck W97A T-cells were able to properly differentiate into either Th1 or Th2 T-cells, an *in vitro* differentiation experiment was performed. CD4+ T-lymphocytes were purified from either WT or Lck W97A mice and cultured in the presence of cytokines and neutralizing Ab to direct cell fate to either Th1 or that of Th2 specificity. Analysis of 3 experiments indicates that there are increased levels of IFN-γ produced with either anti-CD3 or anti-CD3/anti-CD28 stimulation under Th2-differentiating conditions (Fig. 31a,c). This trend is also observed with the addition of IL-2, and under direction to the Th1 phenotype (Fig 31). Some significance is noted for enhanced production of IL-4 with anti-CD3 +IL-2 stimulation, indicating that mutation of

Fig 30. Lck W97A mice have significantly reduced serum IL-4 following infection with *Nippostrongylus brasiliensis* compared to WT mice. *Nippostrongylus brasiliensis* induced serum levels of IL-4. Age and sex-matched mice were infected with 600-700 L3 *Nippostrongylus brasiliensis* larvae. 10μg of biotinylated BVD4-1D11anti-IL-4 antibody was injected i.p. 3-4 hours before each serum collection on days 0, 5, 7, 10 and 12 following *Nippostrongylus brasiliensis* infection. IL-4 levels were determined using luminescent ELISA. Statistical analysis was performed using Student's T–test on cumulative data. n=2.

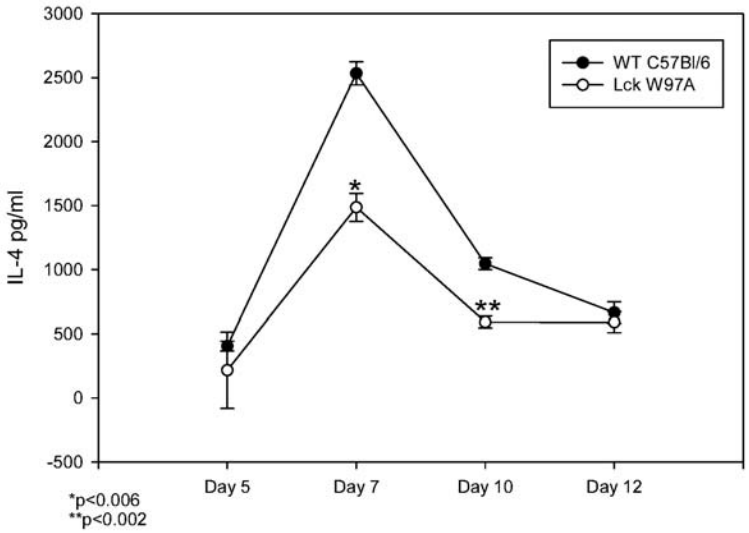
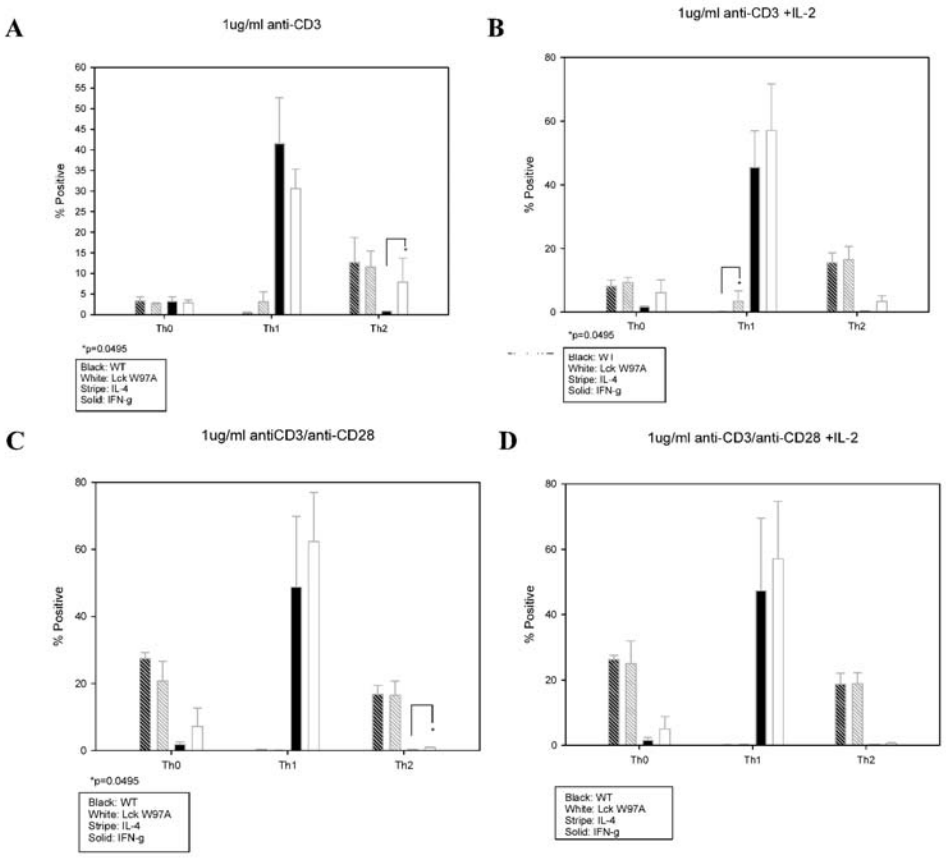


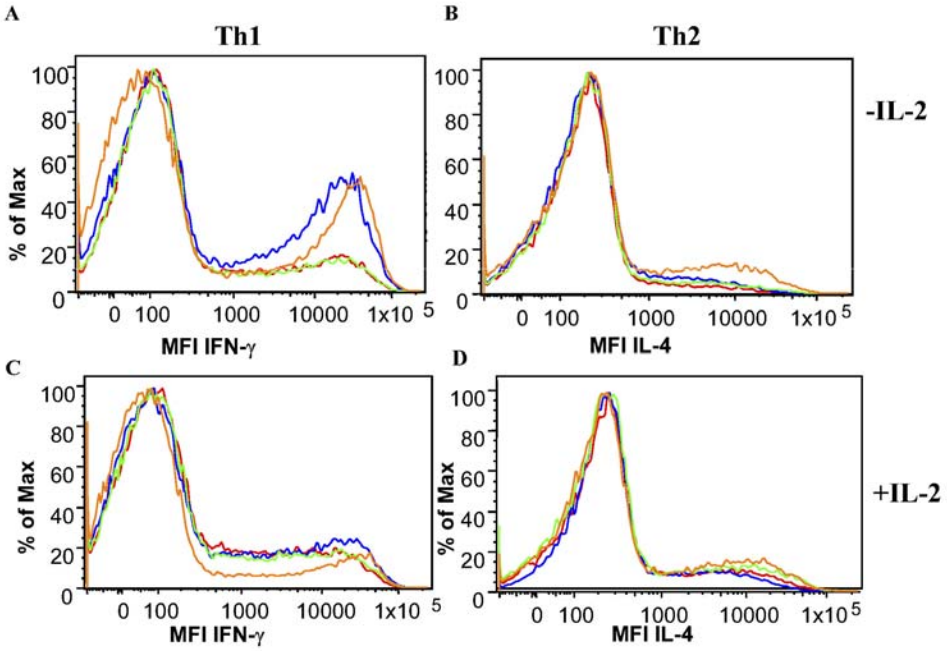
Fig 31. Lck W97A T-cells have similar levels of intracellular IL-4 and enhanced levels of intracellular IFN-γ following *in vitro* Th2 differentiation compared to WT mice. Purified $CD4^+$ T-cells from WT or Lck W976A mice were cultured in the presence of differentiating cytokines and neutralizing antibodies for 4-5 days, and then rested for 2-3 days, before re-stimulation with PMA and ionomycin and staining for intracellular levels of IL-4 and IFN-γ. Statistical analysis performed using nonparametric Wilcoxon assay. Cumulative data shown. n=3.



the Lck SH3 domain does not preclude the differentiation into cells that are able to produce IL-4 (Fig. 31b). This trend is also seen without the addition of IL-2 (Fig. 31a). These data indicate that Lck W97A T-cells are able to differentiate into Th2 cells, as indicated by intracellular staining of IL-4. Furthermore, Lck W97A T-cells produce significantly increased levels of IFN-γ, which may act *in vivo* to inhibit IgE synthesis, and Th2 immunity as a whole. These results indicate a role for the Lck SH3 domain in Th2 immunity and Th2 effector T-cell function.

When looking at the expression levels of IL-4 and IFN-γ, although there are some nuances between groups, the expression levels are similar between WT and Lck W97A CD4+ T-cells following Th1 and Th2 *in vitro* differentiating conditions. Under Th1 conditions, without the addition of IL-2, there is a clear enhancement of the numbers of cells producing IFN-γ as well as its expression in both the WT and Lck W97A T-cells following co-stimulation with anti-CD28 compared with anti-CD3 stimulation alone (Fig. 32). When IL-2 is added, there appears to be a reduction in the number of cells having higher IFN-γ expression so that expression between anti-CD3 and anti-CD3/anti-CD28 stimulations is equivalent. (Fig. 32). Under Th2 differentiating conditions, there is a consistent and comparable level of IL-4 expression, irrespective of the addition of IL-2 or co-stimulation with anti-CD28 (Fig. 32). There is also a slightly higher WT population of cells without IL-2 and with anti-CD28 co-stimulation producing IL-4 (Fig. 32). These data indicate that the expression of IL-4 and IFN-γ is similar between WT and Lck W97A CD4+ T-cells following *in vitro* differentiation.

Fig 32. IL-4 and IFN-γ expression levels are similar between WT C57BL/6 and Lck W97A mice. Analysis of intracellular expression levels of IL-4 and IFN-γ were evaluated using histograms configured using FlowJo software from data in Fig. 32. A) Lck W97A and WT cells gated on CD4 under Th1 differentiating conditions. B) Lck W97A and WT cells gated on CD4 under Th2 differentiating conditions. C) Lck W97A and WT cells gated on CD4 under Th1 differentiating conditions +5ng/ml IL-2 D) Lck W97A and WT cells gated on CD4 under Th2 differentiating conditions +5ng/ml IL-2 . Green line: WT C57BL/6 $CD4^+$ cells stimulated with 1ug/ml anti-CD3, Orange Line: WT C57BL/6 $CD4^+$ cells stimulated with 1ug/ml antiCD3 and 1ug/ml antiCD28. Red line: Lck W97A $CD4^+$ cells stimulated with 1ug/ml anti-CD3. Blue line: Lck W97A $CD4^+$ cells stimulated with 1ug/ml anti-CD3 and 1ug/ml antiCD28. Representative experiment. n=3.



V. Plasmodium, Mosquitos, and the Immune System.

I had the priveledge and honor to perform an internship in the laboratory of Dr. Salaheddine Mecheri at the Institut Pasteur, Paris, France during the course of my studies. In a seemingly unrelated project tangent, I studied the effects of *Anopheles* mosquito saliva on the innate immune system. Recent evidence, however, has made an intruiging link between *Plasmodium* pathogenesis and the SH3 domain of Lck.

Plasmodium, the causative agent of malaria, is one of the deadliest maladies on Earth. As the quest for an efficient vaccine continues, so do we gain new insights into the parameters affecting its pathogenesis. A relatively new piece to the puzzle is the role vector salivary proteins play in the innate and adaptive immune responses to *Plasmodium* protists. The role of saliva from *Anopheles* mosquitos, the only vector for human malaria, with regard to cells of the innate immune system remains unclear. It was the purpose of this project and internship to examine some of the early interactions that occur between vector, protist, and murine host during infection with *Plasmodium berghei.*

A. *Anopheles stephensi* Saliva Increases Macrophage Activation.

In order to examine how mosquito salivary proteins could be affecting cells of the innate immune system, I first examined the activation state of macrophages in response to co-culture with salivary proteins extracted from the salivary glands of the *Anopheles* stephensi mosquito. Elevated expression of MCHII expression on the surface of macrophages is an accepted measure of macrophage activation. LPS is a known activator of macrophages, and was used *in vitro* as a positive control for activation of peritoneal macrophages from C57BL/6 mice. Primary peritoneal macrophages were co-cultured *in*

vitro with LPS and various doses of salivary gland pairs excised from *A. stephensi mosquitoes* to examine the potential regulatory effects of salivary proteins from *A. stephensi* on macrophage activation (Fig. 33). Mosquito saliva was found to slightly increase the activation of macrophages when compared to the activation of macrophages induced with LPS alone (Fig. 33). A characteristic function of macrophages resulting from activation is increased levels of phagocytosis. In order to determine if the slight observed increase in macrophage activation was physiologically important, a novel and efficient method to examine changes in phagocytosis was utilized here with the addition of fluorescent microspheres to *in vitro* macrophage cultures to determine differences in macrophage phagocytosis. In this experiment, salivary gland pairs were used without the co-stimulation of LPS. While pre-incubation of macrophages with saliva significantly increased microsphere uptake, co-incubation of saliva and microspheres resulted in even greater activation and phagocytosis compared to macrophages incubated in the absence of salivary proteins (Fig. 34). Therefore, macrophages are activated by *A. stephensi* salivary gland proteins. This is an important finding, as it indicates that salivary gland proteins alone can encourage the activation of macrophages, and, perhaps pathogen uptake following mosquito probing and pathogen introduction to the host. There are inherent limitations to using microbeads as a true “pathogen” uptake. However, vaccinology frequently uses polymer and latex beads to encourage phagocytosis and uptake of bound Ag for vaccine efficacy with regards to cross-presentation and a specific resulting immune response.

Fig. 33 ***A. Stephensi*** **saliva slightly enhances macrophage activation (MHCII High).** Total salivary gland pair (SGP) proteins from *A. stephensi* mosquitoes were added or withheld in cell cultures with peritoneal macrophages from C57BL/6 mice. Cells were cultured in complete media at 37°C for 20 hours in the presence of 0.1ug LPS. SGP addition to cell cultures was examined in order to determine if salivary gland proteins would affect macrophage activation. Levels of MHC class II high expression was analyzed by FACS as a marker of macrophage activation.

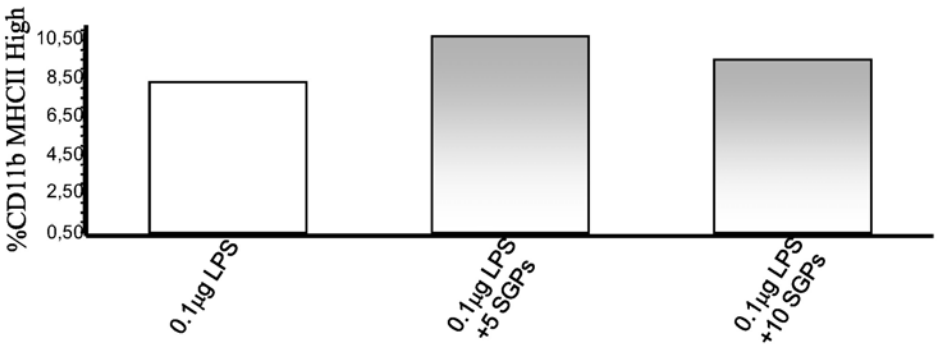
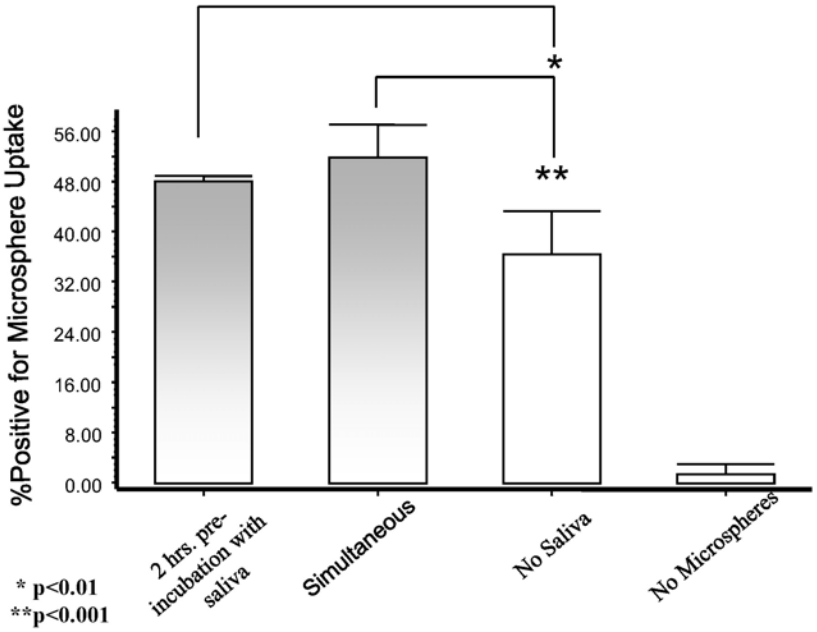


Fig 34. Microsphere uptake by macrophages is enhanced by salivary gland proteins. FITC-fluoresbrite carbocylate microspheres were utilized to examine the phagocytic capacity of peritoneal macrophages from C57BL/6 mice in response to *A. stephensi* salivary gland proteins. Peritoneal macrophages were cultured and (pre)treated with or without saliva for 1hr. in complete media at 37°C with fluorescent microspheres. Macrophages were then harvested and stained with CD11b-PE and analyzed by FACS analysis. Statistics were performed using Student's T-test.



B. Macrophage Infection by Sporozoites. In order to determine the extent to which macrophages are potentially infected by sporozoites, tissue culture microscopy was performed using freshly isolated GFP-tagged sporozoites from *A. stephensi* mosquitoes in complete media. Macrophages were co-cultured with sporozoites for various times (5min.-30min.) and stained for the presence of circumsporozoite protein (PE-tagged CS). Fluorescence microscopy was utilized to determine the location of the sporozoites (green) and the CS protein, which would remain on the surface of cells infected by the sporozoite. Observation at all time points indicate 100% CS positive cells, and the absence of sporozoites in the culture (data not shown). The addition of sporozoites into the cultured macrophages with real-time imaging confirmed that sporozoites rapidly traverse macrophages, leaving CS protein on the surface of the cell, and are quickly relieved of their GFP tag upon phagocytosis by the macrophages. This is, to my knowledge, this first example of this type of rapid traversal and CS shedding by sporozoites and macrophages. Further investigation of alternative tagging and imaging will be required to examine the process of sporozoite infection of macrophages, and the ultimate fate of the sporozoites following phagocytosis.

C. *Anopheles stephensi* Saliva Modestly Decreases Dendritic Cell Activation.

Aside from macrophages, dendritic cells are robust antigen presenting cells (APC) of the innate immune system, and were examined for their potential role as APCs for sporozoite Ag. Bone-marrow derived dendritic cells (DC) from C57BL/6 mice were cultured for 7-8 days in supplemented media. On day 7 or 8 the cells were treated with various doses of LPS, a known activator of DCs, with or without the addition of salivary

proteins, in order to determine the effect of mosquito saliva on DC activation. The addition of mosquito saliva exhibited modest inhibitory effects on DC activation at both high and low doses of LPS stimulation (Fig. 35). Whereas macrophage activation is associated with increased phagocytic function, DC activation results in decreased phagocytic capacity and increased levels of cytokine production. To measure physiologically relevant levels of activation by salivary gland proteins on dendritic cell activity, micropshere uptake by DCs was measured *in vitro* in the presence of salivary gland proteins and/or LPS. Salivary proteins were able to enhance the level of microsphere uptake by DCs *in vitro* (Fig. 36). Higher doses of saliva appear to increase DC activation, as indicated by reduced phagocytosis of the microspheres, but continued to have a lower levels of activation than with LPS alone (Fig. 36). This data, taken together with the effects of saliva on macrophage activity, indicates that mosquito saliva is able to enhance the phagocytic capacity of both macrophages and dendritic cells. When contemplating the use of a vaccine strategy for enhancing the activity of cells of the innate immune system, vector salivary gland proteins should be taken under consideration for efficacy of Ag presentation.

D. Increased T-Cell Proliferation in Response to *Anopheles stephensi*-Pulsed Macrophages. The next step in the progression of this work is to determine if these effects of salivary gland proteins on macrophages and dendritic cells correlate to their Ag-presentation to adaptive immune cells, such as T-lymphocytes. To this end, peritoneal macrophages were pulsed with saliva and co-cultured with CFSE labeled primary T-lymphocytes *in vitro* (Fig. 37). Pre-incubation of macrophages with 10salivary gland pairs (SGP) and ova significantly increased the activation of peripheral T-cells as determined by

Fig 35. *A. stephensi* saliva decreases dendritic cell activation. *A. stephensi* salivary gland protein was added at various concentrations (salivary gland pairs (SGP)/ml of culture) O/N to the cultures of LPS-stimulated bone marrow-derived dendritic cells (BMDC) on day 7 of culture in order to determine the effects of mosquito saliva on BMDC activation. Cells were stained for MHCII expression and analyzed by FACS.

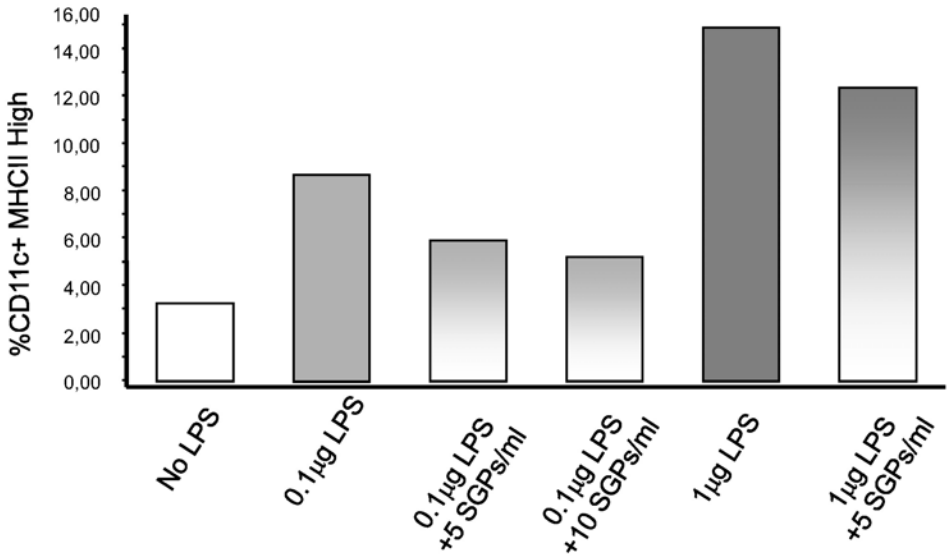


Fig 36. *A. stephensi* saliva increases microsphere uptake by dendritic cells. Bone marrow-derived dendritic cells (BMDDCs) were stimulated or not with LPS for 20 hours and cultured with or without *A. stephensi* mosquito saliva (as salivary gland pairs (SGP)/ml) at various concentrations for 1hr. FITC-fluoresbrite carbocylate microspheres were added at the same time as SGP to examine the effects on the phagocytic capacity of BMDCs in the presence of mosquito salivary gland proteins. Cells were then stained with CD11c and analyzed by FACS.

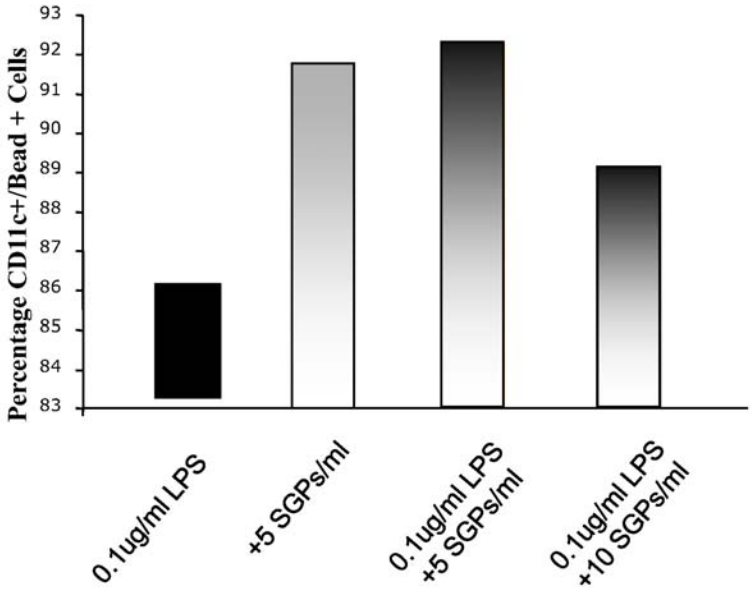
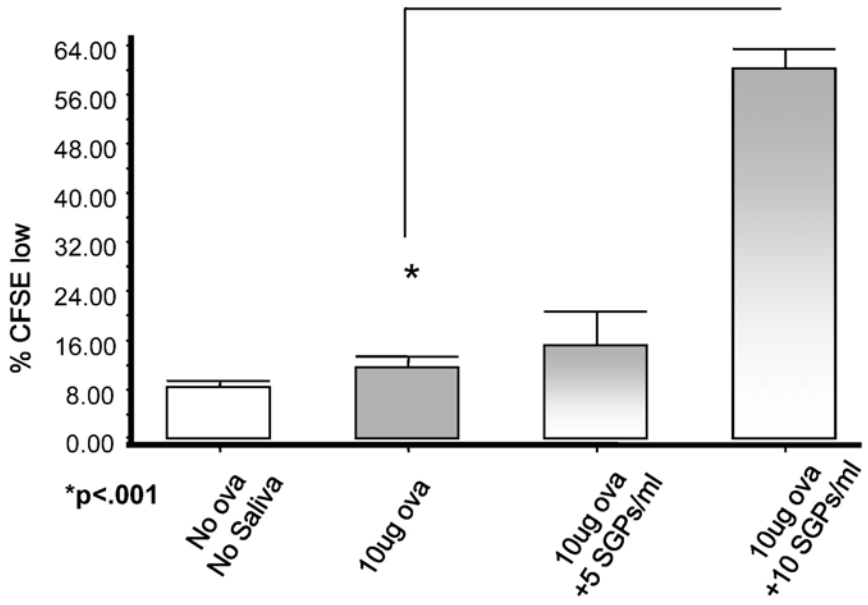


Fig 37. Increased T-cell proliferation in response to *A. stephensi* saliva-pulsed macrophages. Peritoneal macrophages from C57BL/6 mice were pulsed with either ovalbumin alone or ovalbumin in the presence of *A. stephensi* moqsquito saliva at various concentrations (salivary gland pairs (SGP)/ml) for 3hrs. Macrophages were washed and added to cultures of primary C57BL/6 lymphocytes stained with CFSE at a ratio of 1:2 and co-cultured in complete media at 37°C for 48 hrs. T-cell proliferation was analyzed by FACS as CFSE low. Statistics performed using Student's T-test.



significantly increased proliferation (~4 fold enhancement) (Fig. 37). To further investigate the increased ability of macrophages to activate T-cells in response to mosquito salivary proteins, C57BL/6 OT-II mice were either probed, or not, with mosquitoes for 5 minutes (abdominal and footpad exposure, mice were shifted before the mosquitoes were able to take a blood meal, to increase probing, and thus, saliva deposition), and then injected with ovalbumin (ova). Draining lymph nodes (DLN) were harvested three days later and re-stimulated *in vitro* with various doses of ova to determine differences in T-cell responsiveness as a result of exposure to salivary gland proteins delivered via the mosquito vector. Significant increases in the proliferative response by T-cells exposed to salivary gland proteins was observed (data not shown), indicating the *in vivo* relevance of increased T-cell priming by cells of the innate immune system following exposure to vector saliva, as proposed by our earlier in vitro studies with dendritic cells and macrophages.

E. *Plasmodium* and p56Lck.

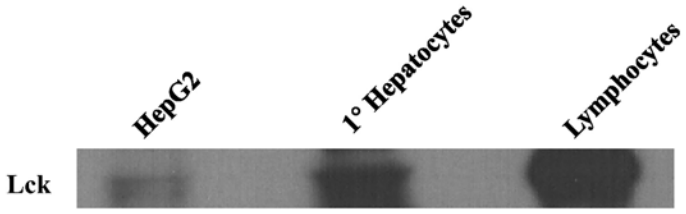
In order to verify recent work claiming that the Src family PTK, Lck, is present in hepatocytes, and that it is involved in the localization of sporozoites within hepatocytes to create a productive infection (Akhouri), I proposed to confirm/disprove this hypothesis by examining the HepG2 liver cell line and primary murine hepatocytes for the presence of Lck.

1. Lck is Present in the HepG2 Cell Line and in Primary Murine Hepatocytes.

Cells from both the HepG2 liver cell line and primary murine hepatocytes were analyzed by Western immunoblot for the presence of Lck. Lck was found in both the pure hepatocyte line, HepG2, and in the primary hepatocytes of C57BL/6 mice (Fig. 38).

These results give credence to the claim that Lck is found in hepatocytes. The presence of Lck in primary murine hepatocytes also encourages the hypothesis that the Lck SH3 is present in these cells, and may potentially act to facilitate sporozoite infection of hepatocytes.

Fig 38. Lck is present in the HepG2 hepatocarcinoma cell line and in primary murine hepatocytes. 1%NP-40 lysates of HepG2 hepatocarcinoma cells, primary murine hepatocytes, and primary murine lymphocytes were analyzed by western blotting for the presence of Lck. n=1.



DISCUSSION

T-cell mediated immunity is of vital interest for the improvement of immuno-therapies and vaccines for a variety of diseases ranging from cancer and autoimmunity to parasite infection. In order to improve and boost immune responsiveness and vaccine efficacy, it is important to understand and be able to control the activation, differentiation and effector function of T-lymphocytes. However, the mechanisms that regulate the outcome of T lymphocyte differentiation, activation and effector function are not well understood.

In order to elucidate the mechanisms for these processes, it is important to understand the regulation of these processes, and the function of signaling molecules within the cell that control them. The Src-family protein tyrosine kinase, p56Lck, is one of the first signaling molecules downstream of the TCR, and is responsible for initiating TCR signaling cascades following TCR ligation. There is a conserved domain homology between the Src family members, including a conserved SH2, SH3, kinase domain and carboxy tail. The unique domain and amino terminus are unique between the Src PTKs and are responsible for directing the specific location of the proteins within the cell. Lck is located mostly at the cell surface membrane. The Lck SH2 domain has been shown to be

important for the initial processes in T-cell activation as a result of TCR engagement, particularly by facilitating the binding of Lck to the TCR zeta chains and the recruitment and activation of ZAP-70 (Straus '96), and is responsible for a variety of proein-protein interactions downstream of proximal signaling events. The role of the SH3 domain, however, and its role in regulating the function of Lck activity are not as well understood. Several binding partners have been identified for the Lck SH3 domain, including the co-stimulatory molecules CD28 (Holdorf) and CD2 (Bell, Holdorf), c-Cbl (Hawash), and PI3-K. Binding to the co-stimulatory molecules CD28 and CD2 enables further recruitment of Lck to the TCR signaling complex during TCR engagement. We have performed experiments here, utilizing a knock-in mouse model containing a mutant *lck* allele containing an alanine substitution of a critical tryptophan residue within the SH3 domain (W97A), in order to examine the role of the Lck SH3 domain in mature T-cell function and immunity, and to examine some of the particular Lck SH3 domain binding partners with regard to these processes. This W97A mutation is known to disrupt normal ligand binding to the SH3 domain (Denny'99, Patel). We have shown in this work that Lck SH3 domain binding to CD28 is not critical for CD28 signaling, as indicated by similar levels of proliferation between WT and Lck W97A T-cells following CD28/PMA stimulation (Fig. 12). The closely related Src family kinase, Fyn, has also been shown to bind CD28 via its SH3 domain (Ong), and whereas this interaction is critical for co-stimulation in thymocytes, it may also be able to compensate for defective Lck in peripheral lymph node T-cells. CD2 co-stimulation is assicated with cell adhesion and has been shown to activate Lck via Lck SH3 domain interactions (Holdorf). However, as preliminary investigation

into cell adhesion (T-/B-cell conjugation, Fig. 25) does not indicate a defect in the ability of Lck W97A T-cells to adhere to B-cells, it is unlikely that even if there were defective CD2/Lck interactions, this interaction is not critical for T-/B-cell adhesion (under these conditions). The ability to control the strength of response to TCR ligation is provided, in part, by the binding of Lck to c-Cbl, an ubiquitin ligase, which induces a negative feedback mechanism by binding and degrading Lck. Using Lck W97A T-lymphocytes we demonstrate that there is significantly reduced T-cell activation following TCR-specific ligation. Thus, while binding of c-Cbl to Lck may be defective in Lck W97A T-cells, defective negative regulation of Lck it is not going to be apparent under these conditions. With regards to PI3-K, while binding of the Lck SH3 domain to PI-3K has been demonstrated, it has also been shown that the Lck SH2 domain is able to bind PI3-K (Susa). As there are no apparent defects in calcium mobilization, and only very slight reductions in PLC-γ1 in Lck W97A T-cells following TCR ligation, it is likely that the Lck SH2 domain is able to compensate for binding to PI3-K. Also, it has been shown that Fyn is also able to bind PI3-K via its SH3 domain, thus this interaction could also be able to compensate for defective Lck SH3 domain binding.

Evidence is presented here that function of the SH3 domain of Lck is necessary for normal T-cell activation and Th2 immunity. Defects in TCR-induced Lck W97A T-cell activation correlate with the selective requirement for Lck SH3 domain function for the proper induction of the MAP Kinase pathway following antigen receptor stimulation. Moreover, defects in Th2 immunity of Lck W97A mice indicate a critical role for the Lck

SH3 domain in the functional response of T-lymphocytes to immunization and helminth infection.

These findings indicate that the Lck SH3 domain provides an important mechanism linking the antigen receptor to intracellular signaling pathways that determine the outcome of T-lymphocyte activation and demonstrate a novel role for the Lck SH3 domain in Th2 differentiation and immunity in an animal model. This is, to my knowledge, the first paper to show a role for the Lck SH3 domain in an infection model with regards to Th2 differentiation and immunity, and to elucidate a potential mechanism for this defect by the dysregulation of the MAPK pathway protein, Erk.

I. Lck SH3 Domain Regulates T-Lymphocyte Phenotype.

We show that in Lck W97A mice there are modest changes in T-cell numbers, particularly the CD8+ T-lymphocyte subset (two- to three-fold reductions), as well as slightly lower CD4 expression on Lck W97A T-cells (Figs. 5-7). While there are only modest reductions in the total number of TCRβ peripheral T-cells in Lck W97A mice, there are significantly reduced percentages (10-15% reduction) of these cells within the lymph node and splenic compartments (Figs. 4,5). Rudd et al. showed significantly reduced numbers of thymocytes in Lck W97A mice, and two- to three-fold reductions in the numbers of CD4+ and CD8+ thymocytes, indicating defective positive selection in Lck W97A mice. The recovery of CD4+ T-cell numbers following T-cell exodus from the thymus can potentially be explained by the finding that although Lck is necessary during thymic selection and maturation of both CD4+ and CD8+ T-cells, the maintenance of

peripheral CD8+ T-cells *in vivo* does not require Lck (Tewari). It is therefore possible that differential requirements exist for Lck signaling necessary for the maintenance of peripheral CD4+ and CD8+ T-cells, and that this process is mediated by the Lck SH3 domain. It is also possible that the Lck SH3 domain plays a differential role in CD4+ T-cells, as Lck has previously been shown to have a biased role in CD4+ T-cell survival, sensitivity to apoptosis, and ability to proliferate in response to a lymphopenic environment compared with CD8+ T-cells (Ferreira). Furthermore, we show that CD4 expression is reduced in Lck W97A mice (Fig. 7). Wiest et al have shown that when you have continued engagement of CD4, and thus activation of Lck, Lck disengages from CD4 and CD4 is subsequently internalized. If our Lck SH3 domain mutant is more basally active than WT (due to loss of inactive conformation stability between SH3-linker region binding), it is possible that there is greater disengagement of Lck from CD4 and more sequestering of CD4 from the cell surface.

Thymic data from these mice has indicated that Lck W97A mice are lymphopenic (Rudd), however, peripheral T-cell numbers show only modest reductions in the number of TCRβ cells in the periphery (Fig. 5). The ability of T-cells to proliferate in response to a lymphopenic environment is termed homeostatic-driven proliferation. Homeostatic proliferation induces a memory-like phenotype in T-cells, where conventional memory T-cell markers are expressed, including CD44 (Cho, Goldrath, Murali-Krishna). Seddon et al. have shown that Lck is of critical importance for homeostatic-driven proliferation of T-lymphoctes *in vivo*, but not for long-term survival. It is possible that the Lck SH3 domain may be involved in this process, and that Lck W97A T-cells might be more at risk for not

being able to proliferate in response to lymphopenic conditions that the ability to survive in the periphery. CD44 expression was examined on Lck W97A T-lymphocytes and was found to be significantly higher on naïve Lck W97A compared with WT T-cells (Fig. 8). It is possible that Lck W97A T-cells may be more activated that WT T-lymphocytes as a result of homeostatic proliferation in response to the reduced thymic output and resulting lymphopoeinic environment in Lck W97A mice. The phenomenon of homeostatic proliferation, and the role that the Lck SH3 domain plays in this phenomenon, could be further examined by introducing Lck W97A T-cells into lymphopoeinic hosts, such as RAG mice, which lack both B and T lymphocytes, in order to delineate differences in this type of proliferative expansion between Lck W97A and WT T-cells.

Furthermore, the CD4+ T-cell population in Lck W97A mice have a slightly reduced CD62L+ (L-selectin) population (Fig. 9) and supports the hypothesis that Lck W97A T-lymphocytes have a more activated phenotype compared to WT, and may indicate homeostatic-driven proliferation in Lck W97A mice, although conflicting data has also been reported that reduced CD62L expression is not a requirement for this process (Murali-Krishna). It is interesting to note that although Lck W97A T-lymphocytes appear to be slightly more activated than their WT counterparts, they have reduced activation as a result of TCR-specific stimulation. Homeostatic-driven proliferation of CD4+ and CD8+ T-cells is believed to result, in part, from reduced competition for several of the γ common chain (γ_c) cytokines (cytokines that bind receptors having a γ_c chain), specifically, IL-7, IL-12 (CD8+ T-cells) and IL-15 (Tan, Kieper) which act to promote homeostatic-driven proliferation. γ_c chain cytokines regulate cell growth via activation of signal transducers

and activators of transcription (STATs) downstream of janus family kinases (JAKs), especially JAK3 (Suzuki), independent of TCR signaling. Moreover, it has been shown that low affinity for the self-MHC/peptides that mediate positive selection in the thymus are utilized to assist homeostatic proliferation of naïve CD4+ and CD8+ T-cells in the periphery (Ernst). As preliminary evidence using Lck W97A T-cells stimulated with Ag result in enhanced activation compared to anti-CD3-sepcific stimulation alone (Fig. 14), it is logical that homeostatic proliferation involving MHC/peptide/TCR engagement would not be as defective as a TCR-specific stimulation. This is confirmed to the extent that Lck W97A T-cells appear to be involved in the process of homeostatic-driven proliferation as they have elevated levels of CD44 expression (Fig. 8b), although there remain significantly fewer CD8+ T-cells in the periphery of Lck W97A mice. Seddon et. al have reported that Lck is required for TCR-driven homoestatic proliferation using an inducible Lck-deficient mouse model. It is interesting to note that homeostatic proliferation is defective in Lck W97A mice, but that there are indications, such as enhanced CD44 expression, of homeostatic proliferation. Thus, the Lck SH3 domain must be involved downstream of TCR-induced homeostatic proliferation, and is involved in the regulation of this process via an unknown mechanism.

To further examine the phenotype of Lck W97A T-cells, and to examine potential activation differences, which are observed in Lck W97A thymocytes (Rudd), we examined T-cell activation following Ab and Ag stimulation *in vitro*, as measured by analysis of proliferation, activation marker induction, and IL-2 production.

II. Lck SH3 Domain Regulates T-Lymphocyte Activation.

Our analysis of Lck W97A T-cell activation indicates substantial alterations of *in vitro* activation of LckW97A T-cells, as indicated by fewer cells undergoing proliferation, reduced induction of the activation marker, CD69, as well as reduced IL-2 production following anti-CD3 TCR-specific stimulation. However, higher doses of anti-TCR stimulation and/or the addition of co-stimulation via CD28 ligation, were both able to enhance the proliferation of Lck W97A T-cells when compared to low-dose stimulation.

Once a T-lymphocyte has received an activation signal via its TCR, the induction of the early activation marker CD69 is transiently observed on the cell surface. IL-2 production, also a marker of early T-cell activation, is a common readout for proper and complete activation of T-cells following ligation with activating stimuli. Activation-induced clonal expansion of naïve T-cells, as with the production of secreted IL-2, is another indicator of complete T-lymphocyte activation in response to activation stimuli. We found a significant increase (~2- fold) in the number of Lck W97A T-cells that could proliferate in response to anti-TCR specific Ab stimulation at higher concentrations (1μg/ml) of anti-CD3 compared to that of low-dose stimulation (0.1μg/ml) (Fig. 10), especially with the addition of CD28 co-stimulation (~5-6- fold) (Fig. 12). IL-2 levels, however, were significantly lower in Lck W97A T-cell cultures stimulated with 1μg/ml anti-CD3 compared to WT (Fig. 11), even though proliferation was mildly mitigated at this concentration.

It is interesting to note that a significant number of Lck W97A T-lymphocytes could be activated, particularly with the addition of CD28 co-stimulatory signals. Lck has

been proposed to play an important role in co-stimulatory signaling through SH3 domain binding to CD28. This conclusion is based on the observations that a proline-rich region in the cytoplasmic region of CD28 is critical for its co-stimulatory function, Lck can bind to this region, and SH3 domain function of p56Lck is important for CD28 signaling in cell lines (Friend, Holdorf'99). Based on this, we expected that co-stimulation of LckW97A T-cells would be defective, but surprisingly we found that stimulation of CD28 effectively augmented anti-CD3 induced proliferation. Furthermore, stimulation with CD28 and phorbol-12-myristate-13-acetate (PMA: a PKC activator) failed to reveal any defect in the response of Lck W97A T-cells (Fig. 12). These findings indicate that SH3 domain recruitment of Lck to CD28 is not necessary for co-stimulatory function, although it is possible that PMA treatment induced intracellular signals that might otherwise require Lck SH3 domain function, or that the closely related Fyn kinase is able to compensate for the loss of Lck SH3 domain function. Alternatively, since binding of Lck to the CD28 proline rich region enhances Lck catalytic activity by relieving the conformation of the protein to the open or active form (Holdorf'99), it is possible that the modest increase in catalytic activity that accompanies mutation of the SH3 domain may replace the role of CD28 binding.

To further examine activation defects in Lck W97A T-cells, examination of the early activation markers CD69 and CD25 (IL-2α) were analyzed. Lck W97A T-cells have a consistently higher basal level of CD25 expression, indicating slightly higher levels of basal activation (Fig. 13). This finding corresponds to previous data involving the ablation of SH3 domain function, where mutation of the Lck SH3 domain reduces stability of the

inactive form of the protein and yields a more basally activated Lck protein compared to WT. Lck W97A CD25 expression remains at modestly elevated levels over a period of 31 hours following stimulation with anti-CD3 antibody, when compared to WT (Fig.13). However, the requirements for induction of CD25 and CD69 must be intrinsically different, as T-cell activation downstream of TCR ligation is defective in inducing CD69, even at high doses of stimulation (20-30% reduced induction at 0.3-5μg/ml 2C11) (Fig. 13). It could be said that although Lck W97A T-cells appear to have a higher basal level of activation, or even *because* they have a higher basal activity, they are not able to become activated to the same degree as WT T-lymphocytes following TCR-specific stimulation by inducing some level of tolerance or even, potentially activation-induced cell death (AICD). I tend not to believe AICD is responsible for the observed differences in cell activation, as indicated by reduced proliferation, etc., as propidium iodide, a stain for dead cells, does not indicate significant differences in mortality between WT and Lck W97A following anti-CD3 stimulation (data not shown).

Although we have examined the levels of TCRβ expression on the surface of Lck W97A T-cells and have found the level of expression to be similar to that of WT (Fig. 7), it might also be of importance to examine the levels of CD3 on the surface of Lck W97A T-cells. Even though a differential level of expression between TCRβ and CD3 has not, to my knowledge, been reported in primary peripheral T-lymphocytes, Wiest et al. have reported differential expression of CD3 in thymocytes (Wiest '94). Reduced CD3 expression could affect the magnitude of TCR engagement with anti-CD3 stimulation, and

thus potentially affect the strength of signal required for the signal threshold for T-lymphocyte activation.

Therefore, although Lck W97A T-cells have defective activation following TCR-specific Ab-induced activation, they are not anergic or unable to respond to activating signals. These findings indicate that Lck SH3 domain function is important for normal T-cell activation, though not absolutely essential.

We next examined TCR signaling function in LckW97A T-cells to understand the basis for the observed changes in the response to receptor stimulation. Thymocyte data does not indicate any defects in ZAP-70 induction in Lck W97A thymocytes following anti-TCR stimulation (Rudd ’06). We examine ZAP-70 induction in purified peripheral T-cells and confirmed that this early signaling event does not seem affected in Lck W97A T-cells following immunoblot analysis (Fig. 18). As this proximal TCR signaling event is not regulated by the Lck SH3 domain, it was important to examine other signaling molecules downstream of this process in order to understand the mechanism behind the defects in Lck W97A T-lymphocyte activation.

The anti-phosphotyrosine (PY) Ab, 4G10, was used in biochemical studies to examine the induction of phosphorylated tyrosine residues following stimulation of Lck W97A T-lymphocytes with anti-CD3 mAb (2C11). Overall basal levels of tyrosine phosphorylation were slightly enhanced, and induced tyrosine phosphorylation of select proteins was reduced following anti-TCR specific stimulation (Fig. 15). As the Lck SH3 domain plays a role in stabilization of the closed, or inactive conformation of the protein, this heightened basal level of phosphotyrosine induction was expected, and corroborates

thymocyte data (Rudd '06). A major band around 35 kDa was significantly reduced in Lck W97A T-cells following TCR ligation (Fig. 15). The scaffold protein LAT, which is approximately 38 kDa, is known to interact with Lck and to play an important role in T-cell activation downstream of ZAP-70 activation. Preliminary analysis of LAT induction in Lck W97A T-cells following stimulation with 2C11 indicates a significant reduction (~5-fold reduced) compared to WT (Fig. 16). This is only a single experiment, and total LAT levels were not examined, so it is not possible to draw any concrete conclusions from this data. However, differences in LAT activation could potentially affect the ability of LAT to form signaling clusters with critical upstream regulators of the MAPK pathway including Grb2/SOS and RAS, as well as bind PLC-γ, a PI pathway protein, leading to differences in either or both of these pathways. As PLC-γ levels and calcium mobilization appear very similar between Lck W97A and WT T-lymphocytes (Figs. 21-22), it is unlikely that this difference, if it is reproducible, is physiologically critical for PI pathway induction and the mobilization of calcium following TCR-specific stimulation. It is possible that the association of SLP-76 with Lck and PLCγ may be able to compensate for diminished LAT activity, and maintain relatively normal levels of PLCγ activation (Qi). LAT is known to be important for T-lymphocyte development, as LAT-deficient mice have a severe block in T-cell development at the DN stage (Zhang). If our data showing reduced P-LAT in Lck W97A T-cells is reproducible, a defect in LAT activity downstream of Lck, or as a result of reduced Lck binding may contribute to the thymic developmental issues noted in Lck W97A mice (Rudd). LAT is known to bind the Lck SH2 domain, but there may be a role for the Lck SH3 domain in this process as well. Immunoprecipitations following TCR-

specific ligation would be useful to examine this interaction and the scope of the defect in LAT activation, if the defect is determined to be significant.

SLP-76, an adaptor protein involved in the PI pathway downstream of ZAP-70 activation, has a size of roughly 76 kDa. A band that could potentially correlate to this protein on our phosphotyrosine blot does not appear negatively affected (Fig. 15), but preliminary analysis of this protein was examined as it has been shown to be a binding partner for Lck, and is important for complete T-cell activation. Western immunoblot analysis of total levels of SLP-76 appear to show no differences between purified lysates of Lck W97A T-cells and those of WT (Fig. 17). Difficulties with our P-SLP-76 Ab have precluded further study of this protein at this time, but would be of interest in the future. Further examination of total SLP-76, as well as potential differences in phosphorylated levels of SLP-76 will be of critical interest with regards to differential functionality in Lck W97A T-cells and SLP-76 activity in peripheral T-cells. Of ultimate importance is the effect on binding between SLP-76 and Lck having a mutated SH3 domain (W97A). Future work, including immunoprecipitation of SLP-76 and Lck (W97A), will determine if there is reduced binding between these proteins due to the ablation of Lck SH3 domain binding capability. Reduced SLP-76 binding to the Lck SH3 domain and subsequent activation have been proposed to play a role in the prevention of activation-induced cell death (AICD) following CD4/TCR stimulation (Sanzenbacher). It is possible, therefore, that if there is reduced binding between Lck and SLP-76 as a result of the W97A mutation, there would be reduced AICD as a result of CD4/TCR stimulation in Lck W97A mice. This

process could be examined by stimulating Lck W97A T-cells *ex vivo* with CD4 and anti-CD3 and examining WT andLck W97A T-cell death.

Activation of the MAPK pathway Erk1/2 kinases is critical for complete T-cell activation and IL-2 production downstream of TCR signaling. Observation of Erk1/2 induction following TCR-specific ligation indicates that activation of this protein is defective in Lck W97A T-cells (Fig. 19). Erk-2 appears to be more negatively impacted (~6- fold reduction) by the Lck SH3 domain mutation that Erk-1 (~4 fold reduction), and neither Erk-1 nor Erk-2 activation is recovered to WT levels at a later time (15 min.) (Figs. 19,20). Given the key role of the MAP Kinase pathway in the control of cell proliferation and gene expression, our findings strongly suggest that the altered activation of peripheral LckW97A T cells is due to defective induction of ERK-1 and ERK-2 following stimulation of the antigen receptor.

This signaling defect appears to be limited to the MAPK pathway, as phospholipase C-γ1 (PLC-γ1) activation, calcium mobilization, and the overall pattern of tyrosine phosphorylation were not substantially affected. Immunoblot analysis of P-PLC-γ1 induction in Lck W97A T-cells following TCR-specific stimulation does not indicate significant differences compared to WT (Fig. 21). To further examine the PI pathway, and to ensure that there were not physiologically significant differences in calcium mobilization, Fura 2, a ratiometric dye, was used to examine calcium mobilization *in vitro.* Analysis of Fura 2 following anti-CD3 cross-linking does not indicate differences in the ability of the Lck W97A T-cells to mobilize stores of calcium in response to a TCR-specific stimulation (Fig. 22). The potential for the binding of ITK and SLP-76 with Lck

for PLCγ activation could be interesting to examine in our Lck W97A T-lymphocytes, as these interactions may have the potential to compensate for losses in LAT. LAT null studies *in* vivo are diifcult to examine, as there is a severe block in T-lymphocyte development in these animals. The potential for SLP-76 and ITK to assist in PLCγ activation, therefore, would be of great interest if the reduced LAT of our preliminary finsdings proves a faithful representation of the signaling in Lck W97A T-cells. Furthermore, CaMKII, a signaling protein activated by intracellular calcium, has been shown to be involved in thymocyte TCR signaling and positive selection (McGargill) as well as calcium-dependent activation of Erk in human T-lymphocytes (Franklin). Franklin et al. propose that CaMKII activation may regulate Lck in a calcium-dependent manner, but that this regulation may not act by affecting the activation of Lck, but rather its localization with binding partners. I was interested in examining potential diffences in Lck and CaMKII interactions as not much is known about how these two proteins interact, even though CaMKII activity is believed to directly impact Lck function. Preliminary analysis of total CaMKII levels in Lck W97A peripheral T-cells indicates slightly elevated levels of this protein (Fig. 23). If normal levels of calcium are mobilized in Lck W97A T-cells, but aberrant CaMKII function is observed in thymocytes as well as peripheral T-cells, it may indicate a role for the Lck SH3 domain in the negative regulation of positive selection and TCR signaling via aberrant SHP-2 interactions or disregulation of CamKII-mediated localization of Lck with its binding partners following calcium mobilization. Further analysis of CaMKII levels in Lck W97A thymocytes is required, as well as examination of SHP-2 regulation in peripheral Lck W97A T-cells. Moreover, CaMKII has been shown, in

vascular smooth muscle cells, to regulate Erk1/2 via both focal adhesion kinase (FAK) dependent and independent mechanisms during cell adhesion (Lu); and Lck is known to phosphorylate and activate FAK (Goldmann). Thus, differential regulation of Lck binding to FAK could affect the interactions of FAK with CamKII resulting in aberrant cell adhesion. However, preliminary examination of T-/B-cell conjugation does not appear to be dysfunctional (Fig. 25). Further examination of cell adhesion and the effects on FAK activity will need to be examined to demonstrate that this interaction is not being negatively affected.

The selective reduction in MAPK pathway activation in mature Lck W97A T-lymphocytes is consistent with previous data from our lab linking Lck SH3 domain function to activation of the MAPK pathway in cell lines and thymocytes (Denny'00, Denny'99). Although the specific target of the Lck SH3 domain that allows it to regulate the MAPK pathway has not been established, Li et al., have recently shown that Lck-deficient J.CaM1.6 cells reconstituted with an LckW97A mutant are defective in activation of Raf-1 following receptor stimulation (Li). Given the key role of the MAP Kinase pathway in the control of cell proliferation and gene expression, our findings strongly suggest that the altered activation of peripheral LckW97A T-cells is due to defective induction of ERK-1 and ERK-2 following stimulation of the antigen receptor.

In order to examine what effect the convergence of multiple signaling pathways would have on the observed defects in MAPK pathway induction and activation in Lck W97A T-cells would have, preliminary investigation of Ag-stimulation, using the OT-1 Tg animal model, was performed. The transgene in these mice encodes a TCR specific for the

SIINFEKL sequence of ovalbumin. *In vitro* investigation of SIINFEKL stimulation on Lck W97A and WT peripheral T-cells indicates only modest reductions in the activation of Lck W97A T-cells compared to that of anti-TCR specific stimulation, as indicated by approximately half the percentage of cells that are able to proliferate in response to the stimulation at low dose of peptide (0.003μg/ml) and almost no difference at higher doses (0.03μg/ml), with IL-2 production being only slightly reduced at higher doses of peptide compared to WT (Fig. 14). It is interesting that higher doses of SIINFEKL, as with higher doses of anti-CD3 alone, appear to recover proliferation defects, and mitigate IL-2 production (Figs. 10,14). The compensation in reduced activation of OT-I-stimulated Lck W97A T-cells compared to that of anti-CD3 stimulation could be facilitated by the combined signaling of multiple receptor engagement in T-lymphocytes employed via Ag-induced ligation. This multi-receptor ligation could lessen the requirement for Lck-specific signaling, compared with that of Ab-mediated TCR-stimulation alone (Fig. 10). It has also been shown that Lck may negatively regulate signals received following peptide/MHC stimulation of T-cells (Lee), which might come in to play at certain doses of peptide stimulation (Criado). Moreover, at higher Ag doses, Fyn may override the importance of Lck for the initiation of TCR signaling (Criado, Sundberg), and may explain why activation would be improved following more potent Ag stimulation in Lck W97A T-cells compared to low peptide doses and Ab-directed stimulation (Figs. 10,14).

In order to further examine other parameters that could affect Ag-recognition and subsequent effector function in response to that activating stimuli, we examined the ability of Lck W97A T-cells to bind and activate B-lymphocytes in response to Ag stimulation.

The current model for the order of events following T-cell/APC interactions includes: 1) the initial Ag-independent cell-cell contact that does not rely on the activity of Fyn, Lck, ZAP-70 or Itk. 2) If Ag is present, this initial step is followed by ligation of the TCRs within the contact zone and attains the threshold level of stimulation (set by Lck) and results in membrane ruffling and cytoskeletal reorganization, also regulated by Lck. 3) The signal is sustained and a more concentrated contact zone forms for improved adhesion and signaling. (Donnadieu, Grakoui, Monks,Wulfing). Lck-deficient T-cells, and those having a mutation of the Lck SH3 domain (cell line data), have significant defects in actin-remodeling and LFA-1 recruitment during T-/B-cell conjugate formation *in vitro*, irrespective of the function of the MAPK protein, Mek1 (Morgan). In order to more fully understand how Lck might be modulating conjugation, the effects of Lck on cytoskeletal reorganization have been examined by performing T:B-cell conjugation experiments. The guanine nucleotide exchange factor (GEF), Vav-1, is an important regulator of Rho family GTPases, which are important for the process of actin remodeling. Defective Lck activity downstream of CD28 signaling has been implicated as having significantly negative effects on Vav-1-dependent Rac-1 activity and NF-AT activation, and thus, defective cytoskeletal remodeling and conjugate formation *in vitro* (Carey, Michel). Our results with *in vitro* conjugation, using Lck W97A OT-2 mice whose Tg TCR is specific for ovalbumin, do not indicate a role for the Lck SH3 domain in regulation of T-cell/B-cell conjugation following Ag stimulation, as there are no obvious reductions in the number of conjugates (double stained for TCRβ and B220) following co-culture of Ag-stimulated murine peripheral T- and B-cells (Fig. 25). This could be due to the strength of Ag stimulation, as well as the

role that antigen-dosing may play in the dependence on Lck for this process (Criado). Also, this is very preliminary work (n=2), and should be repeated. Also, confocal microscopy staining for anti-CD3 (T-cells) and B220 (B-cells) should be performed to confirm conjugate formation is unaffected.

Although LckW97A T cells were activated in response to receptor and co-stimulatory signals, and conjugation did not seem affected under the conditions tested, it remained possible that they had other functional defects, particularly in light of the reduced induction of the MAP kinase pathway. To examine this we assessed the *in vivo* immune response of LckW97A mice to antigen/adjuvant immunization and helminth infection.

III. Lck SH3 Domain Selectively Affects Th2 Immunity.

Although Lck W97A T cells were activated in response to receptor and co-stimulatory signals, it remained possible that they had functional defects, particularly in light of reduced induction of the MAP kinase pathway. To examine this we assessed the *in vivo* immune response of Lck W97A mice to antigen/adjuvant and bacterial immunization as well as infection with the helminth, *Nippostrongylus brasiliensis*. We found that Lck W97A mice have a selective defect in Th2 immunity as characterized by a significant reduction in the serum levels of IgG1, IgE and IL-4, which are known to be associated with, and critical for, the propagation and maintenance of Th2 immune responses (Figs. 26,29,30). There was no effect observed on pre-immune serum Ig levels or the induction of antigen-specific IgG2b and IgG3 isotypes (Fig. 26). Moreover, immunization of Lck W97A mice with heat-killed *Brucella abortus,* a strong inducer of Th1 immunity, shows

no defects in IFN-γ production compared to WT (Fig. 28). Furthermore, Th1/Th2 *in vitro* differentiation studies, performed by culturing purified CD4+ T-lymphocytes in the presence of Th differentiating cytokines, indicate that Lck W97A CD4+ T-cells are able to become either Th1 or Th2-type cells at both high and low doses of 2C11 stimulation as characterized by similar levels of intracellular IL-4 measured compared to WT (Fig. 31). However, the ability of the CD4+ T-lymphocytes to properly polarize into either Th1 or Th2 T-cells appears defective compared to WT. *In vitro* differentiation shows that although there are no differences in the absolute number of IL-4 producing cells in Lck W97A cultures, the polarization is reduced, as there is a much higher percentage of IFN-γ-producing cells under Th2 differentiating conditions compared to WT.

These results indicate that Lck SH3 domain function contributes specifically to the induction of Th2 immunity, and not Th1 immune responses, and is not the result of an initial Ig immune bias. Other contributing factors to the observed reduced Th2 immunity besides a defect in the ability of Lck W97A T-lymphocytes to properly polarize *in vivo,* could include a defect in survival of Th2 cells, or a specific defect in Th2 cell effector function, such as a failure to provide B-cell help via CD40/L interactions. Moreover, it is very likely that enhanced production of inhibitory cytokines by Lck W997A mice, such as IFNγ, could antagonize Th2 immunity by suppressing IgE class-switching (Xu '94).

Lck has previously been implicated in Th2 cell differentiation by studies analyzing the effect of expressing a dominant-inhibitory Lck transgene in peripheral T cells (Yamashita'98). This catalytically inactive mutant would be expected to block Lck functions dependent on catalytic activity. Our results with the Lck W97A mice suggest

that function of the Lck SH3 domain is also required for Th2 differentiation. Full Lck catalytic activity may be needed to phosphorylate a target of the SH3 domain, or it may provide independent functions specifically required for Th2 differentiation.

A substantial amount of evidence has accumulated showing that TCR signaling influences Th differentiation and function (Marsland). Our analysis of Lck W97A mice suggests that the Lck SH3 domain links TCR signaling specifically to induction of the MAP Kinase pathway and that this response is necessary for Th2 differentiation. These results are consistent with an earlier study showing that perturbation of Ras signaling blocked Th2 differentiation (Yamashita'99). In addition to Lck, LAT, the Tec kinases Itk and Rlk, as well as PKCØ have been implicated in Th differentiation (Schaeffer, Marsland). In contrast to our findings, these signaling molecules are proposed to influence Th differentiation by affecting the overall strength of TCR signaling, or by specifically regulating the NFAT or NFκB transcription factors that are required for IL-4 expression and Th2 differentiation. P38, for example, is a member of the MAPK protein family and has been linked with Th1 differentiation and IFN-γ production (Rincon, '00, '03). Direct activation by Lck is believed to occur at Tyr323 on P38, in an "alternative" activation pathway (vs. MKK activation of Thr180 and 182) that follows TCR ligation (Salvador). Even though our findings show that the Lck SH3 domain does not negatively impact IFN-γ production (intracellular) (Fig. 28), P38 activation was analyzed by western immunoblot to examine this pathway in Lck W97A peripheral T-cells. Purified WT and Lck W97A T-cells were stimulated with anti-CD3 and lysates were analyzed by western immunoblot for P38 and P-P38 levels. W97A T-cells had higher basal levels of P-38 than WT, but

underwent a much less dramatic stimulation following anti-CD3 stimulation (Fig. 24). Control P38 lysates were run in parallel to show that basal levels of total P38 were not different between groups (Fig. 24). This experiment was performed twice for all time points with slightly variable results. Although the unstimulated and 30-minute stimulations did not always show elevated levels of phospho-P38 induction in the Lck W97A T-cells (n=3), the 60-minute time point always showed similar, if not slightly elevated levels of phospho-P38 induction in the mutant lymphocytes (n=2). Interestingly, although this demonstrates that there is not a block in P38 activation in Lck W97A T-cells, and that the Lck SH3 domain is not critical for this interaction, it does indicate that higher basal Lck activity in Lck W97A T-cells may be differentially regulating P38 activation, and might help to explain the heightened IFN-γ production observed following Th1/Th2 *in vitro* differentiation (Fig. 31). The reduced levels of IFN-γ early (day 4) in the immune response could indicate defective innate immunity in Lck W97A mice. NK cells, a cellular constituent of the innate immune system, are known producers of IFN-γ and contain Lck. It is possible that the Lck SH3 domain mutation is negatively affecting this cell population and is thus able to alter the early responses to immunization. Examination of serum levels of IFN-γ at early time points following immunization, as well as NK cell population statistics, should be performed to determine the magnitude of the effect that the Lck SH3 domain has on these processes. Depletion of T-cells could be used to further examine the effects that the Lck W97A mutation has on the NK cell population and IFN-γ production. NK cells are also critical regulators of auto-immunity and can act to induce apoptosis of infected cells via the release of granzymes. If the Lck W97A mutation negatively impacts

NK cell activation, there could be increased incidence of auto-immunity and/or decreased killing of infected cells during an immune response.

The concept that the Lck W97A mutation might significantly impact the NK cell population, also leads to the concept that this mutation can have both direct and indirect effects on the innate and adaptive immune systems. If there is heightened IFN-γ production by Lck W97A T-cells (or NK cells) during an immune response, this can act to activate cells of the innate immune system, such as macrophages and neutrophils, leading to the secretion of TNF-α and reactive oxygen species (ROS) that further enhance the inflammatory responses of the host animal (DeMayer, Philip). Recent evidence also suggests that IFN-γ can also stimulate CD8 T-cells directly, thereby enhancing adaptive immunity via heightened CD8-dependent cytotoxicity during viral infections (Whitmire).

There are many factors involved in the proper differentiation of naïve T-cells into Th2 T-cells including antigen recognition, signaling through the TCR, and activation of the interleukin-4 receptor (IL-4R) pathway. The role of the Tec kinase, Itk, in these processes has been best examined using Itk deficient mice (Itk-/-) which have varied deficiencies in Th2 immunity, including significantly reduced levels of secreted IL-4 following infection with various parasites, such as *Nippostrongylus brasiliensis* (Fowell, Schaeffer, review: Schwartzberg). Itk deficient CD4+ T-lymphocytes are able to differentiate into Th2 T-cells, but have defective IL-4 production and GATA3 transcription (Au-Yeung). As Lck is known to activate Itk (at position: Tyr 511) (Heyeck), it is possible that Lck SH3 domain mutant T-cells may have altered activation of Itk, and thus, altered Th2 effector function.

Furthermore, reduced IL-4 production by Lck W97A mice *in* vivo following *Nippostrongylus brasiliensis* infection (Fig. 30) helps to explain the reduced levels of IgE also noted in the serum of infected mice. As IL-4 is known to induce immunoglobulin class switching in B-cells for the synthesis and secretion of IgE, it is probable that defects in IL-4 production result in decreased synthesis of IgE and therefore, reductions in Th2 immunity. Aside from its role downstream of TCR signaling, it is also possible that Lck regulates MAP Kinase activation following IL-4 receptor stimulation, or affects IL-4 synthesis and secretion *in vivo*. Previous work has shown that signaling through the IL-4R pathway and activation of the MAP kinase pathway is critical for subsequent propagation of a complete Th2 immune response (Yamashita'99, So). In the absence of normal IL-4R signaling, Th2 cells are unable to proliferate and provide effective Th2 immunity (Gros, Kopf). In this case, the Lck W97A mutation may not block the initial differentiation of Th2 cells, but may be required for IL-4R signals necessary for a proper Th2 effector response. Moreover, Src family members are known to bind members of the signal transducers and activators of transcription (STAT) family. Lck is known to bind and phosphorylate STAT3 (Lund). STAT6 is a regulator of IL-4 transcription and has a proline rich domain that may interact with the Lck SH3 domain for binding and subsequent activation. Investigation into STAT6 phosphorylation in Lck W97A T-cells should be further examined.

Our results showing that IL-2 production is significantly impaired following TCR-specific stimulation in Lck W97A T-lymphocytes may have a more far-reaching impact on immunity than we have so far explored. As IL-2 is a known activator of not only T-cells,

but T-regulatory T-cells and natural killer cells as well. It would be of great interest to further examine potential differential regulation of these immune cell subpopulations, as well as the effects that differential IL-2 levels may play in Lck W97A mice following immunization or infection. As we note that Lck W97A T-cells produce significantly less IL-2 following anti-TCR ligation, differences in IL-2 production early on during an immune response could significantly alter the establishment of efficient adaptive immunity via defects in T-helper, T-regulatory and natural killer cell activation and differentiation. If IL-2 levels were to be normalized (potentially via cytokine injection *in vivo*), it would be very interesting to see if some of the observed activation and immune deficiencies would still be observed.

Taken together, our data present novel evidence that the SH3 domain of Lck is a critical mediator of normal Th2 immunity *in vivo*, and further provides for a potential mechanism for this defect by the regulation of Th2 cell polarization by the Lck SH3 domain via activation of the MAP kinase protein, Erk.

IV. Mosquito Saliva Affects Innate Immune responses.

During the course of this thesis work, I was able to perform an internship at the Institut Pasteur, Paris, France, in the laboratory of Dr. Salaheddine Mecheri. I was able to study the infection models for the murine strain of malaria, *plasmodium berghei.* It was during this internship that I examined the role that vector saliva was playing in the initial host response to infection.

The aim of this body of work was to understand if and how mosquito saliva might be affecting the cells of the innate immune system, and investigate these potential manipulations with regard to their role in *Plasmodium* infection of C57BL/6 mice with *P. berghei*. The activation of macrophages and bone marrow-derived dendritic cells was examined, as well as the ability of these innate immune cells to induce T-cell activation following incubation with salivary gland proteins. Several methods, both *in vitro* and *in vivo* were employed, as well as the opportunity to design experiments to examine new techniques for the study of these processes.

Direct analysis, included in this work, has shown that *Anopheles stephensi* salivary proteins are able to enhance the activation of dendritic cells and macrophages (Figs. 33, 35). This, in combination with enhanced activation of T-lymphocytes by saliva-activated macrophages (Fig. 37), potentiates the belief that mosquito saliva may enhance the activation of both branches of the immune response, and may play a protective role in response to *Plasmodium* infection.

Mosquito salivary proteins have also been implicated as exacerbating mast cell release of Histamine, leading to cerebral malaria infection. If there is no Histamine-mediated mechanism during infection with Plasmodium, there is protection from cerebral malaria (Beghdadi). Mosquito salivary proteins, therefore, have multiple modes of immune modulation, and therefore, the specific mechanisms must be addressed in the development of an effective malaria vaccine. It is interesting, that extended prophylactic use of anti-malarials results in skin rashes, and that the treatment of these conditions with anti-Histamines not only rids the patient of the skin disorder, but also has been shown to protect

against cerebral malaria (unpublished data). If the mast cell-mediated responses to *Anopheles* salivary proteins could be blocked during vaccine trials, but the immune responses to the salivary proteins allowed to occur, there could, potentially, be enhanced vaccine efficacy.

The next stage to understanding the relevance of these processes involving mosquito saliva would be to examine them *in vivo*, in a murine model of *Plasmodium* infection, and to determine how modulation of these responses confer host protection upon challenge with *Plasmodium berghei*.

V. Lck SH3 Domain May Have a Role in *Plasmodium* Pathogenesis.

In order to examine if previous reports stating that Lck SH3 domain interactions with sporozoites is critical for localization and infection of hepatocytes was also true *in vivo, ex vivo* analysis of the HePG2 cell line and primary murine hepatocytes for the presence of Lck was first confirmed, as Lck has not previously been reported in hepatocytes. Although this is a preliminary investigation, western immunoblot analysis indicates that Lck is present in both the immortalized liver cell line (HePG2) as well as in primary murine hepatocytes (Fig. 38). Further analysis of immunoblots, along with PCR analysis for the presence of Lck in purified primary hepatocytes will be required to confirm this result.

As it appears very likely that Lck is present in hepatocytes, it is extremely intriguing to hypothesize that the SH3 domain of Lck is acting to bind the sporozoite protein TRAP, potentially activate TRAP, and assist with its localization to downstream

critical modulators of its function. The ultimate experiment to either confirm or deny this hypothesis would be to infect Lck W97A mice with infectious sporozoites and determine parasite load. If the mice present with protection from *Plasmodium*, or even decreased pathogenesis, it would indicate that hepatocyte infectivity was indeed affected, and would give further purpose to pursue the Lck SH3 domain as a target for an efficacious vaccine against malaria.

IV. Conclusions and Significance.

In summary, our analysis indicates that Lck SH3 domain function is necessary for normal peripheral T lymphocyte activation and induction of the MAP kinase pathway following T cell antigen receptor stimulation. Our data does not indicate a critical role for the Lck SH3 domain in CD28 function or co-stimulation; however, mice with a defective Lck SH3 domain exhibit a substantial defect in Th2 immunity. These findings suggest that Lck provides a key regulatory function in helper T cell development by providing a selective link to activation of the MAP kinase signaling pathway.

Future work required to better understand the specific parameters for this defect would include the use of inhibitors to specifically target the activity of Lck in WT mice; in particular, the activity of the SH3 domain in isolation. The usefulness of these findings would be of great importance for the management and control of known Lck SH3 domain interactions, specifically with regard to immunization and infection models such as the ability to mitigate the role of Lck in disease immunotherapy for HIV, pneumonia, and even T-cell cancers which have implicated roles for Lck SH3 domain function for infection and

pathogenesis. Current inhibitors for the repression of *lck* transcription (also *yes* and *lyn)*, independent of new protein synthesis, include histone deacetylase inhibitors (HDIs), such as the endogenous butyrate (Hirsch). Understanding how mitigation (versus inhibition of total Lck) by inhibition of Lck SH3 domain function *in vivo* could affect T-cell responsiveness and function is of immense value for the potential use of Lck SH3 domain inhibitors for therapeutic trials. Of especial interest in current research is data indicating that the Nef molecule of the human immunodeficiency virus-1 (HIV-1) is able to bind the Lck SH3 domain and induce Lck activation (potentially via displacement of intramolecular inhibitory conformation by binding with the Lck SH3 domain). Thus activated, Lck is then able to assist in viral protein signaling for HIV-1 replication and invasion of host T-lymphocytes. This interaction, as well as the ability of Nef to affect the association between Lck and the CD4 co-receptor (a target of HIV), is of great interest as a potential target for vaccine development (Trible).

An improved *in vivo* approach to studying the Lck SH3 domain and its relevance to infection and immunity for "proof of theory/principle" studies would be to create a conditional Lck W97A knock-in mouse model. This would bypass any developmental issues involved with the Lck SH3 domain and thymocyte development, so that the defects observed *in vivo* would have no caveats with relation to T-cell development, such as abnormal positive selection as noticed in Lck W97A thymocytes (Rudd '06). By circumventing the requirements for the Lck SH3 domain in T-cell development, peripheral T-cells will have pre-TCR and TCR signaling comparable to WT. Thus, the resulting population of peripheral T-lymphocytes may not have the potential variable reliance on the

function of the Lck SH3 domain as pertains to proximal pre-TCR and TCR signaling (which may be selected for during thymic development in Lck W97A T-cells). Alternative models could also include the creation of a tetracycline inducible system (Gossen'92, '95) in which the presence or absence of tetracycline would be able to manipulate the regulation of the Lck W97A phenotype.

The Lck SH3 domain has also been implicated as a critical component for *Plasmodium* sporozoite infection of host hepatocytes, as well as sporozoite migration from the mid-gut to the salivary glands in the mosquito vector. Activation of the sporozoite protein, TRAP, a critical adhesion molecule for host invasion, and which is also required for localization of the sporozoite within the liver cell, has been shown to rely on Lck SH3 domain for binding and localization within the cell. It has also been shown that Lck is able to activate TRAP once it is bound to the Lck SH3 domain (Akhouri, Sultan). Studies involving the co-infection processes involving HIV and *Plasmodium* are intensely investigating immune targets for vaccine development, which will be able to simultaneously interrupt/prevent infection and disease pathogenesis. The Lck SH3 domain presents a unique and intriguing target for new approaches to simultaneous-treatment vaccine development, as it has been implicated as critical for both of these life-threatening disease pathologies.

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