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The Role of ITK and RLK in CD8+ T Cell Development and Function: a Dissertation

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THE ROLE OF ITK AND RLK IN CD8+ T CELL DEVELOPMENT AND FUNCTION

A Dissertation Presented

By

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Abstract

Itk and Rlk are members of the Tec kinase family of non-receptor protein tyrosine kinases that are preferentially expressed in T cells. Numerous previous studies have demonstrated that these proteins play an important a role in the regulation of signalling processes downstream of TCR activation in CD4+ T cells, particularly in the phosphorylation of PLCγ1. In addition, Itk and Rlk have both been shown to be important for CD4+ T cell development, differentiation, function and homeostasis following TCR activation. In the absence of Itk and Rlk, CD8+ SP thymocytes and T cells develop a memory/previously activated phenotypic profile, however, very little is known about the influence of Itk and Rlk on CD8+ T cell development and function. This study illustrates a previously unappreciated role for Itk and Rlk in the regulation of cytokine signals during CD8+ SP thymocyte maturation, and in the development of the memory CD44hi profile of Itk -/- and Itk -/- Rlk -/- CD8+ SP thymocytes and CD8+ T cells. This study also provides the first detailed study of the role of loss of Itk and particularly both Itk and Rlk in CD8+ signalling and function and shows that these Tec kinase family members play an important role in the maintenance of CD8+ T cell fitness and function, particularly in the ability of CD8+ T cells to accumulate in response to infection. Collectively, my studies demonstrate a critical role for Itk and Rlk in the generation of optimal CD8+ T cell responses. They also raise the novel observation that these proteins may be involved on the regulation of cytokine signals in T cells.

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Attributions

Chapter II

The work in this chapter was done in collaboration with Julie Lucas. Dr. Joseph Maciaszek also assisted us with aspects these experiments.

Chapter III

This study would not have been possible without the kind provision of LCMV Armstrong virus, LCMV epitope specific peptides and tetramers by Dr. Raymond Welsh and the Welsh Lab. Dr. Michael Brehm, Dr. Sung Kwon Kim, and Keith Daniels also provided invaluable assistance with various aspects of my experiments. The Vaccinia – OVA construct was a kind gift from Dr. Kenneth Rock. The OVA specific peptide SIINFEKL was also kindly provided by the Welsh Lab.

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To the memory of all who struggled and died
that I may live in freedom.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Development of the T Cell Repertoire

Thymic Organogenesis:

The thymus is the seat of T cell development and differentiation and without this organ there would be no thymocytes available for seeding of the peripheral lymphoid compartments. That the thymus was the source of the cells that were found in the lymph and peripheral organs was reported by Jacques Miller in 1961 at a time when not much was know about this mysterious organ that sat above the heart and gave rise to the cells that travelled throughout the body. Since this time, significant progress has been made in characterising this organ that is now so central to T cell biology and function. Perhaps not surprisingly, the relationship between the thymus and thymocytes is a symbiotic one, with the thymocyte needing the thymus to complete its maturation and differentiation and the thymus relying on the thymocyte maturation process to become a fully differentiated and mature organ (1, 2).

This symbiotic relationship begins at about d10 of murine gestation with the formation of the thymic anlage. A primitive epithelial thymic primordium is generated when neural crest derived mesenchymal cells fuse with ectoderm and endoderm from the pharyngeal pouch. These cell types are the precursors of the thymic stromal cells that makeup the skeleton of the developing thymus (3). Thymocyte progenitors enter the thymic primordium from the fetal liver via surrounding blood vessels at about d11 of murine gestation. As the thymus is not yet vascularised, the thymocyte progenitors have to leave adjacent pharyngeal vessels and traverse the perithymic mesenchyme and the

basement layer surrounding the thymic rudiment. This process is mediated by chemoattractants secreted by the stromal cells of the thymus. The progenitors proliferate and differentiate, allowing the thymus to develop from an undefined mass of epithelial cells and lymphocytes into a highly ordered structure containing distinct cortical and medullary regions surrounded by a subcapsular region, interspersed with blood vessels and containing many distinct cell types (1, 2). In keeping with the symbiosis between thymus and thymocyte, in the absence of thymocyte progenitors, the thymus is incapable of developing its highly ordered structure. In animal models containing natural mutations that impair T cell development, the cortical and medullary regions fail to develop. This development is restored by the re-introduction of thymocytes into the thymic rudiment (4, 5).

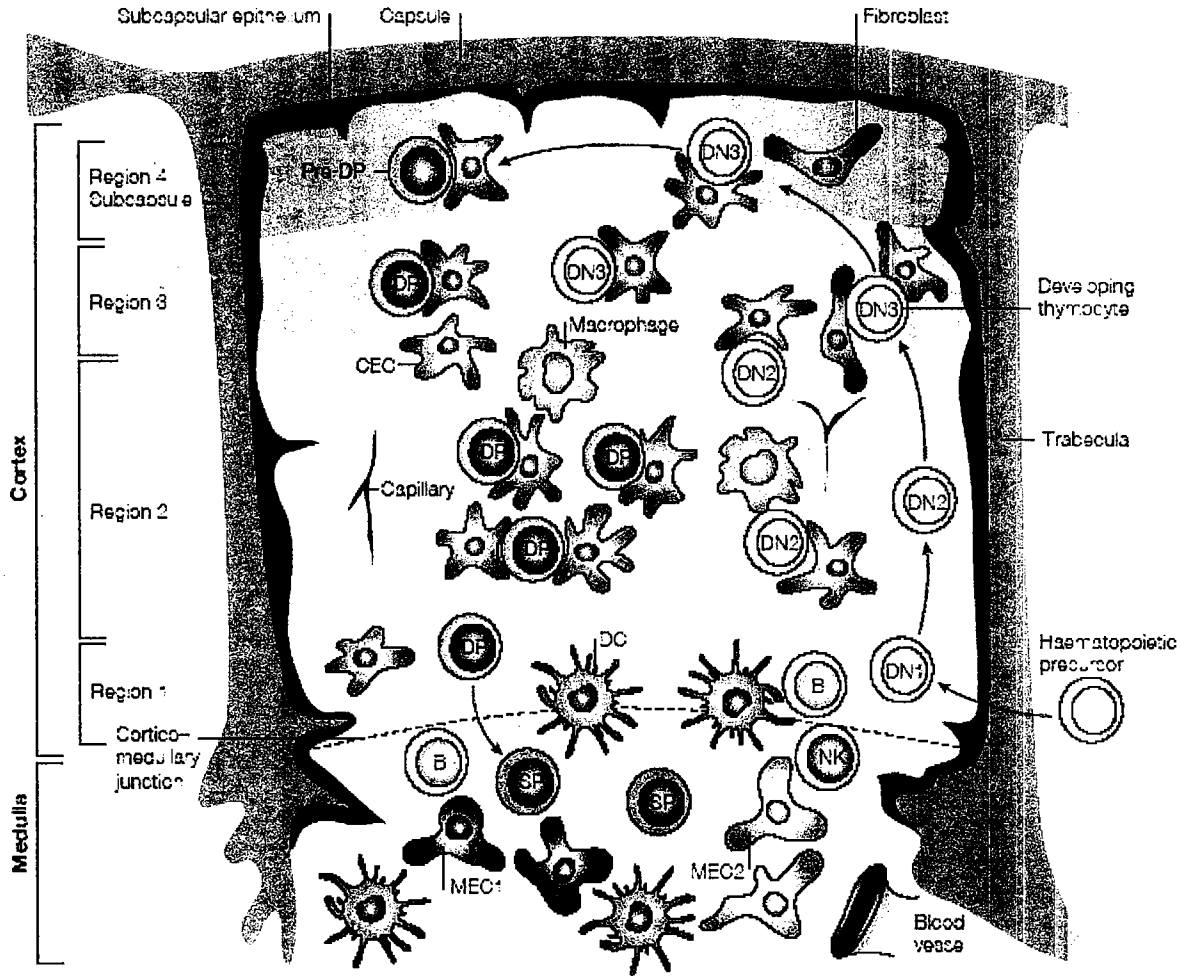
Extensive phenotypic analysis of the cell types composing the thymus has revealed that the cortex of the thymus contains mainly cortical epithelial cells and macrophages, while the medulla of the thymus contains mainly bone-marrow derived cells such as dendritic cells and macrophages. Interdigitating dendritric cells are localized mainly at the boundary between the cortex and the medulla known as the cortic-medullary junction (1, 6). These epithelial and bone-marrow derived cells express the MHC Class I and MHC Class II molecules that are necessary for thymocyte development. Blood vessels permeate the thymus, extending deep into the medullary and cortical regions of the thymus. Thymocyte progenitors, derived from the bone-marrow later in neonatal life, utilize these blood vessels to gain access to the thymus. The thymic progenitors enter the thymus at the cortico-medullary junction. From here they traverse

the cortex and travel to the subcapsular region, maturing in the process. Immature thymocytes from the subcapsular region then re-migrate across the thymus, first across the cortex and then into the medulla, developing into mature thymocytes along the way (7) (Figure 1.1).

Figure 1.1: Thymic Organization and Thymocyte Migration

Thymocyte progenitors enter the thymus at the cortico-medullary junction via blood vessels located in and around the medulla of the thymus. The cells then migrate inward toward the subcapsular epithelium. Crosstalk between the developing thymocytes and the thymic epithelium promotes their maturing and differentiation from DN (CD4- CD8-) or TN (CD3- CD4- CD8-) progenitors into pre-DP (CD4+ CD8. As the DP cells become mature SP thymocytes they migrate into the medulla where they remain until their exit to the periphery.

THYMUS ORGANIZATION AND THYMOCYTE MIGRATION



C.Blackburn and N. Manley, Nature Reviews 2004 (4):278

The forces that regulate the formation of this highly complex organ still remain a mystery. Although incredible progress has been made in identifying the individual cell types involved in the formation of the thymus, many questions remain regarding the processes that regulate the generation of the distinct microenvironments, the communication between thymocytes and the various cell types that populate these microenvironments and how this impacts the complex route the thymocytes take through the thymus and their subsequent maturation and differentiation.

T Cell Development:

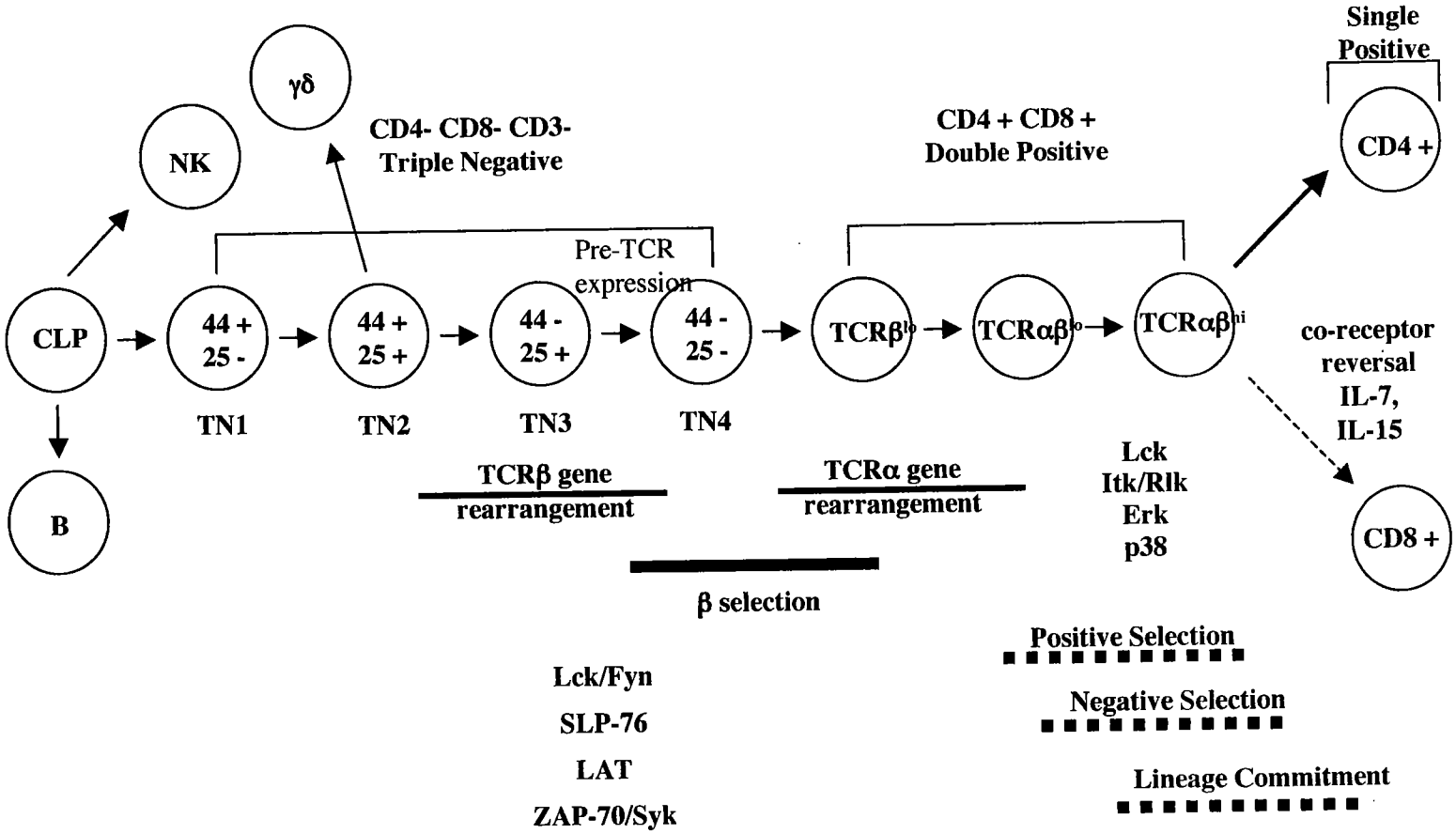
T cell development is a highly complex process that takes place in a series of ordered steps concurrent with the differentiation and organization of the thymic architecture. Upon entry into the thymus from the bone-marrow, the common lymphoid progenitor lacks expression of the T Cell Receptor (TCR), CD3, a component of the TCR complex and the co-receptors CD4 and CD8 and is designated a triple-negative TN immature thymocyte. T cell maturation is chronicled by the sequential expression of these and other cell surface molecules, and can be divided into three broad developmental stages based on the surface expression of these receptors Figure (1.2) (8). During the most immature TN stage of thymocyte development, thymocytes express the molecules CD44 and CD25, and can be further sub-divided into four developmental stages based on expression of these receptors: TN1 (CD44+ CD25-), TN2 (CD44+ CD25+), TN3 (CD44- CD25+), TN4 (CD44- CD25-). As the immature thymocytes progress through the latter two stages (TN3 – TN4) of development, they undergo VDJ rearrangement of their TCRβ genes. This developmental process takes place as the progenitors make their

second pilgrimage across the thymus from the subcapsular region to the cortico-medullary junction (7). Productive rearrangement of TCRβ genes and expression of TCRβ along with pre-Tα (the Pre-TCR complex) on the surface of the thymocyte leads to β selection, upregulation of the CD4 and CD8 co-receptors, and the generation of immature CD4+ CD8+ double positive (DP) thymocytes. This initiates the second phase of thymocyte development. Following pre-TCR expression and differentiation to the double positive stage of thymocyte development, thymocytes undergo repeated re-arrangement of their TCRα chain genes until a productive rearrangement is made. This TCRα chain replaces the surrogate pre-Tα chain, generating a functional mature α/β TCR. Double positive thymocytes are found mainly in the cortex of the thymus as they migrate from the subcapsular region back to the medulla (7). Thymocytes that do not generate productive TCRβ rearrangements undergo apoptosis. The DP thymocytes are now capable of undergoing their third and final phase of maturation, the processes of positive and negative selection and lineage commitment, to become mature CD4+ or CD8+ single positive (SP) thymocytes (8, 9) (Figure 1.2).

Figure 1.2: Thymocyte Development

T cell development begins with entry of the common lymphoid progenitor (CLP) into the thymus. These cells are CD3- CD4- CD8- and are designated TN cells. Thymocytes progress through several stages of TN development characterized by expression of CD44 and CD25 termed TN1 – TN4. These cells are permanently committed to the T cell lineage with the initiation of TCR γ and δ gene rearrangement at the TN1 – TN 2 stages. TN2 cells that successfully rearrange their TCR γ and δ genes commit to the γδ lineage T cell lineage. Rearrangement of the TCRβ gene begins at the TN2 – TN3 stage and signals commitment to the αβ T cell lineage. A functionally rearranged TCRβ gene is expressed at the cell surface coupled with the pre-Tα chain to form the pre-Tα complex. Progression to the DP (CD4+ CD8+) stage is preceded by rearrangement of the TCRα gene. Expression of a functional TCR α gene signals allelic exclusion and the functional TCR α replaces pre-Tα and pairs with TCRβ chain to form the mature αβ TCR on the surface of DP cells. Signal through TCR αβ promote positive and negative selection and lineage commitment leading to the generation of mature CD4+ and CD8+ SP thymocytes.

THYMOCYTE DEVELOPMENT



Thymocyte Selection:

The T cell repertoire is sufficiently diverse to allow for the generation of immune responses to a wide array of foreign pathogens. This diversity is generated by the processes of VDJ recombination and positive and negative selection that enable the generation of a diverse T cell repertoire, while simultaneously protecting us from autoimmunity by removing potentially self reactive T cells from the T cell repertoire (8).

Positive selection refers to the process whereby double positive (DP) thymocytes are induced to become either CD4+ or CD8+ single positive thymocytes, depending on their interaction with either MHC Class I or MHC Class II respectively. Positive selection is thought to occur primarily in the cortical area of the thymus where the TCR expressed on T cells encounter self-peptide/MHC molecules expressed on thymic epithelial cells. Negative selection is thought to occur primarily in the medullary area of the thymus, where the maturing T cells encounter peptide/MHC complexes expressed on medullary epithelial cells and bone-marrow derived stromal cells (2, 10, 11). Supporting this view are data showing that most of the thymocytes present in the medullary region of the thymus are either CD4+ or CD8+ single positive (SP), while the cortical areas contain mainly CD4+ CD8+ double positive thymocytes (DP).

T cell selection is governed by the efficacy of the signal received through the TCR. If this interaction is of a moderate affinity allowing for the generation of a signal within a unique signalling threshold, T cells are positively selected. However, if a double positive T cell encounters self-peptide/MHC complexes to which they have too strong of an avidity, a deleterious signal is generated which leads to the death of that potentially

autoreactive T cell (**8**). T cells also undergo death by neglect if they do not encounter self-peptide/MHC complexes that can generate a signal within the threshold that allows for positive selection. Following the selection process, the thymocytes are now committed to being either CD4+ SP or CD8+ SP, exit the thymus from the medulla and migrate into the peripheral lymphoid areas where they are now capable of generating immune responses to a wide array of pathogens, while maintaining silence to self.

Lineage Commitment:

Lineage commitment is the decision making process that a DP thymocyte undergoes in becoming a CD4+ or CD8+ SP thymocyte. This decision occurs concurrent with the process of positive selection and is governed primarily by the interaction of TCR and the CD4 or CD8 co-receptors expressed on the surface of the thymocyte with either Class I or Class II MHC molecules expressed on thymic epithelial cells. Classically, engagement of TCR and CD8 with MHC I results in the down-regulation of CD4 co-receptor expression and generation of a CD8+ Class I restricted T cell, while CD4+ SP thymocytes arise from the interaction of TCR and CD4 with MHC II molecules and the resultant extinguishing of CD8 co-receptor expression.

Cytokines and Signalling Molecules in CD4 and CD8 SP Selection and Lineage Commitment:

It has always been of much interest that signals received through the TCR can result in such distinct outcomes – positive selection, cell survival and lineage commitment versus negative selection and cell death. However, not all stages of

thymocyte development are dependent solely on signals transmitted through the pre-TCR or the TCR αβ complex. The earliest lymphoid progenitors (TN1 and TN2 thymocytes) express receptors for stem cell factor (SCF) and IL-7 and are dependent on the cytokines SCF and IL-7 for maintenance and survival in this early TCR independent stage of thymocyte development. The importance of these cytokines is demonstrated in genetically deficient murine models. For example, mice lacking IL-7R have an overall reduction in thymic cellularity with diminished production of αβ thymocytes and a complete absence of γδ thymocytes. This lack of αβ thymocytes is due to a block in TN thymocyte development at the TN1 – TN2 stage and it was subsequently established that IL-7 (acting through IL-7Rα and the common γchain) was necessary for the up-regulation of anti-apoptotic molecules such as Bcl-2, and for the regulation of transcription and VDJ recombination at the TCRγ genetic locus (12). This dependence on cytokine signals occurs before the maturing thymocyte undergoes the processes of positive and negative selection and lineage commitment. When the maturing thymocyte reaches the TN3 – TN4 stage of thymocyte development, it expresses the pre-TCR complex on its surface and enters the receptor dependent phase of thymocyte development. At this stage, expression of IL-7R is downregulated and is not re-expressed until the thymocyte becomes SP (13). The thymocyte is now primarily dependent on signals transmitted first through its immature antigen receptor complex (pre-TCR at TN3 – TN4) and later its mature α/β TCR (at the DP – SP transition).

Thymocytes express many of the same family of signalling molecules downstream of the pre-TCR and the α/β TCR on CD4+ or CD8+ SP thymocytes, as

peripheral T cells do downstream of their mature α/β TCR complex (14). This observation led to the analysis of the role of many of these molecules in thymocyte development and maturation, most successfully so in genetically deficient murine models. Among the membrane proximal molecules found to be important in CD4+ and CD8+ SP thymocyte selection and/or lineage commitment were the Src kinases Lck and Fyn, the Syk kinase ZAP-70 and the Tec kinases Itk and Rlk (15-18). Absence of the src kinases Lck and Fyn in developing thymcoytes results in a reduction in the development of SP thymocytes and abrogation of positive selection of CD4+ and CD8+ SP thymocytes (17, 19). Lck is also important in the CD4/CD8 lineage choice, as it has been shown that modulation of Lck expression and activity can affect the decision that a DP thymocyte makes when committing to either the CD4+ or CD8+ SP lineage (20, 21). Loss of ZAP-70 also results in a complete block in CD4+ and CD8+ SP thymocyte development, and as such does not affect the CD4+ versus CD8+ lineage choice (22, 23). Also implicated in modulation of the signalling thresholds that regulate positive and negative selection are the Tec kinase family members Itk and Rlk. Loss of Itk or both Itk and Rlk mostly affects positive selection of CD4+ and CD8+ SP thymocytes and also has a minor effect on negative selection of CD8+ SP thymocytes. So far, there is no evidence to suggest that loss of either or both of these kinases affects the CD4/CD8 lineage decision (15, 18, 24) (Figure 1.2).

Recently, several reports have provided evidence of a role for the common γc cytokines IL-7 and IL-15 in the later receptor dependent phase of thymocyte development and differentiation, mainly in the lineage CD4/CD8 lineage decision. The involvement of

IL-7 in the regulation of the CD8+ SP lineage decision was initially reported by Brugnera and colleagues (25). These authors, using a system meant to test the developmental potential of signalled DP thymocytes, showed that thymocytes exposed to stimulatory cultures containing anti-CD3 antibodies or the pharmacological agents PMA and Ionomycin down-regulated expression of the CD8 co-receptor to become CD4+ $CD8^{lo}$ intermediates. If subsequently cultured under non-stimulatory conditions, these thymocytes preferentially became CD4+. However, if cultured in media with IL-7, these thymocytes downregulated expression of CD4 and preferentially re-expressed the CD8 co-receptor to become CD8+ SP thymocytes. Participation of IL-7 in the CD8+ SP lineage decision was further demonstrated by Chong and colleagues who showed that thymocytes lacking the negative regulator of cytokine signalling SOCS-1, preferentially became CD8+ SP thymocytes when stimulated through the IL-7R (26). Ilangumaran and colleagues implicated IL-15 in regulation of the CD8 lineage decision by demonstrating that thymocytes from SOCS-1 -/- IFNγ -/- mice were skewed towards the CD8+ SP lineage in fetal thymic organ culture (FTOC), and were particularly sensitive to IL-15 stimulation *in-vitro* (27).

The Cellular Immune Response

After surviving the developmental and selective pressures of the thymic microenvironment, the mature, self-tolerant CD4+ and CD8+ SP thymocytes emigrate from the thymus to the peripheral lymphoid organs. These T cells circulate throughout the host and its network of lymphoid and non-lymphoid tissues in constant surveillance for the presence of foreign antigens and infectious agents.

The LCMV Model of the Cellular Immune Response

A hallmark of the mature immune system is its ability to mount a response to a variety of pathogens. One of the most studied of these pathogens is the murine Lymphocytic choriomeningitis virus (LCMV). LCMV is an enveloped ambisense RNA virus and is the prototypic member of the arenavirus family. It contains two ambisense RNA's, the L and S RNA's which each encode two genes. The L RNA encodes the L protein, a RNA dependent RNA polymerase required for transcription of the LCMV genome and the Z protein, while the S RNA encodes for nucleoprotein (NP) and glycoprotein (GP). LCMV replicates mainly in the cytoplasm of cells and spreads by budding from the plasma membrane of infected cells. It has a wide host range and can replicate in many different cell types including the cells of the central and peripheral lymphoid system. Infection of mice with LCMV and the immune response it generates has been used for years as a model to characterize the various cell types and mechanisms involved in the evolution of the cellular immune response to pathogens. There has also been extensive characterization of the epitope specific responses generated following LCMV infection, and there are many tools currently available that allow the antigen specific response to be followed at a single cell level, that makes LCMV a valuable tool for study of the cellular immune response (28, 29).

Innate Immunity:

Generally infection with LCMV results in a transient infection to which there is a strong sterilizing host immune response that clears the virus by eight days post infection,

and leads to the development of immunologic memory. Following LCMV infection or infection with other intracellular pathogens, infected cells produce type 1 IFNs, IFNα and IFNβ. These cytokines with their potent anti-viral properties are at the frontline of the host's defense to viral pathogens. Numerous cells types of the innate, non-specific immune system including NK cells, NK T cells and various granulocytes are recruited to the site of infection by the various cytokines and chemokines secreted by infected cells. NK cells are a major line of defense against viral infection. They are activated by the type1 IFN's and possess cytolytic granules and specific cell surface receptors, which enable them to recognize and directly eliminate infected cells. NK cells also secrete the inflammatory and anti-viral cytokines IFNγ and TNFα. These cytokines have direct anti-viral properties, as well as enhancing the activation state of macrophages and dendritic cells. Activation of macrophages and dendritic cells, also known as professional antigen presenting cells (APC's) is the link between the innate and adaptive immune response to infection (30, 31).

Adaptive Immunity: The Antigen Specific T cell response

Macrophages and dendritic cells, which are derived from bone-marrow precursors, along with B cells, are unique in their ability to present antigen and to catalyze antigen specific T cell responses. These cells express high levels of the co-stimulatory molecules B7.1/B7.2 and CD40, in addition to MHC Class I and MHC Class II molecules. In response to the inflammatory cytokine milieu produced by an ongoing infection, macrophages and dendritic cells up-regulate expression of their co-stimulatory

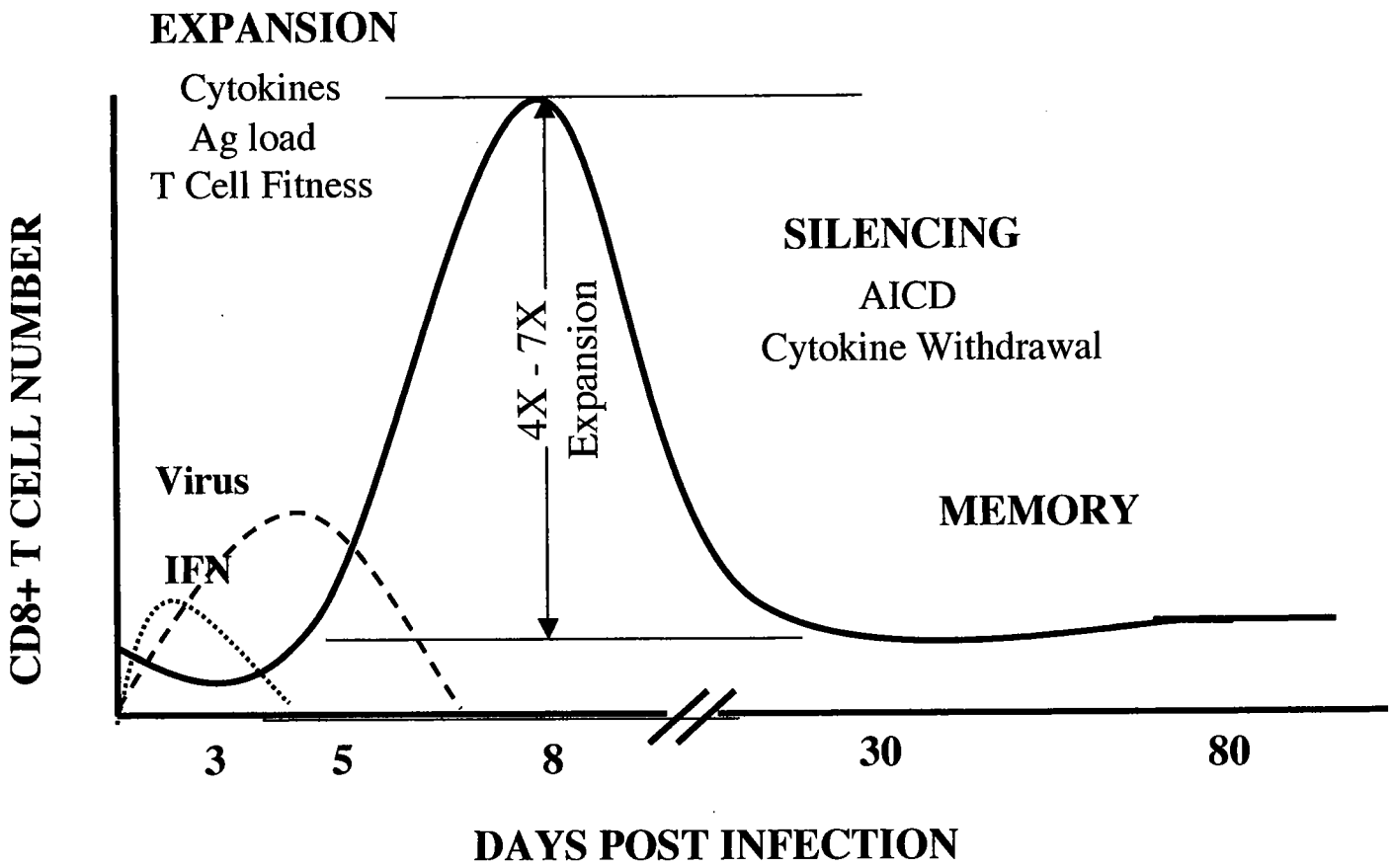
and MHC molecules. This enhances antigen presentation to CD4+ and CD8+ T cells and initiates activation of the antigen specific T cell (adaptive) immune response (31).

Infection with the Armstrong strain of LCMV results in a characteristic CD4+ and CD8+ T cell response that can be divided into three distinct phases. 1. The expansion/effector phase during which antigen specific T cells increase in number and function to control viral spread. 2. The silencing phase in which the immune system returns to homeostasis. 3. The memory phase, in which antigen specific cells surviving the response are maintained as a defense against secondary infection (28) (Figure 1.3).

Figure 1.3: The LCMV Model of the Cellular Immune Response

Early in infection with LCMV, the innate arm of the immune response is activated with production of the Type I IFNs (IFNα and IFNβ) and activation of NK cells. Viral replication peaks about day four post-infection and virus is cleared by approximately day 8 post infection. Infection with LCMV results in a characteristic expansion (approximately 5 to seven fold) of the CD8+ T cell subset that is detectable by day five post-infection and peaks at day eight post-infection. This expansion is a programmed event subject to many factors intrinsic and extrinsic to the CD8+ T cell such as response to cytokines and the duration and strength of the TCR signal. The peak of the infection is followed by a decline in the numbers of antigen specific cells as the immune system returns to homeostasis. This process is mediated in part by the process of activation induced cell death and also by cytokine withdrawal as the inflammatory response subsides. The cells remaining at the end of the immune response become long-lived memory T cells.

CD8 T CELL RESPONSE TO LCMV INFECTION



T Cell Expansion

Although there is expansion of both the CD4+ and CD8+ T cell compartments, the sterilising T cell response to LCMV infection is mediated mainly by CD8+ T cells. Antigen specific T cell expansion is first detectable at about five to six post infection. This is followed by a massive expansion of antigen specific CD8+ T cells that peaks at approximately eight days post infection (Figure 1.3). At this point LCMV specific CD8+ T cells account for approximately fifty percent of the CD8+ T cell compartment (32, 33). The mechanism behind this massive proliferation of CD8+ T cells is not well defined. It was initially thought to be partially dependent on the provision of IL-2 to the CD8+ T cells, in either a paracrine manner by CD4+ T cells or in combination with autocrine delivery by the CD8+ T cells themselves (34-36). More recently, it has been shown that the CD8+ T cell response to infection is an autonomous event initiated following contact with antigen. Evidently, CD8+ T cells are more sensitive to this program of division and differentiation than CD4+ T cells, and undergo a more extensive program of proliferation than CD4+ T cells (37-39). CD4+ T cell expansion to LCMV infection occurs with a delayed kinetic as compared to CD8+ T cell expansion. The magnitude of the response is such that the CD4/CD8 ratio changes from about 2:1 in naïve mice to about 1:2 in acutely infected mice at the peak of the CD8+ T cell response. CD4+ T cells rebound both in total number and function as the CD8+ T cell response declines (28, 40). The antigen specific response is directed to a diverse array of epitopes, many of which have been defined for T cells in a variety of murine strains. In the C57BL/6 mouse specifically, the immunodominant CTL epitopes that have been defined include np 396, gp 33, gp 34, and

gp 276 all of which are D^b restricted. Subdominant CTL responses are mounted to the epitopes gp 92 and np 205 which are D^b and K^b restricted respectively. Fewer CD4+ epitopes have been defined. The CD4+ immunodominant epitope identified so far is gp 61, while the only subdominant epitope described so far is np 309, which are both I-A^b restricted (28, 41, 42).

Activated CD4+ T cells and CD8+ (CTL's) possess a variety of effector functions with which to combat infection. Upon activation CD4+ T cells secrete the antiviral cytokines IFNγ and TNFα in addition to IL-2. CD8+ CTL's in addition to secreting the anti-viral cytokines IFNγ and TNFα, contain the cytolytic granules perforin and granzyme B that are necessary for cell-mediated cytolysis. Resolution of LCMV infection is dependent on the CD8+ (CTL) response, as mice deficient in CD8+ T cells fail to resolve an LCMV infection (43). This resolution is also perforin dependent and somewhat IFNγ dependent, as LCMV infection of perforin deficient mice results in a persistent infection despite the generation of a robust CTL response, while infection of IFNγR deficient mice results in delayed clearance of virus (44, 45).

Immune Silencing

Following the peak of expansion of the T cell response, there is a rapid decline in the numbers of both the CD4+ and CD8+ epitope specific T cells as the immune system undergoes homeostasis. This results in the generation of a long-lived population of CD4+ and CD8+ LCMV specific memory T cells. Multiple mechanisms have been proposed to explain the regulation of this phase of the immune response, but a definitive

mechanism remains elusive. Immune silencing is mediated in part by the process of activation induced cell death (AICD). Virally activated CD8+ T cells upregulate expression of the TNF family of pro-apoptotic Fas, Fas L and TNF receptor molecules. As a consequence, these activated CD8+ T cells are more sensitive to apoptosis. However, immune silencing cannot be wholly explained by expression of these molecules, as loss of these molecules does not abrogate silencing of the immune response. This process is also not dependent on regulation of expression of anti-apoptotic molecules, as it is not rescued by over expression of Bcl-2 (46-48). Other Bcl-2 family members have also been implicated in modulation of the pro-apoptotic and anti-apoptotic pathways that regulate cell death. For example, studies have shown that the pro-apoptotic molecules Bax and Bak are part of an obligate machinery that controls thymocyte deletion and peripheral homeostasis. Bax and Bak are activated by the pro-apoptotic BH3 - only family members Bim and Bid. Bim and Bid in turn are inhibited by binding to a pocket created by intermolecular associations between the BH1 – BH3 subunits of Bcl-2 and Bcl-XL. The survival of the cell is then controlled by the ratio of expression of pro-apoptotic to anti-apoptotic molecules. Recently Bim, a member of the BH3 only family of pro-apoptotic proteins, was implicated in regulation of the immune silencing of the influenza response. Bim was shown to be important for the cytokine withdrawal induced apoptosis that occurs as the cytokine milieu generated during the immune response wanes (49). Immune silencing has also been demonstrated to be facilitated in part by the migration of antigen responsive cells to non-lymphoid tissues, although this would not account for the total decline in the antigen specific population in

lymphoid tissues (50). It has also been suggested that the contraction of the immune response is a programmed event that is intrinsic to the T cell although no mechanisms have been proposed to explain how this process may be regulated (51). So the regulation of this phase of the immune response is still somewhat shrouded in mystery.

T Cell Memory

The decline of the antigen specific response leaves in its wake a population of long-lived antigen specific memory CD4+ and CD8+ T cells. These T cells are capable of mounting a faster more efficient response to subsequent infections (52, 53). The LCMV model system has been extensively used to study the mechanisms by which memory T cells are generated and maintained. Although memory T cells were originally believed to be descendant from a separate lineage to that of effector T cells, current opinion largely holds that memory T cells are derived from the linear differentiation of effector T cells (54-58). Generally, in the murine system, a memory CD4+ or CD8+ T cell is defined by elevated expression of the surface molecules CD44 and CD62L (CD44hi CD62Lhi). Memory CD8+ T cells additionally possess high levels of CD122 (IL-2Rβ) and Ly-6C. Current work has also shown that memory T cells are not a homogenous population and can be divided into several subsets with distinct functional capabilities, on the basis of cell surface markers. CD44hi CD4+ and CD8+ memory T cells can be further subdivided based on differential expression of CD62L and the chemokine receptor CCR7. Memory T cells are subdivided into effector memory T_{EM} (CD44hiCD62LloCCR7-) and central memory T_{CM} (CD44hiCD62LhiCCR7+) populations. These cells are found in different sites in the body, with T_{EM} cells localising

preferentially to the blood, spleen and non-lymphoid tissue, and T_{CM} cells localised to lymphoid compartments. Additionally, it was demonstrated that T_{EM} cells respond to antigen and make cytokines more rapidly than T_{CM} cells, although these generalities do not hold in all systems and sometimes differ between CD4+ and CD8+ memory T cells (50, 54, 57). Despite these inconsistencies, there is a general consensus as to the existence of these memory T cell subsets.

An important characteristic of the memory T cell population is their ability to be maintained in the host for extended periods of time, sometimes offering protection for the lifetime of the host (55, 59, 60). This characteristic has been a subject of intense investigation for vaccinologists searching for the answers to the generation of a long-lived protective response to vaccination. One of the first questions asked was whether memory T cells needed a constant tickling through their TCR and as such a constant exposure to residual antigen to survive in the host. However, several studies have shown that memory T cells can be maintained independently of antigen stimulation, as memory T cells transferred into MHC deficient hosts can survive long-term (61, 62). Subsequent investigation has shown however that these cells are not as functionally efficient as memory T cells maintained in the presence of MHC (63).

Memory T cells, mainly CD8+ CD44hi memory T cells, have been shown to be dependent on the common γc cytokines IL-15 and IL-7 for maintenance (58). The requirement for IL-15 is partially due to the high expression of IL-2Rβ on CD8+ memory T cells (64-67). The involvement of cytokines in the maintenance of the CD4+ CD44hi memory subset is more controversial, as there have been studies that both support and

oppose a role for common γ chain cytokines, in particular IL-7, in the maintenance of CD4+ memory T cells (65, 68-70). Additional studies characterizing memory T cells show that these cells have a higher rate of turnover in the periphery as compared to naïve T cells, and higher expression of the anti-apoptotic molecules Bcl-2 and Bcl-XL(58).

Although many advances have been made in the past few years in characterizing memory T cell development and maintenance, many questions still remain as to the impact of extrinsic and intrinsic T cell factors on the generation and maintenance of memory T cells. For example, much still remains to be defined about the biochemical and epigenetic changes that CD4+ or CD8+ effector T cells undergo in becoming memory T cells, the hierarchy of the signalling pathways that modulate these changes, how these changes are inherited by memory T cells, how they differ between CD4+ and CD8+ memory T cells, and how they may contribute to the heterogeneity in phenotype and function of the memory T cell subsets. As such, this leaves much work to be done in refining of our understanding of this subset of T cells so important to our immunological stability.

T Cell Activation

As detailed in the preceding paragraphs, T cells are a vital component of the body's defense against pathogenic challenge. The initiation of this response follows a complex interaction of the TCR with antigen presented by self-MHC molecules and with accessory co-stimulatory molecules expressed on the surface of specialized antigen presenting cells. Along the pathway to immune function, the T cell activates a complex array of genes beginning as early as ten minutes following contact with antigen, that can

over the course of seven to ten days result in a complex array of regulatory and differentiative events that follows the T cell into immunological memory (71).

One of the earliest questions in T cell signalling biology was how the first detectable consequences of T cell receptor cross-linking, the mobilization of intracellular stores of calcium and the phosphorylation of membrane proximal and cytoplasmic molecules, were linked to transcription factor activation and subsequent IL-2 gene transcription and production by activated T cells. Elucidation of these mechanisms contains the key to understanding how T cells integrate signals received through the TCR and convert them into functionally distinct responses such as cytokine production, proliferation and cytotocixity. Unlocking these mechanisms would potentially provide a tool with which to manipulate the immune system to generate protective immune responses to a wide array of pathogens. Extensive work has gone into understanding this connection between the interaction of the T cell with antigen presenting cells and the subsequent induction of protective T cell immune responses and has resulted in our current understanding of the positive and negative regulatory events controlling T cell activation.

Src and Syk Kinases

Contact and interaction of the TCR with MHC/foreign peptide on the surface of APC's leads to capping of the TCR and formation of a structure known as the immunological synapse (IS). The TCR/MHC complex is at the center of this synapse and recruits to itself all the signalling molecules necessary to activate T cell function (72, 73).

One of the earliest positive regulatory events following crosslinking of the TCR is the phosphorylation and activation of the src kinase family members Lck and fyn. These kinases initiate signalling events downstream of the TCR by phosphorylating immunoreceptor activation motifs (ITAMS) in the cytoplasmic tails of the TCR associated CD3 molecules. Lck is very important to T cell activation as the absence of Lck results in minimum phosphorylation of ITAMS. Fyn is not able to fully compensate for the absence of Lck (74). The main purpose of ITAM phosphorylation is the recruitment of the syk kinases ZAP-70 and Syk to the T cell receptor. ZAP-70 and Syk dock to the phosphorylated ITAM motifs via their SH2 domains. The importance of ZAP-70 in T cell function is demonstrated by the severe immunodeficiency of ZAP-70 null humans and mice. ZAP-70 null humans and mice lack mature peripheral CD8+ T cells and have TCR unresponsive CD4+ T cells (23, 75). After T cell activation, Lck phosphorylates ZAP-70. The phosphorylation and autophosphorylation of ZAP-70 creates docking sites for other SH2 domain containing signalling molecules. Among the SH2 containing signalling molecules recruited to ZAP-70 is the linker of activated T cells (LAT). LAT is a substrate for ZAP-70 and after being phosphorylated by ZAP-70 serves to recruit other signalling molecules such as SLP-76, PLCγ, PI3K (p85), Grb-2, Sos, Vav and the Tec family kinases Itk and Rlk (76). Recruitment of LAT is a seminal event in T cell activation as it results in the formation of a signalosome complex downstream of the T cell receptor that is critical to the continued transduction of signals from the TCR (14).

PLCγ

Phospholipase C γ1 (PLCγ1) regulates the hydrolysis of inositol phospholipids and the generation of polyinositolphosphates and diacylglycerols (77). Specifically, recruitment of PLCγ1 to the signalosome complex facilitates the phosphorylation and activation of PLCγ. This phosphorylation activates PLCγ to catalyse the hydrolysis of phosphotidylinositol 4,5 bi phosphate (PIP2) present in the cell membrane into inositol 1,4,5 trisphosphate (IP3) and diacyl glycerol (DAG). IP3 binds to receptors on the endoplasmic reticulum causing release of intracellular stores of calcium. The rise in intracellular calcium eventually results in the opening of calcium channels in the cell membrane leading to an influx of extracellular calcium and the generation of a sustained calcium flux. Generation of a sustained calcium flux is necessary for the initiation of many effector functions such as cytokine gene transcription, proliferation and cytotoxic function. For example, one of the first consequences of the generation of a sustained calcium flux is the translocation of the transcription factor NFAT into the nucleus where it participates, along with the c-fos/c-jun (AP-1) transcription factor complex, in the transcription of cytokine genes like IL-2, IFNγ and TNFα (78). Generation of DAG links the TCR to two distinct signalling pathways, the protein kinase C (PKC) and Ras/Raf Map kinase (MAPK) signalling cascades.

Activation of PKC results in the eventual activation and translocation of the transcription factor NFkB to the nucleus where it promotes cell proliferation, cell survival and the transcription of effector cytokine genes (79). Activation of PKC also promotes the phosphorylation and activation of the c-jun N terminal MAP - kinase JNK.

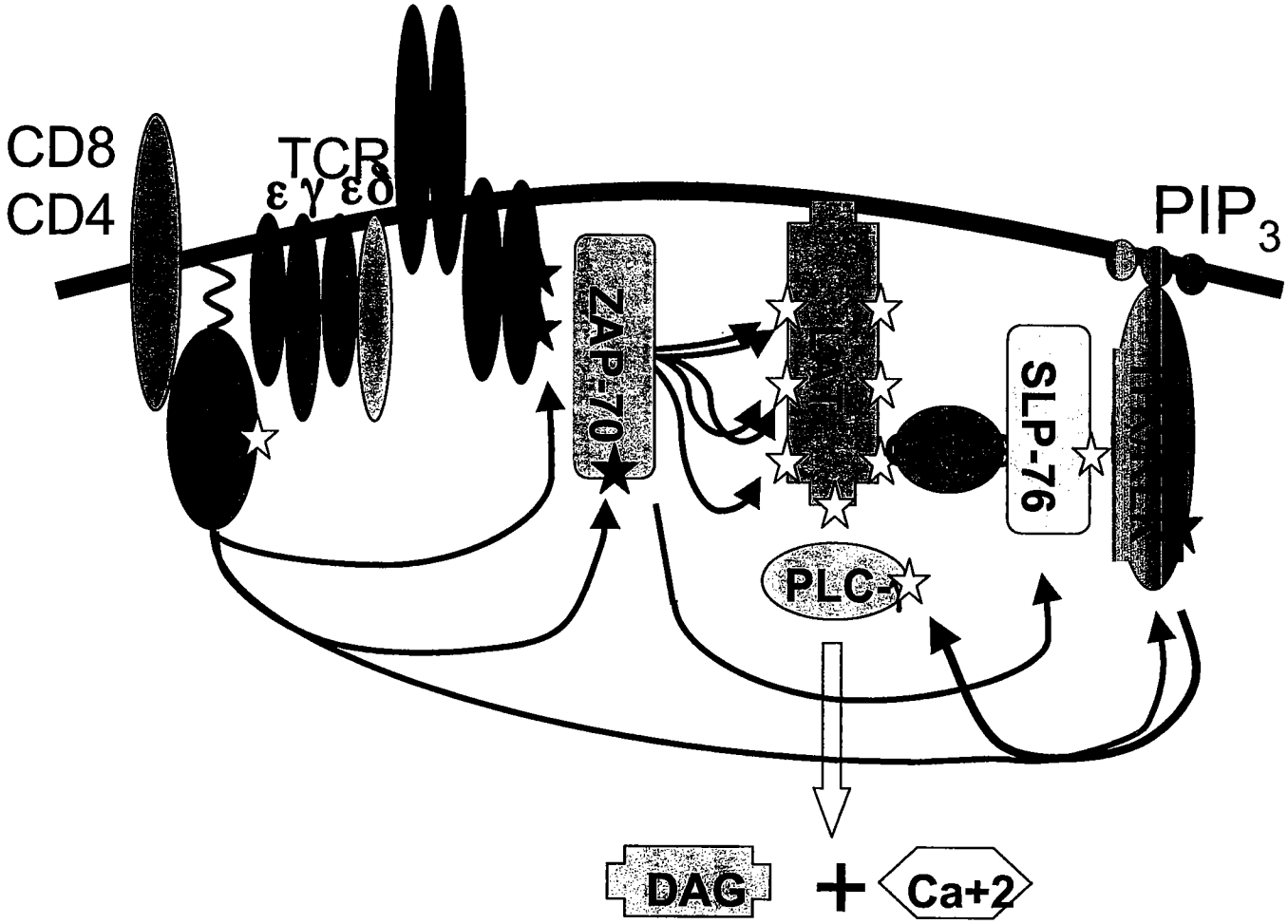
Activation of both isoforms of JNK (JNK1/2) results in the phosphorylation and activation of the c-jun and c-fos transcription factors that form the AP-1 complex (80). Generation of DAG also activates the Ras/Raf MAP-kinase pathway. Activation of the Ras guanine exchange factor and its partner Raf leads to the activation of the ERK and p38 MAP-kinases (81) (Figure 1.4).

Figure 1.4: Itk and Rlk in T Cell Receptor Signalling

Following TCR crosslinking, the src kinase Lck is activated. Lck then phosphorylates ITAM residues on the intracellular domain of the TCR associated CD3 molecules, facilitating the recruitment of ZAP-70. Lck subsequently phosphorylates ZAP-70, and recruits the adaptor molecule LAT – Linker of activated T cells. The recruitment of LAT and its subsequent phosphorylation by Lck initiates the formation of a signalling complex as signalling molecules such as SLP-76, Vav, Grb2, Sos and PLCγ are recruited to the phosphorylated SH2 domains of LAT. The Tec kinases Itk and Rlk are also recruited to the LAT nucleated signalling complex, where they are phosphorylated and activated by Lck. Upon activation, Itk and Rlk mediate a central event in T cell activation, the phosphorylation and activation of PLCγ1. PLCγ is now able to catalyse the generation of the second messengers DAG and IP3 from PIP2 in the cell membrane.

The generation of DAG leads to the activation of the PKC and NFkB pathway and to the activation of the ERK, JNK and p-38 MAP-kinase pathways which result in the generation of the AP-1 transcription factors jun and fos. IP3 binds to receptors on the mitochondrial membrane, facilitating an increase in the intracellular stores of calcium and the generation of a sustained calcium flux which results in the activation and translocation of the transcription factor NFAT to the nucleus, where it participates along with NFkB and the AP-1 complex in the transcription of genes for multiple effector functions including the transcription of cytokines such as IL-2, IFNγ and TNFα and T cell proliferation.

TCR SIGNALLING



Tec Kinases

Activation of PLCγ is a critical event in the activation of a T cell, and it serves as a major intersection following crosslinking of the TCR to link the TCR to multiple distinct cellular signalling pathways. The activation of PLCγ in T cells is mediated in part by the Tec kinase family members Itk and Rlk.

The Tec kinase family of non-receptor protein kinases is the second largest family of cytoplasmic protein kinases (82). There are six known members, five of which are variably expressed in cells of the hematopoietic lineage. Specifically, Tec, the first member of this family to be identified, is expressed in most hematopoietic cells as well as in endothelial cells and the liver (82, 83). Btk, the third Tec family member identified, can be found in most hematopoietic cells including B cells and mast cells (84). The other Tec kinases, Itk (the second Tec family member to be identified) and the more recently cloned Rlk have a limited pattern of expression and are restricted to thymocytes, T cells, NK cells and mast cells with Itk being detectable in the thymus by d14 of foetal development, before the generation of DP thymocytes (85-87).

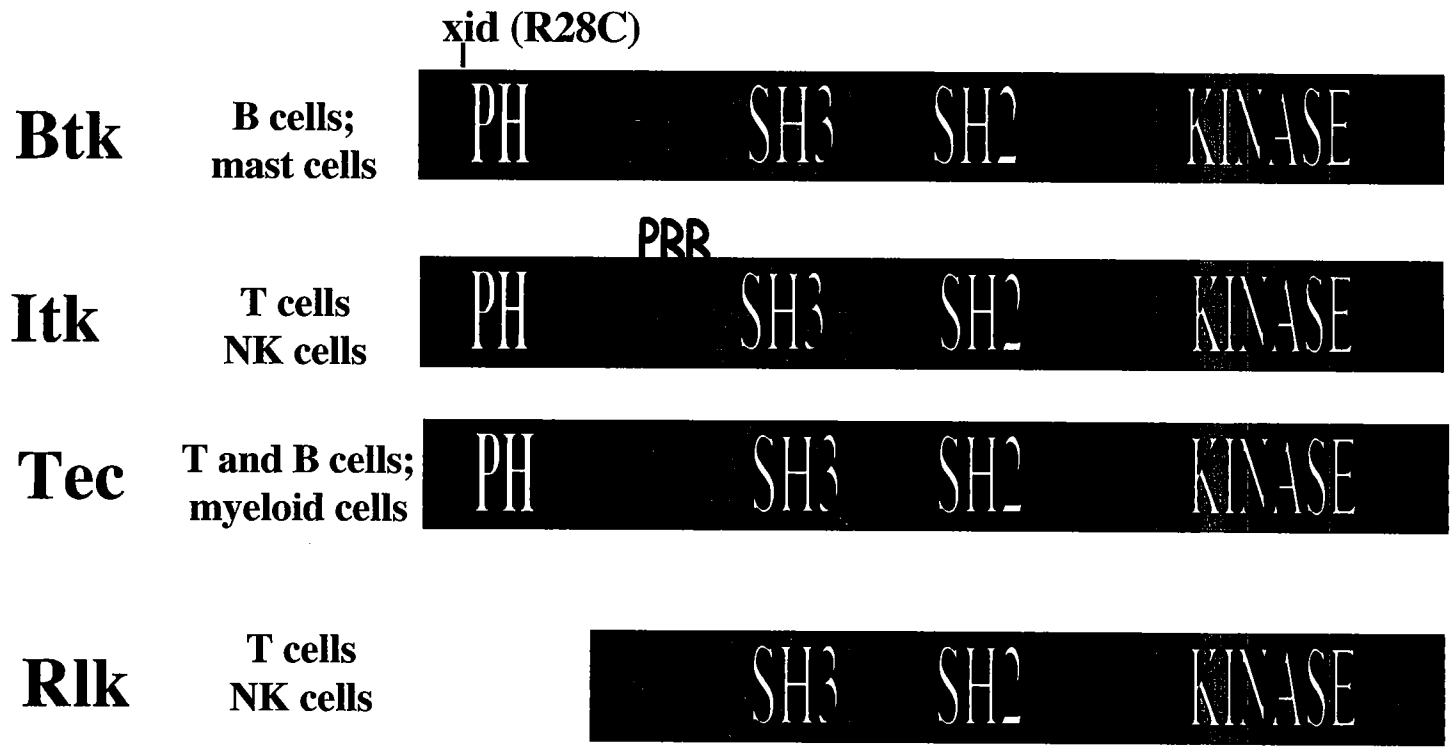
Structurally, the Tec kinases possess a modular organization that is reminiscent of the family of Src kinases. Tec, Btk and Itk have unique N terminal regions followed by Src homology 3 (SH3) and SH2 protein interaction domains and a catalytic tyrosine kinase domain. Unlike the Src kinases however, the Tec kinases have an N- terminal (plextrin homology) PH domain, which is important for localization to the cell membrane, an adjacent Tec homology domain (consisting of a Btk homology region

(BH) and one or two proline rich regions (PRRs)) instead of the N-terminal myristolation sites and regulatory C terminal tyrosine residues of the Src kinases (82). Rlk has two main differences to its other family members. The first is that it does not possess an N-terminal PH domain, having instead a string of N terminal cysteine residues, and it has no BH domain, having only the proline rich regions. The second is that Rlk has an alternative translational start site that generates a shorter form of Rlk that can translocate to the nucleus (88, 89) (Figure 1.5).

Figure 1.5: Structure of the TEC Kinase Family Members

Itk and Rlk are members of the larger Tec family of non-receptor tyrosine kinases. The Tec kinase family consists of five members that are expressed in haematopoeitic cells, four of which are shown here. These include Tec and Btk. The kinases are highly structurally homologous, each possessing a C terminal catalytic kinase domain, an SH2 and SH3 domain, a TH domain containing proline rich repeats (PRR). At the N terminus, the Tec kinase family, with the exception of Rlk, possesses a plextrin homology (PH) domain that promotes the localisation of the Tec kinases to the cell membrane following TCR activation. Rlk instead has a string of N terminal cysteine repeats that perform the same function.

THE TEC FAMILY OF TYROSINE KINASES



The potential importance of the Tec kinases in the regulation of lymphocyte signalling and function was realised following the discovery that mutations in the tyrosine kinase Btk were associated with a disorder in B cell development and function in humans. This rare genetic disorder, X-linked agammaglobulinemia, caused a severe reduction in serum immunoglobulin levels (90). A similar but less severe phenotype (xid) was caused by a point mutation in the PH domain of murine Btk (91). The importance of Btk in the regulation of B cell function, and the limited pattern of expression of Itk, Rlk and Tec to cells of the immune system and to T cells in particular, led to attempts to determine the importance of these Tec kinase family members in T cell function.

The Role of Itk and Rlk

Itk and Rlk in T Cell Development

To understand the role of Itk in T cell signalling and function, mice genetically deficient in Itk and either Rlk or both Itk and Rlk were generated (18, 92, 93). The initial analyses of Itk -/- mice revealed that Itk was critical for the development of CD4+ cells in the thymus and in the periphery. The thymus and lymph nodes of mice lacking Itk were deficient in both percentages and total numbers of CD4+ SP thymocytes and mature CD4+ T cells, and the CD4:CD8 ratio was reversed, from 2:1 in wildtype mice to approximately 1:1 in Itk -/- mice. Although the fraction of CD8+ SP thymocytes was increased in the thymus of the Itk -/- mice, there was no effect on the percentages or total numbers of peripheral CD8+ T cells. To further study the effect of Itk deficiency on thymocyte development, the Itk -/- mice were crossed to TCR transgenics. Upon

crossing the Itk -/- mice to the AND TCR transgenic which is MHC Class II restricted and recognizes a pigeon cytochrome C peptide and to the HY TCR transgenic which is MHC Class I restricted and is specific for male antigen, it became evident that Itk was important in the positive selection of CD4+ and CD8+ SP thymocytes during thymic development (18). Negative selection, which was assessed in the well-characterized male HY transgenic model, did not appear to be different between the wildtype and Itk-/- mice. The role of Itk in CD4+ T cell selection was further assessed by Julie Lucas *in the lab* who showed, using a series of MHC II restricted TCR transgenics with different avidities for the selecting peptide, that increasing the avidity of the TCR increased the efficiency of positive selection, and that the efficiency of this process was regulated by the presence of Itk (24). This model, which was based on the hypothesis that the processes of positive and negative selection is influenced by qualitative differences in signalling downstream of the TCR, implicated Itk in modulation and amplification of the TCR signal (24).

The role of Rlk in T cell development was a little more difficult to discern. Mice lacking only Rlk did not have any visible defects on T cell development. However, crossing the Rlk -/- mice to the Itk -/- mice resulted in an exacerbation of the defects seen in the absence of Itk. Positive selection of CD4+ SP thymocytes and CD4+ peripheral T cells were more impaired in AND TCR transgenic Itk -/- Rlk -/- mice as compared to AND TCR transgenic Itk -/- mice. In contrast to a previous report, examination of the loss of Rlk and both Itk and Rlk on negative selection revealed that negative selection of HY transgenic CD8+ T cells was impaired in the absence of Itk. This defect was exacerbated by the absence of both Itk and Rlk. Surprisingly, despite this deficiency in

positive selection and possibly due to the effect of impaired negative selection, the total numbers of CD4+ SP thymocytes in Itk -/- Rlk -/- mice is similar to that seen in wildtype (WT) mice. In addition, both the percentages and total numbers of CD8+ SP thymocytes in the Itk -/- Rlk -/- mice are increased compared to both Itk -/- and WT mice. This increase in total numbers in the CD4+ SP and CD8+ SP compartment reduces the CD4:CD8 ratio from 2:1 in WT mice to 1:1 in Itk -/- Rlk -/- mice, similar to that seen in Itk -/- mice (15, 93, 94). In addition to defects in positive and negative selection, the T cells in the Itk -/- and Itk -/- Rlk -/- mice have an altered phenotype. Approximately 50% of the CD4 T cells and 80% of the CD8+ T cells in the periphery are CD44hi. Paradoxically, a portion of the CD8+ SP thymocytes in the thymus of these mice are also CD44hi (24). Questions pertaining to the origin and function of these cells are the subject of the second chapter of this thesis.

Itk and Rlk in T Cell Signalling and Function

Mice deficient in Itk or in both Itk and Rlk were used to investigate the importance of these Tec kinases in TCR signalling. Itk -/- CD4+ and CD8+ T cells proliferated poorly in response to allogeneic and anti-CD3 stimulation. This proliferative defect was rescued by stimulation with the phorbol ester PMA which activates PKC and the calcium ionophore Ionomycin, indicating that Itk was acting in a membrane proximal manner, prior to activation of PLCγ (18). Experiments done to place Itk in the signalling cascade downstream of the TCR showed that Itk was directly phosphorylated by Lck, the src kinase that is activated immediately following TCR crosslinking, and that this phosphorylation increased the kinase activity of Itk (95, 96). These analyses were

extended by Karen Liu who showed that Itk -/- CD4+ T cells were deficient in the phosphorylation of PLCγ and that this resulted in diminished production of the second messenger IP3 and in the subsequent generation of an impaired calcium flux (92). As a consequence, Itk -/- CD4+ T cells proliferated poorly in response to stimulation with anti-CD3 and anti-CD28 as compared to wildtype mice. These studies along with many others were seminal in elucidation of the current model of Itk in T cell receptor signalling. Included in these were studies by Stephen Bunnell showing that Itk was recruited to the cell membrane in a PH domain dependent manner following TCR activation (97). In addition, these data showed that the SH2 and SH3 domains of Itk were able to bind to multiple proteins demonstrated to be important for TCR signalling such as SLP-76, ZAP-70, LAT and PLCγ (97). In this model, Itk is recruited to the cell membrane in a PI3K and PH domain dependent manner, where after being phosphorylated by Lck, it forms part of a multiprotein signalling complex nucleated by LAT. Recruitment to this complex places Itk in proximity to its major substrate PLCγ, and promotes the activation of PLCγ dependent signalling pathways (Figure 1.4).

Similar studies were done to elucidate a role for Rlk in T cell signalling. In-vitro studies done to identify binding partners for the Rlk SH3 and SH2 domains, placed Rlk in the same signalosome complex with Itk where it was implicated in the phosphorylation of SLP-76 and in promotion of IL-2 cytokine secretion (98). Rlk has also been shown to cooperate with Itk in the phosphorylation of PLCγ, the generation of a sustained calcium flux and in the activation of the ERK MAP-kinase pathway, indicating that these kinases have redundant roles downstream of TCR signalling (93). Consistent with these

observations, activation of Itk -/- Rlk -/- CD4 T cells resulted in increased impairment in the generation of a sustained calcium flux and in the subsequent activation and nuclear translocation of the NFAT and AP-1 transcription factors. These observations were in keeping with data showing that activation of Itk -/- Rlk -/- T cells with anti-CD3 resulted in a proliferative response that was even more impaired than that of Itk -/- T cells. The Itk -/- Rlk -/- T cells were also more deficient in secretion of IL-2. Rlk is required for activation of IL-2 cytokine secretion, as Rlk -/- T cells produced two-fold less IL-2 that wildtype T cells, but loss of Itk or both Itk and Rlk completely abolished IL-2 cytokine secretion. Other studies have similarly shown that activation of Itk -/- and Itk -/- Rlk -/- CD4+ T cells results in impaired secretion of other effector cytokines like IFNγ and IL-4 (93, 94, 99, 100).

Itk and Rlk in the Immune Response

CD4 T Cell Immunity

Initial studies on the physiological relevance of a deficiency in Itk and Rlk on T cell function in-vivo were focused on those requiring a CD4+ T cell or TH mediated response for the generation of protective immunity. Deborah Fowell, who initiated these studies in Richard Locksley's lab, undertook studies to characterize the effects of loss of Itk on the activation of cytokine genes. She showed that in response to stimulation with anti-TCR, anti-CD28 antibodies and APC's, Itk -/- cells on a BALB/c background were deficient in the generation of the TH2 cytokine IL-4 as well as in IL-5 and IL-13 secretion (99). This defect was shown to directly result from the impairment of NFATc nuclear localization following TCR activation and the subsequent deficient activation of

IL-4 gene transcription. Additionally, Itk -/- mice on the BALB/c background preferentially mounted a protective TH1 response to infection with the intracellular parasite Leishmania major, instead of the non-protective TH2 response usually generated to this pathogen by BALB/c mice (99). The Itk -/- mice were essentially resistant to this pathogen as control of the infection was associated with a marked reduction in parasite load as well as a strong CD4+ T cell mediated IFNγ response. In keeping with this inability to mount a TH2 response, Itk -/- mice infected with the nematode Nippostrongulus brasilienis failed to generate the TH2 response necessary for clearance of this pathogen. These experiments demonstrated that loss of Itk had a direct effect on the ability of activated T cells to mount an efficient immune response. They also implicated Itk in the regulation of mechanisms controlling the epigenetic modification of cytokine gene expression during T cell activation and differentiation, since NFATc has the ability to recruit coactivators capable of modulating chromatin structure (101, 102). Interestingly, the region of the chromosome to which Itk is localized also contains genes for the TH2 cytokines IL-5, IL-4 and IL-13. Recently it has been shown that mice lacking a conserved noncoding sequence designated CNS 1, which is located between the IL-4 and IL-13 genes, are impaired in their ability to generate a TH2 response. Like the Itk -/- mice, CNS 1 -/- mice on a BALB/c background are resistant to Leishmania major. Even more intriguing are the observations that the CNS1 -/- mice are impaired in basal modification at the IL-4 and IL-13 genetic loci and that this impairment is similar to that found at the IL-4 and IL-13 genetic loci of Itk -/- mice. This data strongly implicates Itk in the regulation of the mechanisms controlling the epigenetic modification at cytokine

gene loci (103). Underscoring the importance of Itk in the regulation of TH2 responses is the observation that mice lacking Itk have an impaired response in an experimental asthma model (104). Itk is also potentially important in the downregulation of the CD4+ T cell mediated immune response. Andrew Miller demonstrated that Itk -/- CD4+ T cells stimulated with APC plus peptide fail to upregulate expression of the death molecule FasL (100). This results in enhanced expansion of the CD4+ T cells upon secondary stimulation, and in failure of superantigen (SEB) induced deletion that is dependent in part on the Fas/FasL interaction.

To examine the role of Rlk in the generation of an effective CD4+ T cell mediated immune response, Itk -/- Rlk -/- and Itk -/- mice were infected with the helminth Schistosoma mansoni. As had been shown for infection with L. major, Itk -/- mice failed to mount a TH2 response to S. mansoni resulting in reduced secretion of the TH2 cytokines IL-4, IL-5 and IL-10 (94). Paradoxically, Itk -/- Rlk -/- mice infected with S. mansoni mounted an effective TH2 response and developed lung granulomas similar in size and volume to those seen in wildtype mice. This response was surprising because given the defects in signalling in Itk -/- Rlk -/- CD4+ T cells, it was expected that the Itk -/- Rlk -/- CD4+ T cell response would be even more impaired than that of Itk -/- CD4+ T cells. Investigation of the expression of TH2 lineage determining transcription factors revealed that Itk -/- Rlk -/- CD4+ T cells were impaired in their ability to downregulate expression of GATA-3 following TCR stimulation, suggesting that Itk -/- Rlk -/- CD4+ T cells had a greater tendency to become TH2 cells (94).

Complicating the analysis of the role of Itk and Rlk in TH2 mediated responses is the TH2-like environment of the Itk -/- and Itk -/- Rlk -/- mice. Analysis of the serum immunoglobulin levels of Itk -/- mice revealed that naïve Itk -/- mice had approximately 20 fold higher levels of IgE. In addition, analysis of the splenic architecture of these mice showed the presence of enlarged germinal centers and eosinophilia, which is indicative of a TH2-like environment (92). Like Itk -/- mice, the serum IgE levels in Itk -/- Rlk -/- mice are also elevated and the splenic architecture is perturbed with enlarged germinal centers. It is probable that in the absence of Itk and both Itk and Rlk, there is dysregulated cytokine production by another cell type that expresses Itk and/or Itk, possibly NK T cells or mast cells that results in the TH2 environment seen in these mice. These possibilities and the mechanisms behind them are currently under investigation.

Itk and Rlk are also important for the generation of TH1 mediated responses. Infection of Itk -/- and Itk-/- Rlk -/- mice with the intracellular pathogen Toxoplasma gondii resulted in a graded response in these Tec deficient animals. T cells from Itk -/- mice produced IFNγ in amounts similar to those of wildtype mice, while Itk -/- Rlk -/- T cells made significantly less IFNγ (93). Additionally, the Tec deficient mice were less able to contain the infection than wildtype mice as Itk -/- Rlk -/- mice had increased numbers of brain cysts and a higher mortality rate later in infection. Itk -/- mice had an intermediate phenotype with less numbers of brain cysts and a lower mortality rate than Itk -/- Rlk -/- mice. Together, these experiments demonstrate that the Tec kinases are essential components of the TCR signalling apparatus required for transmission of TCR

mediated signals, and show that non-receptor tyrosine kinases are of physiological relevance in the initiation of an antigen driven T cell response.

CD8 T Cell Immunity

Involvement of the Tec kinases in the regulation of CD8+ T cell immune responses is less well characterized than that of the CD4+ T cell response. An initial study by Martin Bachmann attempted to address this question by analyzing the anti-viral CD8+ T cell response of Itk -/- mice to infection with LCMV (WE strain), vaccinia virus (VV) and vesicular stomatitis virus (VSV) (105). Analysis of the LCMV specific CTL response showed that the lytic ability of CD8+ T cells from acutely infected Itk -/- mice were approximately two-fold reduced as compared to that of CD8+ T cells from wildtype mice, and that the development of the CTL response was delayed by approximately one day in the Itk -/- mice. Additionally, the secondary CTL response of CD8+ T cells from acutely infected mice restimulated in culture for five days before analysis, was also impaired in the Itk -/- mice. Despite this impairment in the CTL response, the Itk -/- mice were able to clear the virus by eight days post infection.

The observations made in this study are the only published data available that address the role of Itk in the CD8+ T cell mediated response. However, these data do not thoroughly explore a role for Itk in CD8+ T cell function as analysis of the role of Itk in the different phases of the antiviral CD8+ T cell response was lacking, including the effect of Itk on the expansion, contraction and memory CD8+ T cell response and the antigen-specific cytokine response. Additionally, no mechanisms as to the cause of the impaired anti-viral responses made by the Itk -/- mice were addressed. The role of Rlk in

the CD8+ T cell response is currently unknown, as no experiments have been published that directly address the role of Rlk in CD8+ T cell function either *in-vitro* or *in-vivo*.

Similarities and differences in activation of CD4 versus CD8 T Cells

The lack of available knowledge on the involvement of the Tec kinases in CD8+ T cell function is symptomatic of a wider dearth of knowledge of the mechanisms regulating CD8+ T cell effector function. There is a wealth of information available regarding the signalling processes and mechanisms governing CD4+ T effector cell subset differentiation and the epigenetic modifications that take place at cytokine loci during differentiation. However, there is a disconcerting lack of data that similarly examines the signalling processes and mechanisms governing CD8+ T effector T cell differentiation and the remodeling of cytokine genes, despite ample evidence to support a thesis that these processes may be differentially regulated in CD4+ versus CD8+ T cells.

The most visible difference between CD4+ and CD8+ T cells is in their effector function. Upon infection with an intracellular pathogen, CD4 T cells tend to differentiate into TH1 cells in which they secrete IFNγ, IL2 and TNFα and facilitate the development of a CD4+ TH1 and CD8+ T cell (CTL) response. Alternatively, when faced with a parasitic antigen, CD4+ T cells differentiate into TH2 cells, secrete IL4 and aid in the development of the humoral immune response. Although CD8+ T cells have been shown to differentiate into TH2 – like cells (Tc2), the mechanisms governing this process are not well characterized (106). Unlike CD4+ T cells, CD8+ T cells preferentially contain granules with the cytolytic molecules perforin and granzymes, and develop into cytolytic

T cells (CTL's) that induce apoptosis of infected target cells. Like CD4+ cells, CD8+ T cells produce the effector cytokines IFNγ and TNFα in response to infection.

Despite similarity in production of the TH1 cytokines IFNγ and TNFα, CD4+ and CD8+ T cells utilise differential regulatory mechanisms to control transcription and expression of these genes (107, 108). For example, production of IFNγ from CD4+ T cells requires expression of T-bet, a transcription factor that is a master regulator of TH1 development. CD8+ T cells do not require T-bet to produce IFNγ, but instead seem to require expression of a newly discovered regulator of CD8+ T cell differentiation, eomesodermin to produce effector cytokines and to develop cytolytic function. CD4+ and CD8+ T cells also have differential requirements for co-stimulatory signals to be efficiently activated. CD4+ T cell activation depends more on the provision of co-stimulatory CD40/CD40L and OX-40/OX-40L signals for example, while CD8+ T cell activation is somewhat dependent on 4-IBB/4-IBBL interactions than CD4+ T cell activation. Additionally, CD8+ T cells are more sensitive to signals through the TCR than CD4+ T cells, and will undergo a more extensive program of proliferation than CD4+ T cells following activation (39). CD4+ and CD8+ T cells also differ in their requirements for signalling molecules downstream of TCR activation, and the consequences of mutation or removal of these signalling molecules is different for CD4+ and CD8+ T cells (109-111).

These data all suggest that CD4+ and CD8+ T cells, despite some of their similarities, have distinct requirements for activation and elaboration of effector function. It is possible that CD4+ and CD8+ T cells integrate the signals transduced downstream of

the TCR differently or that they integrate different combinations of signalling pathways following activation. Whatever the possibilities, much work remains to be done to distinguish the factors that may differentially regulate CD4+ and CD8+ T cell activation and function.

Work presented in this Thesis

The Tec family of non-receptor protein tyrosine kinases is important in the propagation of signals downstream of the TCR and BCR. The kinases are also important in the translation of these signals into physiological T cell and B cell functions respectively. The Tec kinases primarily expressed in T cells, Itk and Rlk are necessary for the optimal function of CD4+ T cells in-vitro, and for the differentiation of CD4 T+ cells into functional TH1 and TH2 subsets in-vivo. In spite of all the information available about the role of Itk and Rlk in CD4+ T cell signalling and function, there is very little known about the comparative role of these kinases in CD8+ T cell signalling and function.

The work presented in this thesis addresses first the role of the Tec kinases Itk and Rlk in the development of the CD8+ SP thymocyte compartment in the thymus of the Itk -/- and Itk -/- Rlk -/- mice. These cells have a "mature, activated" phenotype characteristic of peripheral cells and we look at several potential mechanisms by which this phenotype might occur. Second, I look at the biochemical consequences of Itk and Rlk deficiency on CD8+ T cell signalling and function in-vitro, and finally, using a model of LCMV infection, I look at the physiological impact of loss of Itk and Rlk on the

development of an antigen-specific anti-viral response. These analyses represent the first extensive analysis of the Tec kinases in CD8+ T cell signalling and function.

CHAPTER 11

THE ROLE OF ITK AND RLK IN CD8+ T CELL DEVELOPMENT

INTRODUCTION

Thymocyte development occurs in discrete stages in the thymus. The thymus is a highly ordered and compartmentalized organ, and thymocyte development is thought to occur in a progressive manner in three distinct regions of the thymus. Generally, immature thymocyte progenitors enter the thymus via blood vessels located in the corticomedullary junction. These earliest progenitors are CD44+ CD25- (TN1). They progress from being CD44+ CD25- (TN1) when they enter the thymus, to being CD44+ CD25+ (TN2), CD44- CD25+ (TN3) and lastly CD44- CD25- (TN4). The most immature TN thymocytes are found in the subcapsular region. DP thymocytes are housed mostly in the cortex, while the most mature CD4+ and CD8+ SP thymocyte subsets reside in the medulla. The development of CD4+ and CD8+ T cells in the thymus from immature thymocyte progenitors depends on the ability of these early precursors to interpret and integrate a variety of signals from the thymic environment. These signals and the manner in which they are interpreted by the developing thymocyte can be divided into two major phases of thymocyte development. In the earlier phase, thymocytes lack expression of the TCR and the CD4 and CD8 co-receptors, and are designated TN. Signals received at this stage are receptor independent. At this stage of development the thymocytes express c-kit and IL-7R and are dependent on the cytokines SCF and IL-7 for progression to the TN3 – TN4 stage of TN differentiation and maturation (112, 113).

Progression through each stage of TN thymocyte development occurs as the maturing thymocytes migrate inward from the corticomedullary junction to the

subcapsular region of the thymus, such that the later TN stages of development, TN3 – TN4, occur as the thymocytes enter the subcapsular region of the thymus.

As the thymocytes enter the TN3 and TN4 stages of development they rearrange the TCRβ gene and coexpress TCRβ along with the surrogate alpha chain pre-Tα, to generate the immature pre-TCR complex. Expression of TCRβ marks the beginning of the later phase of thymocyte development, when the signals that the thymocyte must integrate to further differentiate are primarily receptor dependent. At this point the thymocytes undergo β selection, which results in the proliferation of cells with a productive β chain re-arrangement, allelic exclusion at the β chain locus, and the initiation of TCRα gene re-arrangement. Generation of a productive TCRα gene rearrangement initiates replacement of the surrogate pre-Tα receptor with a mature TCRα chain and expression of the mature α/β TCR complex on the surface of the thymocyte. In addition, the TN4 thymocytes that successfully navigate this checkpoint express the co-receptors CD4 and CD8 to become DP thymocytes. At this point, the thymocytes are mainly localized to the cortex of the thymus. DP thymocytes in turn give rise to either CD4+ or CD8+ SP thymocytes, and progression through this stage of development is accompanied by migration of the CD4+ and CD8+ SP thymocytes into the medulla of the thymus (8).

In the medulla, some SP thymocytes acquire the CD44lo and HSAlo phenotypic and functional characteristics of mature peripheral cells. Studies characterizing the phenotype of recent thymic emigrants have shown that these cells are a mixture of mature and intermediate phenotypes, as some cells continue their maturation in the periphery,

while others do not. The processes that regulate the maturation of medullary thymocytes as well as the length of time for which thymocytes are housed in the medulla and the signals that initiate their exit into the periphery are still largely unknown, although chemokine signals have been implicated both in the traffic of positively selected CD4+ and CD8+ SP thymocytes into the medulla, and their subsequent migration to the periphery (114, 115).

The differentiation of thymocytes from the immature DP to mature CD4+ or CD8+ SP stage is regulated by the processes of positive and negative selection and lineage commitment. These processes run concurrently and are not mutually exclusive and have been the subject of several recent reviews (116-118). A common thread connecting them is their dependence on the strength of the signal generated through the mature α/β TCR complex for the development of mature CD4+ or CD8+ SP thymocytes

Signalling pathways downstream of the TCR have been implicated for some time in both the promotion of thymocyte selection and in the CD4/CD8 lineage choice (116). This threshold or quantitative signalling model has demonstrated that commitment to the CD4+ thymocyte lineage depends on stronger signals through the TCR, while signals of lower intensity favour development of the CD8+ SP lineage. This difference in signalling is thought to be dependent in part on the TCR proximal signalling molecule Lck. The quantitative signalling model is similarly applicable to positive and negative selection. Generally, it has been shown that positive selection of thymocytes is mediated by signals of moderate or intermediate intensity, while stronger signals lead to negative selection and thymocyte deletion (8).

In keeping with this hypothesis, modulation of TCR signalling strength can shift the thresholds that regulate positive and negative selection. Several signalling molecules proximal to the TCR have been implicated in modulation of TCR signalling. Among them are the MAP-kinases ERK, JNK and p-38. Activation of the ERK MAP-kinase signalling pathway is important for the positive selection of thymocytes, as impairment of ERK activation selectively affects positive but not negative selection. The JNK and p-38 pathways are seemingly more important for the regulation of thymocyte negative selection (117, 119).

Another TCR proximal signalling molecule implicated in the setting of the thresholds that regulate positive versus negative selection is the Tec kinase family member Itk. Like Btk, which is very important in signalling downstream of the B cell antigen receptor, Itk, which is selectively expressed in murine T and NK cells, is important for signalling downstream of the TCR (85, 92).

A defining characteristic of mice lacking Itk is the impaired positive selection of CD4+ SP thymocytes. In these mice, the percentages as well as the total numbers of CD4+ SP thymocytes are lowered, and the CD4:CD8 ratio, usually 2:1 in wildtype thymi is reversed. Data from Julie Lucas have shown that the absence of Itk lowers the positive selction efficieny of MHC Class II restricted transgenic T cells with high avidity for their selecting ligands, as compared to wildtype littermate controls (24). This efficiency is further lowered as the affinity for selecting ligand decreases, implicating Itk in the setting of the thresholds that modulate positive and negative selection.

Another characteristic of T cell development in Itk -/- mice is that there is an increase in both the percentages and total numbers of CD8+ SP thymocytes in the thymus (24). Curiously these thymocytes have a mature and activated phenotype similar to that of peripheral previously-activated or memory T cells in that they are CD44hi and HSAlo. This accumulation of CD44hi HSAlo CD8+ SP cells in the thymus is followed by an accumulation of CD8+ CD44hi CD62Lhi Ly6Chi cells in the periphery of Itk -/- mice.

Given the importance of Itk in the modulation of the signalling threshold for positive selection, we initially hypothesized that the generation of the increased numbers of CD8+ SP thymocytes in the Itk-/- mice may be due to a switch in lineage commitment from the CD4+ to the CD8+ SP lineage. We investigated this possibility using MHC II restricted TCR transgenics on a variety of selecting backgrounds, however we never observed an increase in either the percentages or total numbers of CD8+ SP thymocytes in any of these mice, in the absence of Itk (24).

The absence of a defect in lineage switching to explain the presence of these "activated" cells in the thymus of Itk -/- mice led to several other considerations. We first theorized that these cells had developed in the thymus as a result of a defect in thymocyte development, distinct from a defect in lineage switching. Alternatively, our second theory proposed that the CD8+ CD44hi cells in the thymus and periphery of Itk -/- mice, had developed in the periphery of these mice as a result of impaired homeostatic mechanisms, either in response to the mild lymphopenia induced by the reduction in CD4+ T cell numbers, or in response to an environmental or self-antigen in the periphery of the Itk -/- mice and recirculated to the thymus. This hypothesis was in part based on

data from other groups showing that CD44hi cells in the periphery were capable of migrating to the thymus (120, 121). None of the data generated supported these first hypotheses, and this led to our last hypothesis in which we proposed that the CD8+ CD44hi SP thymocytes in the Itk -/- developed as a result of altered cytokine signals received during lineage maturation. This last hypothesis was based on recent data looking at the role of cytokines, specifically IL-7 and IL-15, on the later stages of thymocyte differentiation, in particular CD8+ SP thymocyte maturation. These data show that IL-7 and IL-15 are important for the development of CD8+ SP thymocytes, and that in the absence of proper regulation of these cytokine signals, CD8+ SP thymocytes can become CD44hi (25-27).

In this chapter we address the question of the origin of the CD8+ CD44hi SP thymocytes in Itk -/- and Itk -/- Rlk -/- mice. We performed a series of experiments to determine whether these cells develop in the thymus as a result of aberrant signals during thymocyte development or alternatively, are cells that initially develop in the periphery as a result of impaired homeostatic mechanisms, and have recirculated to the thymus. Our accumulated data suggest that the CD44hi HSAlo CD8+ SP thymocytes in Itk -/- and Itk -/- Rlk -/- mice develop in the thymus most likely due to altered cytokine signals received during CD8+ SP lineage maturation. These cells accumulate in the thymus and retain their activated phenotype upon migration to the periphery.

RESULTS

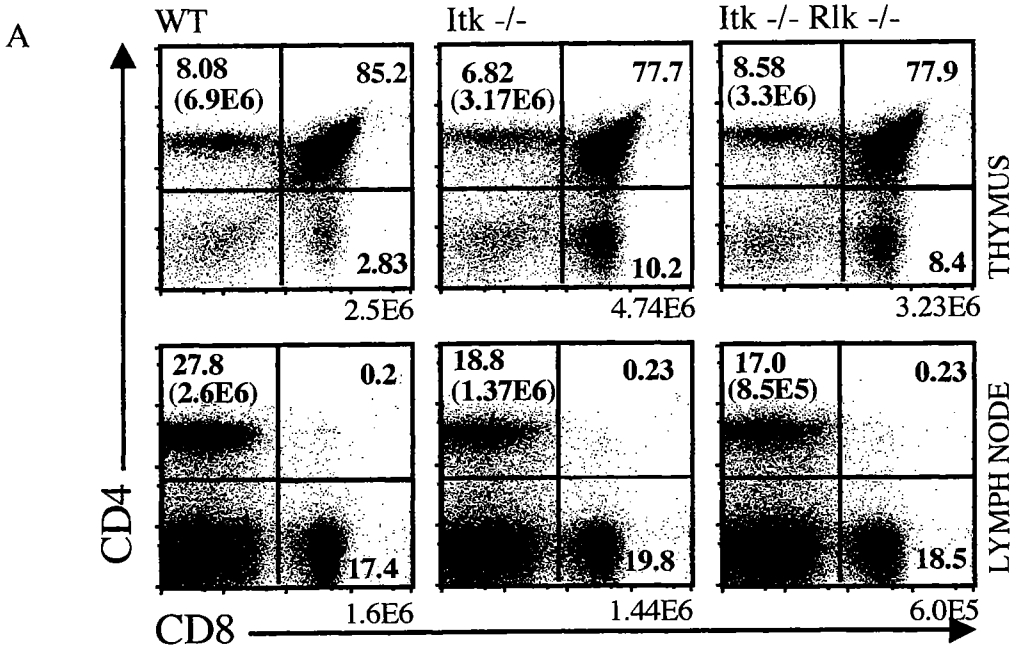
CD8 T Cells in the thymus and periphery of Itk -/- and Itk -/- Rlk -/-mice have an activated phenotype

Immediately observable in mice lacking Itk is the impairment in the positive selection of the CD4+ SP thymocyte subset. This defect causes an almost twofold reduction in the percentage and total numbers of CD4+ SP cells in the thymus and the periphery, resulting in a reversal of the CD4:CD8 ratio from 2:1 to 1:1 or 1:2. This defect in positive selection of CD4+ T cells is also observed in Itk -/- Rlk -/- mice. In contrast, the proportion and total numbers of CD8+ SP thymocyte and peripheral T cell subsets in the Itk -/- and Itk -/- Rlk -/- mice are not reduced compared to that of wildtype mice. Instead, while the fraction of CD8+ T cells in the periphery of Itk -/- and Itk -/- Rlk -/- is very similar to that of wildtype mice, the thymi of Itk -/- and Itk -/- Rlk -/- have a significant increase in the percentages and total numbers of CD8+ SP cells (Figure 2.1A). In addition, analysis of activation marker profiles on CD8+ thymocytes from Itk -/- and Itk -/- Rlk -/- mice revealed that these cells have an "activated and mature" phenotype. These thymocytes are CD44hi and HSAlo, making them very similar to the previously activated "memory" cells that usually reside in the periphery. Like the CD8+ SP thymocytes, the CD8+ T cells in the periphery of Itk -/- and Itk -/- Rlk -/- mice are also CD44hi, in addition to having increased expression of other activation markers such as CD122 (IL-2Rβ)(Figure 2.1B).

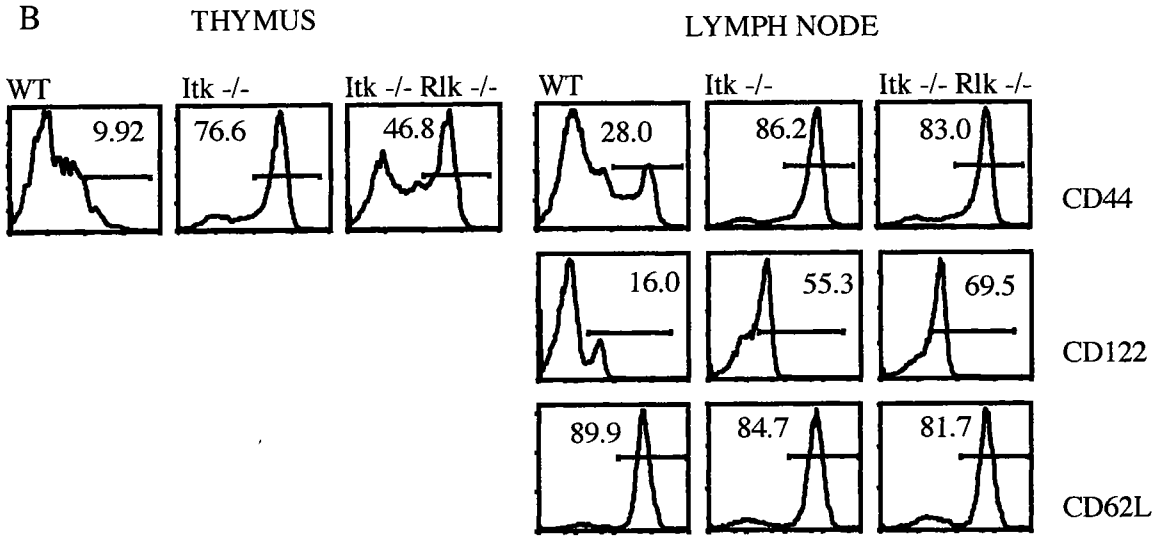
Figure 2.1: CD4/CD8 distribution and activation marker profile of WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells

Whole thymocyte and lymph node suspensions from WT, Itk -/- and Itk -/- Rlk -/- thymus and lymph nodes were stained with antibodies against CD4 and CD8 (A). CD8+ SP thymocytes were than analyzed for expression of the activation marker CD44 and peripheral T cells were analysed for expression of the activation markers CD44, CD122, and CD62L (B). This experiment is one of more than five experiments done to look at CD4 and CD8 expression and activation marker profiles in the WT, Itk -/- and Itk -/- Rlk -/- mice.

CD4/CD8 PROFILES IN Itk -/- and Itk -/- Rlk -/- LYMPHOID ORGANS



ACTIVATION MARKER EXPRESSION ON Itk -/- and Itk -/- Rlk -/- CD8 CELLS



Histograms gated on CD8+ T cells

The CD8 CD44hi cells of Itk -/- and Itk -/- Rlk -/- mice are not present at birth and do not develop in FTOC

As thymocytes develop and mature they gain and lose CD44 expression and this modulation of CD44 expression is used to characterize the various stages of the TN thymocyte subset early in thymocyte differentiation (8). The most mature SP subset of thymocytes is CD44lo as they prepare for exit to peripheral lymphoid organs. However, as the mature SP thymocytes enter the periphery, which in the early stages post-gestation are devoid of T cells, they undergo proliferation in response to the lymphopenia of the peripheral organs. The result of this lymphopenia-induced proliferation is up-regulation of the marker CD44, usually identified with "activated" cells (122). The frequency of these cells gradually diminishes as cells continually migrate from the thymus into the periphery, filling up space and preventing further proliferation of the newly emigrated cells from the thymus. These CD44hi cells remain to constitute the fraction of "previously-activated" cells typical of peripheral lymphoid organs (123). Although CD44hi cells do not typically develop in the thymus, they can gain access to the thymus as a result of recirculation from the periphery (120, 121).

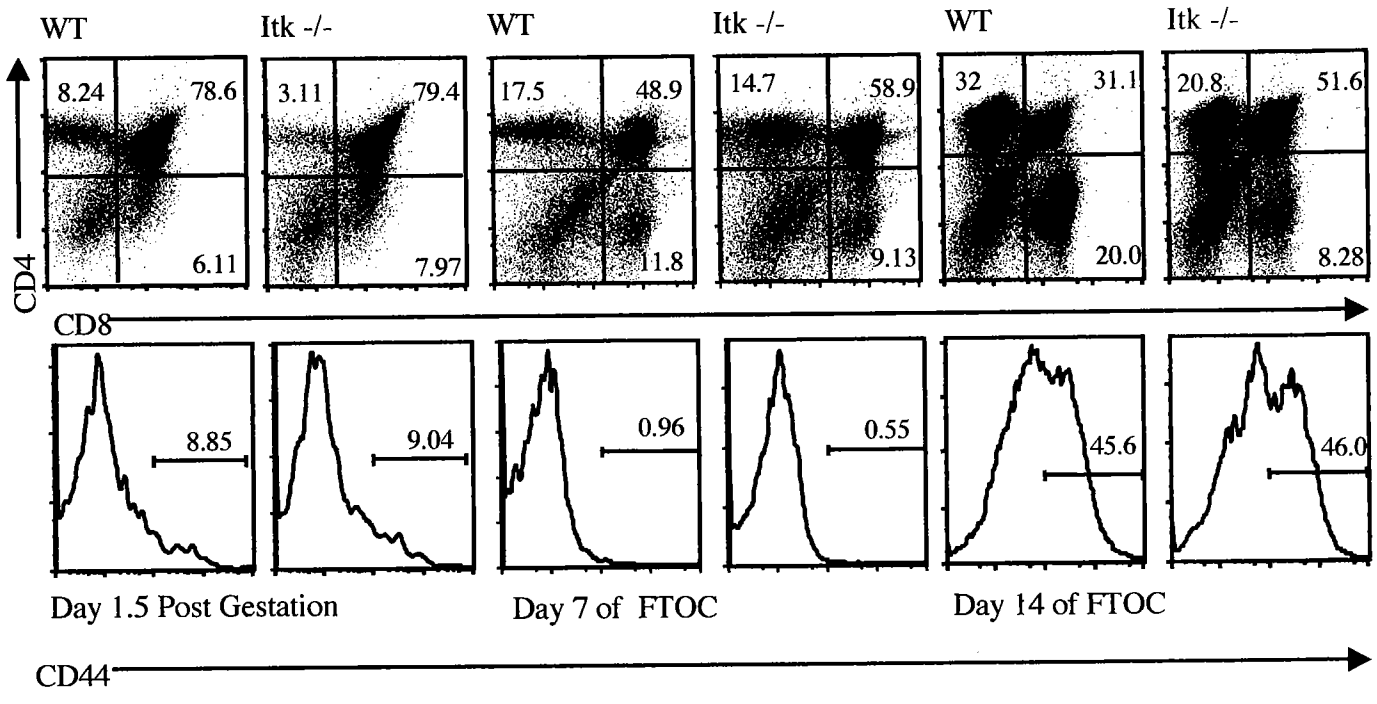
In our first hypothesis we proposed that the CD44hi cells observed in the thymi of the Itk -/- and Itk -/- Rlk -/- mice had developed as a result of a defect in thymocyte development. As a first test of this hypothesis, we looked at the CD44 expression on thymocytes from neonatal mice immediately after birth (Figure 2.2). In addition, we looked at the ability of these cells to develop in fetal thymic organ culture to determine whether the CD44hi phenotype of the Itk -/- CD8+ SP cells developed during the initial

stages of thymocyte differentiation. As shown in Figure 2.2, CD8+ CD44hi cells were not present immediately after birth in the thymi of Itk -/- mice. In keeping with this observation, CD8+ CD44hi cells did not develop in FTOC when thymi were cultured for seven days. Extension of the culture period for an additional seven days resulted in the generation of CD44hi cells, but these cells were also present in the WT cultures, and there were no appreciable differences in CD44 expression between WT and Itk -/- CD8+ SP thymocytes (Figure 2.2). These experiments showed that long-term FTOC induced expression of CD44, but did not help resolve the question of whether CD8+ CD44hi SP thymocytes observed in Itk -/- thymi developed in the thymus as a result of altered developmental processes in the thymus of Itk -/- mice. We next looked the kinetic progression of CD8+ CD44hi expression during CD8 thymocyte development to detrermine at what point the CD44hi phenotype developed in Itk -/- CD8+ SP cells.

Figure 2.2: Neonatal and FTOC derived CD8+ CD44 Expression

Whole thymocyte suspensions were generated from day 15 and day 16 fetal thymi that were cultured for either seven or fourteen days at 37°C to generate CD4 and CD8+ SP thymocytes. Whole thymocyte suspensions were also generated from thymi isolated from neonatal day 1.5 thymi. These cells were than analysed for expression of CD4, CD8 and CD44. This is one experiment of two done to look at thymocyte development by FTOC. WT: N = 2, Itk: -/- N = 7.

CD44HI CELLS ARE NOT PRESENT IN WT AND Itk -/- POST NATAL THYMI AND DO NOT DEVELOP IN SHORT TERM OR LONG TERM FTOC



Histogram gated on CD8+ cells

CD8 CD44hi cells are first detectable in day 4 post natal thymi and spleen of Itk -/- mice

Given the inconclusive results of the fetal thymic organ cultures, we next performed a longitudinal study of CD8+ SP thymocyte and peripheral T cell development to determine whether the accumulation of CD8+ CD44hi cells observed in the thymi of Itk -/- mice developed first in the thymi or in the periphery of the Itk -/- mice. The CD44 expression on CD8+ TCRhi SP thymocytes and peripheral CD8+ T cells were followed from birth (approximately d1.5) to adulthood (approximately 8 weeks) of age (Figure 2.3A).

Our study revealed that CD8+ SP thymocytes accumulated both in percentage and total numbers in the thymus of Itk -/- mice as compared to WT mice. This accumulation was not mirrored by a similar accumulation of CD8+ T cells in the periphery of the Itk -/- mice. In fact it appeared that the Itk -/- CD8+ SP thymocytes were slightly delayed in their entrance into the periphery. A delay that became noticeable at approximately three weeks post – partum in the periphery of the Itk -/- mice (Figure 2.3A).

Our analysis also showed that CD8+ CD44hi cells developed by four days after birth in WT and Itk -/- mice. Although Itk -/- thymi seemed to have a higher proportion of CD8+ CD44hi SP cells than WT thymi at this time, development of CD8+ CD44hi expression occurred equivalently on the peripheral CD8+ cells of WT and Itk -/- mice (Figure 2.3B).

The preferential accumulation of CD8+ SP thymocytes in the thymi of Itk -/- mice as compared to WT mice suggested that the CD8+ phenotype typical of the Itk -/- mice had developed first in the thymus. However, since the appearance of the CD8+ CD44hi

cell phenotype in the thymus was mirrored by the appearance of similar cells in the periphery of WT and Itk -/- mice, these data did not provide a definitive determination of the origin of the CD8+ CD44hi cells.

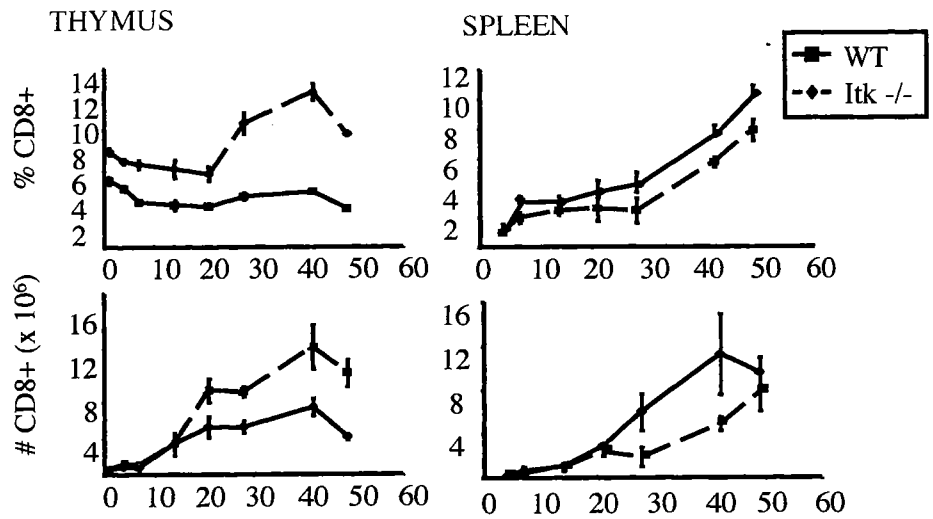
Figure 2.3: Day 4 Neonatal and Longitudinal Analysis of CD8+ CD44 Expression

(A) The percentages and total numbers of CD8+ SP thymocytes and peripheral T cells developing in WT, Itk -/- and Itk -/- Rlk -/- were followed from birth to adulthood. Itk -/- thymi accumulate greater percentages and total cell numbers of CD8+ SP thymocytes than WT mice. This accumulation does not occur in the peripheral lymphoid compartment. Single cell suspensions were generated from thymi and spleens isolated from WT, Itk -/- and Itk-/- Rlk-/- mice from birth to approximately six – weeks of age, and cells were stained with antibodies to CD4, CD8, CD44 and TCR to follow the development of CD8+ CD44 expression. These experiments were blind and genotypes were determined following experiments. Two experiments were done per timepoint. There were a minimum of two mice of a single genotype per WT or Itk -/- per timepoint.

(B) In this figure, CD44 expression is detected on WT peripheral CD8+ T cells by neonatal day 4. However, CD44 expression is also upregulated on Itk -/- CD8+ SP thymocytes and peripheral T cells at this time. This is one of two experiments done at day 4. WT: N = 5, Itk -/- N = 3.

CD8 + CELLS ACCUMULATE IN THE THYMUS BUT NOT THE PERIPHERY OF Itk -/- MICE

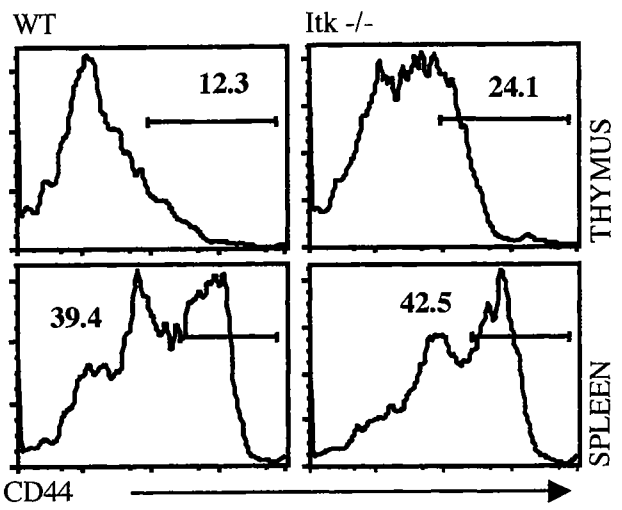
A



Average percentages and absolute numbers of CD8+ cells in the thymus and spleen of Itk +/- and Itk -/- mice analyzed at different times post gestation.

CD44 EXPRESSION IS UPREGULATED BY NEONATAL DAY 4 IN WT AND Itk -/- THYMUS AND PERIPHERY

B



Histogram gated on CD8+ TCRhi cells

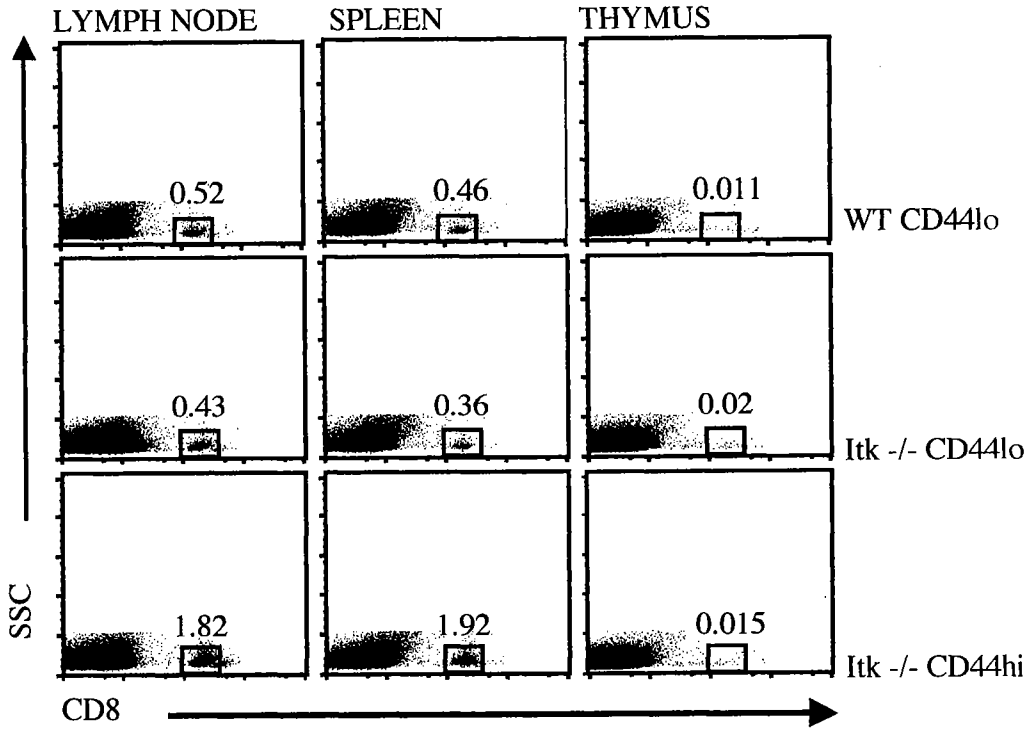
Adoptively transferred Itk -/- CD44hi CD8+ T Cells do not migrate to the thymi of WT hosts

Mature CD44hi cells resident in the periphery of mature mice are capable of re-entering the thymus. This occurs either as an end result of continual migration of these cells through the vasculature of the body or following an activation event that promotes recirculation of activated cells (120, 121). Given this possibility, we wondered whether the "mature, activated" cells present in the thymus might have recirculated from the periphery. To investigate this possibility, we adoptively transferred WT and Itk -/- CD45.2 CD44hi and CD44lo thymocytes into CD45.1 congenic WT mice and looked for trafficking of the transferred cells to the thymus of the congenic mice. As shown in Figure 2.4, we could not detect localization of the transferred cells in the thymus of host mice, even though these cells were readily observed in the peripheral lymphoid organs of the host mice. This result suggested that the presence of CD8+ CD44hi cells present in the thymi of Itk -/- mice was not due to the ability of the CD8+ SP CD44hi cells that had developed in the periphery of the Itk -/- mice to migrate to the thymus.

Figure 2.4: Itk -/- Cells do not Migrate to the Thymus

Single cell suspensions were generated from thymi isolated from WT and Itk -/- mice. CD4 and DP thymocytes were complement depleted and the remaining cells sorted for CD8+ and CD44 expression. 2 x 10^6 WT and Itk -/- CD8+ CD44lo thymocytes and 4 x 10^6 CD8+ CD44hi Itk -/- thymocytes were adoptively transferred into separate WT congenic host mice. The presence of transferred CD45.2 donor cells in spleen, lymph node and thymus was followed from day 1.5 post transfer to approximately 16 days post transfer. This experiment shows day 7 post adoptive transfer. This is one experiment of two and there were two to three mice per timepoint per WT and Itk -/- animal.

CD8 CD44^{HI} Itk -/- T CELLS DO NOT MIGRATE TO THE THYMUS



gated on CD8+ CD45.2 donor cells

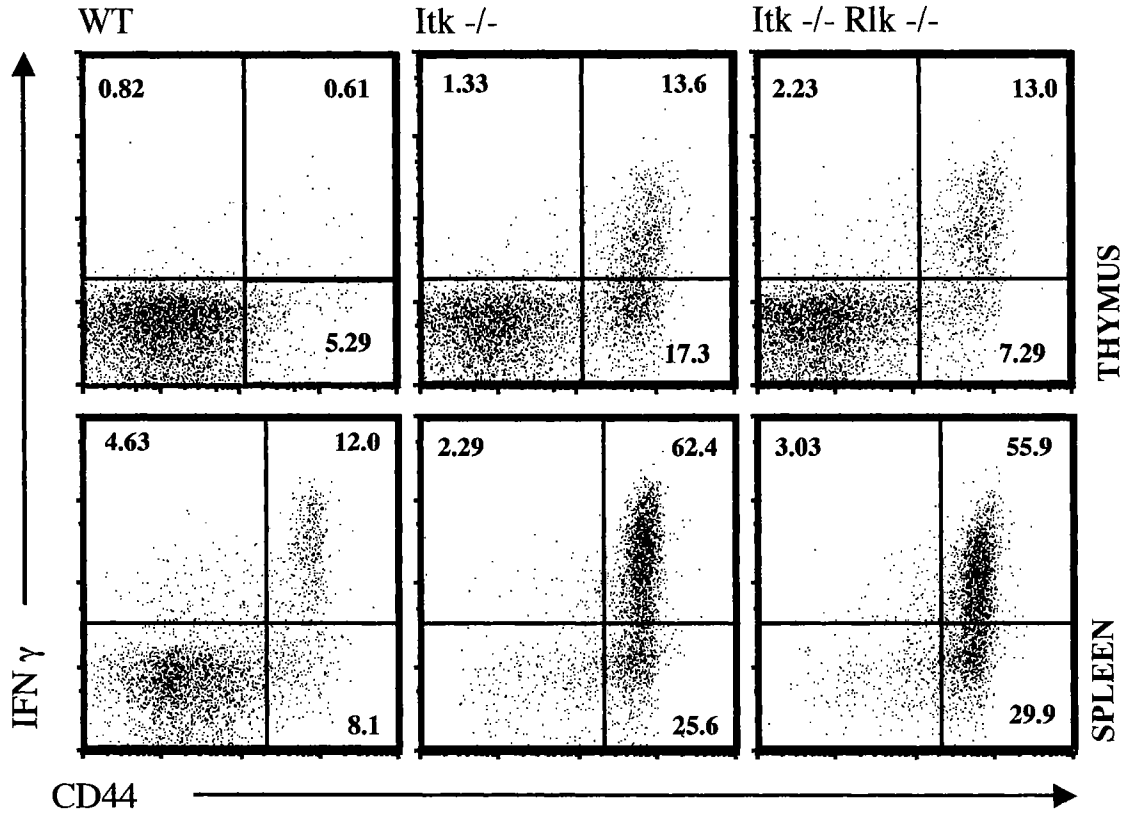
CD8+ CD44hi SP thymocytes in Itk -/- and Itk -/- Rlk -/- thymi are functionally mature

A hallmark of mature, peripheral CD8+ CD44hi T cell function is their ability to secrete effector cytokines in response to activation. To determine whether the CD8+ CD44hi SP cells in the thymi of Itk -/- and Itk -/- Rlk -/- mice were functionally as well as phenotypically similar to mature peripheral cells, we looked at the ability of these cells to secrete the effector cytokine IFNγ. For this we stimulated the cells with the phorbol ester PMA and the calcium ionophore Ionomycin, and then looked at cytokine production by intracellular staining. As shown in Figure 2.5, the Itk -/- and Itk -/- Rlk -/- CD8+ CD44hi SP cells were able to produce IFNγ directly ex-vivo in response to stimulation. As these cells are not present in WT thymi, there was no cytokine production from the WT thymocytes. However, while useful in indicating that the CD8+ CD44hi cells in the thymus of Itk -/- and Itk -/- Rlk -/- mice were functionally as well as phenotypically mature, this experiment did not resolve the question of the origin of these cells.

Figure 2.5: Cytokine Production by Itk -/- and Itk -/- Rlk -/- CD44hi Thymocytes

Whole thymocyte and spleen cell suspensions from WT, Itk -/- and Itk -/- Rlk -/- mice were stimulated with PMA and Ionomycin for 5 hours in the presence of Brefeldin A. Cells were then stained extracellularly for CD4 and CD8 expression and then intracellularly for IFNγ expression. The IFNγ production by gated CD8+ cell population was then assessed. This is one experiment for the stimulation of the thymocytes and one of three experiments done on isolated peripheral CD8+ T cells from WT, Itk -/- and Itk -/- Rlk -/- mice.

CD8 CD44HI CELLS IN THYMUS AND PERIPHERY OF Itk -/- AND Itk -/- Rlk -/- MICE PRODUCE IFNγ



gated on CD8+ cells

CD8+ CD44hi cells develop in the thymus but not the periphery of Itk -/- IL-15 -/- mice

In recent years, the common gamma cytokines IL-15 and IL-7 have been implicated in CD8+ lineage maturation (25, 27). As our previous experiments had failed to conclusively pinpoint whether or not the CD8+ CD44hi SP phenotype in the thymus of Itk -/- mice had developed first in the thymus or the periphery, we next considered the possibility that the CD8+ CD44hi SP cells in the thymus of Itk -/- and Itk -/- Rlk -/- mice had developed as a result of altered cytokine signals received during CD8+ T cell lineage maturation. This hypothesis was based on data from multiple labs showing the involvement of cytokine signals in CD8+ SP thymocyte development. Particularly interesting were data from two animal models, the SOCS 1 -/- and the SOCS 1-/- IFNγ -/- mice. These animals have increased percentages of CD8+ cells and a majority of the CD8+ cells in the thymus and the spleen express high levels of CD44 as well as CD122 and Ly-6C. In addition, the CD8+ SP thymocytes in these mice were found to be hyperresponsive to the common γ chain cytokines IL-7 and IL-15 (26, 27, 124, 125). This was particularly interesting to us in light of the mounting evidence of a role for IL-7 and IL-15 in the generation and maintenance of "memory" CD8 T cells in the periphery (64-66).

To look at the effect of IL-15 on the development of the CD8+ CD44hi SP cells in the thymus and on the maintenance of these cells in the periphery of Itk -/- mice, we crossed the IL-15 -/- mice to Itk -/- mice. We hypothesized that if IL-15 was indeed important for the generation of the CD8+ CD44hi phenotype of the CD8+ SP thymocytes in the Itk -/- mice, then removing Il-15 should resolve that phenotype. Since IL-15 is

necessary for the maintenance of peripheral CD8+ CD44hi cells, we did not expect to see a population of CD8+ cells in the periphery of the Itk -/- mice also deficient in IL-15.

As shown in Figure 2.6, Itk -/- that are also deficient in IL-15, while practically devoid of CD8+ T cells in the periphery, develop a population of CD8+ SP cells in the thymus. These data were the first to definitively demonstrate that the CD8+ SP phenotype observed in Itk -/- mice had developed first in the thymus (Figure 2.6). In addition, the percentage of CD8+ SP thymocytes in the Itk -/- IL-15 -/- mice is now very similar to that found in WT mice, and much reduced compared to the percentage of CD8+ SP thymocytes in Itk -/- mice, indicating that the removal of IL-15 had a dampening effect on the accumulation of CD8+ SP thymocytes normally observed in the thymus of Itk -/- mice.

Interestingly, the loss of IL-15 did not completely resolve the CD44hi phenotype of the Itk -/- CD8+ SP thymocytes, as there was only an approximate 40% reduction in the fraction of CD8+ SP thymocytes residing in the thymus of the Itk -/- IL-15 -/- mice that are CD44hi as compared to littermate control Itk -/- IL-15 +/- mice. As shown in Figure 2.6A, while approximately 78% of the CD8+ SP thymocytes in the Itk -/- mice are CD44hi, only 43% of these cells are CD44hi in the thymus of Itk -/- IL-15 -/- mice. These data suggest that another cytokine, possibly IL-7 may also be involved in generation of the CD44hi phenotype of the Itk -/- CD8+ SP thymocytes.

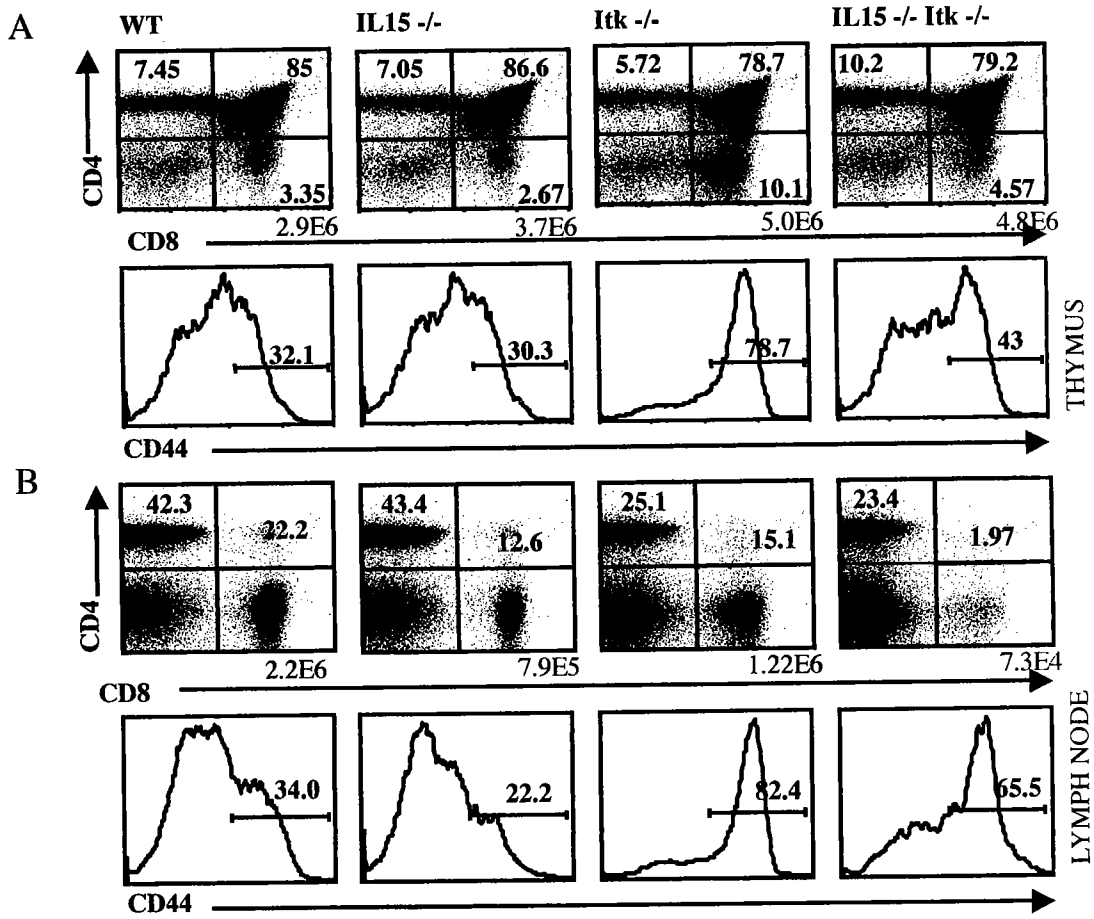
These data, in addition to demonstrating that the CD44hi phenotype of the Itk -/- CD8+ SP thymocytes developed first in the thymus, also suggested that the CD8+ CD44hi cells in the thymus of the Itk -/- mice were, by some unknown mechanism,

altered in their response to IL-15 late in thymocyte differentiation. The incomplete resolution of the CD44hi phenotype of the CD8+ SP cells in the thymus of Itk -/- IL-15 -/- mice suggested the involvement of another cytokine, possibly IL-7, in the generation of the CD44hi phenotype of the CD8+ SP cells in the thymi of Itk -/- mice.

Figure 2.6: CD8+ CD44hi cells develop in Thymus of Itk -/- IL-15 -/- mice

Thymus and lymph node cells isolated from WT, Itk -/-, IL-15 -/- and Itk -/- IL-15 -/- mice were stained for expression of CD4, CD8 and CD44. The CD4 and CD8 distribution in WT, Itk -/-, IL-15 -/- and Itk -/- IL-15 -/- thymi (A) and lymph node (B) was assessed. CD44 expression was then analysed on gated CD8+ populations. Numbers shown are total CD8+ T cell numbers. This is one experiment of three.

CD8 CD44^{HI} CELLS DEVELOP IN THE THYMUS OF, BUT ARE ABSENT FROM THE PERIPHERY OF Itk -/- IL-15 -/- MICE



Histograms gated on CD8+ TCR+ cells

CD8+ CD44hi cells in Itk -/- and Itk -/- Rlk -/- mice are hyperresponsive to the common γc cytokines IL-15 and IL-7

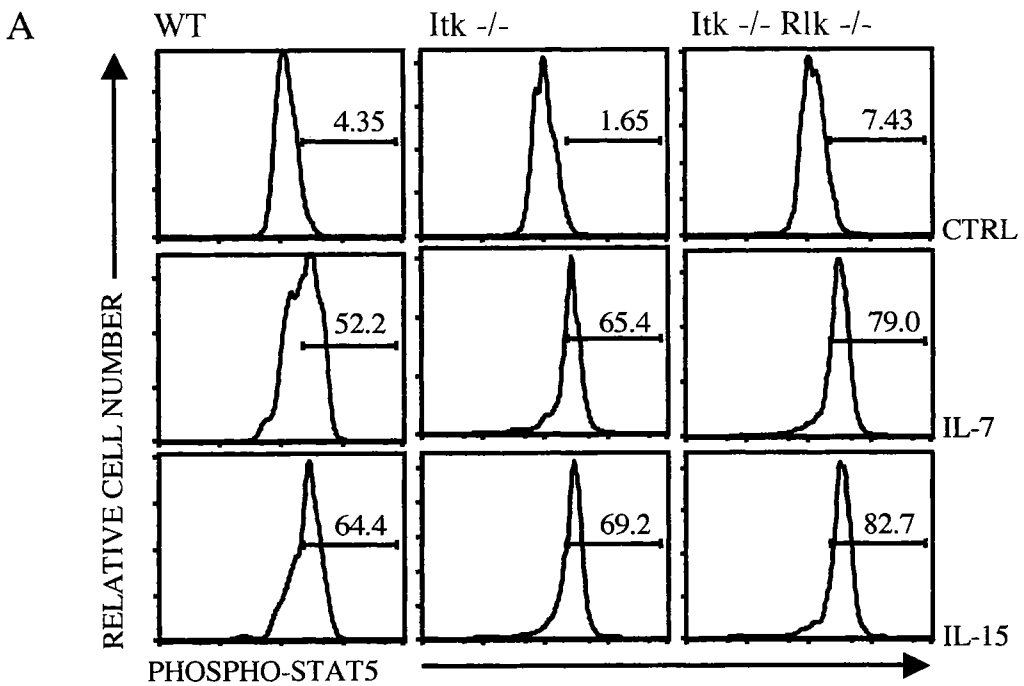
The phenotype of the Itk -/- IL-15 -/- mice as compared to Itk -/-, suggested that IL-15 was involved in the development of the CD44hi phenotype of the CD8+ SP thymocytes in Itk -/- mice. Additionally, the thymic and peripheral CD8+ T cell phenotype of Itk -/- and Itk -/- Rlk -/- mice is very similar to that of the CD8+ SP thymocytes and peripheral cells from murine models in which the regulation of cytokine signalling is perturbed (27, 124-126). We therefore asked whether the CD8+ SP thymocytes and peripheral T cells from Itk -/- and Itk -/- Rlk -/- mice were similarly deficient in the regulation of signalling processes downstream of IL-7 and IL-15 stimulation. To do this, we cultured thymocytes from WT, Itk -/- and Itk -/- Rlk -/- mice in a 1/20 dilution of IL-7 supernatant or 40 ng/ml of IL-15 and looked at the effect of these cytokines on CD8+ thymocytes. Whole thymocyte suspensions from WT, Itk -/- and Itk -/- Rlk -/- mice were cultured in IL-15 for 30 minutes at 37^{0}C. The cells were then stained for CD4, CD8 and CD44 expression. As an assessment of cytokine responsiveness, we looked at the phosphorylation of STAT-5 in CD8+ CD44hi SP cells in response to IL-15 signalling. STAT-5 is the principal STAT molecule downstream of common γc cytokine signalling, and is maximally phosphorylated approximately thirty minutes following exposure to cytokine (127). As shown in Figure 2.7, after thirty minutes of culture IL-7 and even moreso IL-15 promoted phosphorylation of STAT-5 in WT CD8+ SP CD44hi cells. However, CD8+ CD44hi SP cells from Itk -/- and Itk -/- Rlk -/- thymi had higher levels of phosphorylated STAT-5 than CD8+ CD44hi SP cells

from WT thymi. These cytokine response experiments suggest that Itk -/- and Itk -/- Rlk -/- CD8+ SP cells are altered in their ability to regulate signals downstream of IL-7 and IL-15.

Figure 2.7: Response to common γc cytokine stimulation

Whole thymocyte suspensions from WT, Itk -/- and Itk -/- Rlk -/- mice were cultured in the presence of IL-7 and IL-15 for 30 minutes in media containing 1mg/ml BSA and 0.5 % FCS. STAT-5 phosphorylation was assessed by intracellular staining. This is one experiment of three.

Itk -/- and Itk -/- Rlk -/- CD8 CD44hi THYMOCYTES ARE HYPERSENSITIVE TO IL-15 AND IL-7 STIMULATION



DISCUSSION

Itk and Rlk are members of the Tec kinase family of signalling molecules and are known to have a somewhat redundant function downstream of TCR signalling (93). In addition to their importance in peripheral T cell signalling, Itk and Rlk are also important in signalling downstream of the TCR in maturing thymocytes. Their importance as amplifiers of the strength to the signal downstream of the TCR has been extensively demonstrated in a variety of murine transgenic models in which the positive selection of CD4+ SP cells and to a lesser extent CD8+ SP cells is impaired in Itk -/- mice and more so in Itk -/- Rlk -/- mice (15, 18, 24). This defect in positive selection is thought to occur primarily as a result of the lowering of the strength of the TCR signal that occurs in the absence of Itk and both Itk and Rlk. This diminishes either the strength or the duration of the signal that a maturing DP thymocyte needs to differentiate into a CD4+ or CD8+ SP cell, thereby lowering the efficiency of this process.

An interesting characteristic of development in the Itk -/- and Itk -/- Rlk -/- thymus, in which the positive selection and development of the CD4+ T cell subset is more affected than that of the CD8+ subset, is that there is an accumulation of CD44hi CD8+ SP cells (Figure 2.3). This phenotype is carried over to the CD8+ T cells in the periphery of Itk -/- and Itk -/- Rlk -/- mice which in addition to being CD44hi and CD62Lhi, also express increased levels of the activation markers CD122 (Figure 2.1B). This increase in CD8+ SP cells in the thymus is not due to lineage switching, as has been previously demonstrated in published data from this lab (24). In these experiments, the author, using a variety of pigeon/moth cytochrome C MHC Class II restricted transgenics

with a range of avidities for the selecting ligand, showed that although lowering the avidity of the MHC/TCR interaction affected the positive selection of Itk -/- transgenic mice more than that of WT transgenic mice, at no point was there increased development of CD8+ SP cells on the Itk -/- background.

In this study we explore the origin of the CD8+ CD44hi SP cells that develop in the thymus of Itk -/- and Itk -/- Rlk -/- mice and a potential role for Itk and Rlk in the lineage differentiation process. Our data show that CD8+ SP thymocytes accumulate in the thymus of Itk -/- mice, presumably in the medullary compartment, and have a slight delay in their accumulation in the periphery, suggesting a delay in migration to the periphery (Figure 2.3). In addition, our data demonstrate that CD8+ SP thymocytes from mice lacking Itk and moreso from mice lacking both Itk and Rlk are altered in their ability to regulate their response to cytokine signals, specifically in their response to the common γc cytokines IL-7 and IL-15.

Classically, cytokines have only been shown to influence thymocyte development at the earliest TN1 – TN2 stages of thymocyte differentiation. At this early stage of thymocyte differentiation thymocytes express receptors for SCF and IL-7, and are critically dependent on these cytokines for progression to the later TN3 – TN4 stages of thymocyte development (112, 113).

More recently, data from the Singer laboratory have suggested that IL-7 is also important for support of the CD8+ lineage differentiation process (25, 128). This model of CD8+ lineage differentiation termed "co-receptor reversal", proposes that when DP cells are signalled either in response to MHC Class I or MHC Class II expressing antigen

presenting cells, they downregulate expression of their CD8 co-receptor to become CD4+ CD8lo intermediates. If these intermediates are subsequently subjected to CD4 selecting signals that is they receive a persistent signal from an MHC Class 11 restricted APC, they maintain CD4 expression and develop into CD4+ SP thymocytes. In the absence of a persistent or sustained signal, the CD4+ CD8lo intermediates silence expression of CD4, and become dependent on the cytokine IL-7 to maintain expression of CD8. Importantly, in the presence of a persistent signal, signalled DP thymocytes cultured in the presence of IL-7 did not upregulate CD8 co-receptor expression or extinguish CD4 co-receptor expression. The signalled DP thymocytes cultured in the presence of IL-7R blocking antibody and anti γc antibody, abrogated development of CD8+ SP cells silencing of CD4 co-receptor expression. In addition to suggesting a role for IL-7 in CD8+ lineage differentiation, the need for anti γc antibody to fully block CD8+ SP development suggests that other common γc cytokines, possibly IL-15 may potentially be involved in this process. Although this system is very artificial, it nonetheless provides intriguing evidence for a role for cytokines in this later CD8+ SP stage of thymocyte development.

Additional evidence for a role for cytokines in CD8+ lineage determination has come from studies in the SOCS-1 -/- and SOCS-1 -/- IFNγ -/- mouse models. SOCS-1 is expressed at all stages of TN thymocyte differentiation. However, as thymocytes become DP, the level of SOCS-1 increases dramatically. Equally dramatic is the quenching of SOCS-1 expression that occurs as DP cells undergo the processes of positive selection and lineage commitment to become CD4+ SP and CD8+ SP thymocytes. CD4+ SP cells subsequently lose expression of SOCS-1, but SOCS-1 expression is maintained in CD8+

SP cells (26, 129). Analysis of the peripheral lymphoid compartments and the thymi of SOCS-1 deficient animals show that a significant proportion of the CD8+ SP cells in the thymus and peripheral lymphoid organs of SOCS-1 -/- and SOCS-1 -/- IFNγ -/- mice is CD44hi. These cells are hyperresponsive to stimulation with IL-7 and IL-15. For example, thymi of SOCS-1 -/- and SOCS-1 -/- IFNγ -/- mice cultured in the presence of these cytokines generate more CD8+ SP cells than WT thymi. In addition, CD8+ thymocytes from SOCS-1 -/- mice maintain an elevated level of STAT-5 phosphorylation over a longer time-period than CD8+ SP thymocytes from WT mice (26, 27). These experiments demonstrate a potential role for IL-7 and IL-15 in regulation of the CD8+ lineage differentiation process, and show that the inability to regulate the signalling of these cytokines can accelerate the maturation of CD8+ SP thymocytes just as overexpression of IL-7 and IL-15 can promote the phenotypic and functional maturation of peripheral CD8+ T cells. CD8+ SP thymocytes and peripheral CD8+ T cells from Itk -/- and Itk -/- Rlk -/- mice are phenotypically similar to that of SOCS-1 -/- and SOCS-1 -/- IFNγ -/- mice.

Additionally, the peripheral CD8+ T cells in Itk -/- and Itk -/- Rlk -/- mice are phenotypically similar to peripheral CD8+ lymphocytes from IL-7 and IL-15 transgenic mice, where these cytokines are overexpressed (126, 130), and to the CD8+ lymphocytes from STAT-5 transgenic mice, where there is constitutive activation of the signalling pathways downstream of the common γc cytokine receptors (131). These studies prompted us to investigate the effect of the common γc cytokines on the development of the CD8+ CD44hi cells in the thymus and periphery of the Itk -/- and Itk -/- Rlk -/- mice.

We initiated our investigation by generating Itk -/- IL-15 -/- mice. Analyses of the CD8+ cell populations in the thymus and the periphery of these mice led us to conclude that the CD8+ CD44hi cells in the Itk -/- mice most likely arose during thymocyte development, specifically during CD8+ lineage maturation. This conclusion was based on the data showing that a smaller fraction of CD8+ CD44hi SP thymocytes were generated in the thymus of the Itk -/- IL-15 -/- mice, as compared to Itk -/- mice, and that very few CD8+ CD44hi cells remained in the periphery of the Itk -/- IL-15 -/- mice (Figure 2.6). This lessened the probability that the CD8+ CD44hi cells remaining in the Itk -/- IL-15 -/- mice had recirculated from the periphery. In addition, our studies on the response of Itk -/- and Itk -/- Rlk -/- CD8+ SP thymocytes to signals from IL-7 and IL-15 revealed that Itk -/- and moreso Itk -/- Rlk -/- CD8+ SP thymocytes are hypersensitive to IL-7 and in particular to IL-15 signals (Figure 2.7). The Tec kinase family has been previously implicated in cytokine signalling, but these experiments were mostly with other Tec kinase family members and in non-T cell types (132, 133). Our experiments demonstrate a role for the T cell specific Tec kinase family members in the regulation of cytokine signalling in T cells.

The crosstalk between Tec kinases specific to signalling downstream of the TCR and proteins connected to the regulation of the SOCS-1 dependent control of cytokine signalling are unknown. However, there has been data suggesting that the proper transduction of signals downstream from the TCR is necessary for maintaining the level of SOCS-1 protein expression. For example, in cells lacking the negative regulatory transcription factor Jun D, there is a decrease in SOCS-1 at the RNA level. This

phenotype is followed by an increase in the secretion of cytokines from T cells lacking JunD. As such, it is possible that in the absence of Itk and Rlk which regulate the expression of the jun and fos family members, expression of JunD is decreased, thereby affecting SOCS-1 expression (134). However, it is debatable whether this mechanism is operable during the later TCR dependent phase of thymocyte development.

Alternatively, SOCS-1 expression in Itk -/- and Itk -/- Rlk -/- CD8+ SP thymocytes may be regulated post – translationally by a protein such as Pim-1. Pim – 1 is a serine threonine kinase and is expressed primarily in cells of the hematopoietic lineage. Expression of Pim –1 mRNA is markedly induced following TCR crosslinking. Pim-1 is also induced in response to cytokine signalling and is involved in the regulation of SOCS-1 protein stability. Pim –1 has a role in thymocyte development as it is expressed during the late TN stage of thymocyte development where it participates in the transition from the TN4 – DP stage of thymocyte development (135-137). Diminished production of Pim-1 in the absence of Itk and Rlk would potentially lead to the impaired stability and function of SOCS-1, lowering SOCS-1 expression in CD8+ SP thymocytes and resulting in hyperresponsiveness to cytokine stimuli

As such, it is possible that the levels of SOCS-1 protein or mRNA are lower in Itk -/- and Itk -/- Rlk -/- CD8+ SP thymocytes. Although the signals regulating the lowering of SOCS-1 expression in CD8+ SP thymocytes as they develop from DP to SP thymocytes are unknown, we propose that the some of the signals needed to maintain SOCS-1 expression in CD8+ SP thymocytes are TCR dependent. There are several mechanisms by which SOCS-1 expression may be diminished in CD8+ SP thymocytes.

We hypothesize that in Itk -/- and Itk -/- Rlk -/- CD8+ SP thymocytes undergoing lineage differentiation in response to reduced TCR mediated signals, the strength of these signals are further reduced. We propose that this reduction in the strength of TCR signalling may directly affect SOCS-1 expression in CD8+ SP thymocytes, leading to a hypersensitive response to IL-7 or IL-15 during the lineage differentiation process.

Our experiments also showed that CD8+ SP thymocytes accumulate in the thymus of Itk -/- mice. The most plausible anatomical location of this accumulation is in the medulla of the thymus. The medulla of the thymus is the point at which most mature CD8+ SP thymocytes are thought to leave the thymus before migrating to the periphery as recent thymic emigrants. Most recent thymic emigrants have the phenotypic properties of mature peripheral cells, as they are CD44lo and HSAlo. However, some recent thymic emigrants have also been shown to undergo this phenotypic maturation upon exit to the periphery. These data suggest that the thymocytes leaving the thymus are a mixed population of mature and maturing cells, and the mechanisms governing the dwell time of SP thymocytes in the medulla and maturational stage at which these cells leave the medulla and enter the periphery are largely unknown, although chemokines are thought to play a large part in this process (114). As such, it is possible that the accumulation of CD8+ SP thymocytes in the thymus of Itk -/- mice, in addition to their slight delay in entrance to the periphery can cause them to be subjected to the maturational processes that occur in the medulla for a longer period of time than CD8+ SP cells in the thymi of wildtype mice, causing them to completely downregulate HSA and upregulate CD44, all before exit to periphery. This is an interesting and attractive

hypothesis. However, whether this excessive exposure to maturational signals in the thymus would alter the response to cytokine signals enough to generate the results obtained in Figure 2.7 is a matter of debate. It is also possible that the phenotype seen in the Itk -/- and Itk -/- Rlk -/- mice are a combination of these and previously mentioned events. These studies are the subject of ongoing experiments.

CHAPTER III

ITK AND RLK IN CD8 T CELL SIGNALLING AND FUNCTION

INTRODUCTION

T cell antigen receptor (TCR) signalling plays a critical role in the generation of adaptive immune responses. Signals mediated by the TCR are required to activate naïve antigen-specific T cells, thereby initiating gene expression programs that result in T cell proliferation and the acquisition of distinct T cell effector functions. This same TCR signalling pathway is then required for the reactivation of effector T cells following their migration out of lymphoid organs to the site of infection. Within the two major classes of T cells, CD4+ helper T cells and CD8+ cytolytic T cells, there is substantial, if not complete, overlap in their expression of TCR signalling proteins. In spite of this overlap, CD4+ and CD8+ T cells translate the signals mediated by these proteins into functionally distinct responses. Previous studies have also demonstrated that, even when CD4+ and CD8+ T cells share effector functions such as the secretion of the cytokines IFNγ and TNFα, the two cell types have distinct methods of regulating the expression of these genes. For example, production of IFNγ from CD4+ T cells requires expression of T-bet, a transcription factor that is a master regulator of TH1 development. CD8+ T cells do not require T-bet to produce IFNγ, but instead seem to require expression of a newly discovered regulator of CD8+ T cell differentiation, eomesodermin to produce effector cytokines and to develop cytolytic function(107-110). Until recently, most studies of TCR signalling were performed in tumour cell lines, and thus did not lend themselves to a comparison of signalling pathways between CD4+ and CD8+ T cells. However, with the availability of numerous lines of knockout mice, it has now become feasible to assess

the precise function(s) of individual signalling proteins in the two major subsets of T cells.

Our studies have focused on the Tec family tyrosine kinases, Itk and Rlk. Previous data have demonstrated an important role for Itk downstream of the TCR. Specifically, Itk is critical for the activation of Phospholipase C-γ1 (PLCγ1) in response to TCR stimulation, and thus, plays a role in calcium mobilization as well as in the activation of the ERK and JNK MAP-kinases (18, 92, 93, 100). Itk has also been shown to be important for actin polymerization and cytoskeletal reorganization following TCR stimulation (138). In contrast to Itk, the precise role of Rlk in T cell signalling is less clear. While Rlk interacts with many of the same T cell signalling proteins as Itk, e.g. the SLP-76, GADS, LAT, PLC-γ1 complex, Rlk-deficient T cells have only minimal defects in TCR signalling (93, 98). Nonetheless, a combined deficiency in Itk plus Rlk results in a substantial exacerbation of the signalling defect observed in Itk-/- T cells, suggesting that at the very least, Rlk function is somewhat redundant with that of Itk.

The biochemical defect in Itk-/- T cells leads to impaired T cell activation and effector functions. Primarily, the studies performed to date have focused on CD4+ T cell responses. For instance, purified CD4+ T cells from Itk-/- and Itk-/- Rlk-/- mice produce greatly reduced amounts of the T cell growth factor, IL-2, and thus proliferate poorly to mitogenic stimuli (93). In addition, Itk-/- CD4+ T cells show reduced effector functions, including substantial defects in secretion of effector cytokines IL-4 and IFN γ, as well as impaired activation-induced cell death responses due to reduced induction of Fas ligand expression (99, 100). In the case of effector cytokine secretion, again Itk-/- Rlk-/- CD4+

T cells show more profound defects than CD4+ T cells lacking only Itk. Overall, these defective responses have been attributed to substantial defects in the activation of latent transcription factors NFAT and NF-kB, as well as to reduced induction of additional transcription factors such as Egr-2 and Egr-3 (94, 100). To date, no comparable studies have been performed with isolated Itk-/- or Itk-/- Rlk-/- CD8+ T cells.

A relatively small number of studies have addressed the ability of Itk-/- and Itk-/- Rlk-/- mice to mount protective responses to pathogenic infections, and again these studies have primarily focused on CD4+ T cell responses. For instance, Fowell and colleagues first showed the somewhat paradoxical finding that Itk-/- Balb/c mice mounted a protective TH1 response to infection with Leishmania major, in contrast to wild type Balb/c mice that succumb to this infection. These investigators concluded that this unexpected resistance to L. major by Itk-/- mice was due to the inability of Itk-/- CD4+ T cells to produce IL-4, a conclusion that was further supported by their finding that Itk-/- mice could not generate a protective TH2 response to the parasite, Nippostrongylus brasiliensis. Supplementing these studies, Schaeffer and colleagues found that Itk-/- mice were also unable to generate a TH2 response to the helminthic parasite, Schistosoma mansoni. However, in this study, the Itk-/- mice also succumbed to infection by Toxoplasma gondii, an intracellular protozoan that is normally cleared by a TH1-mediated immune response. Together, these data indicate that Itk-/- mice are particularly susceptible to parasitic infections requiring TH2 cytokine-mediated responses, but in some cases, are also unable to mount protective TH1 cytokine-dependent responses. Finally, this latter study examined the response of Itk-/- Rlk-/-

mice to these two pathogens, T. gondii and S. mansoni. Interestingly, while the Itk-/- Rlk-/- mice succumbed even more rapidly to the T. gondii infection than the Itk-/- mice, and produced only very low levels of the critical effector cytokine IFNγ, these doubly-deficient mice showed a paradoxical ability to clear the parasitic infection by S. mansoni (93, 94).

One limitation of the studies described above is the inability to track the pathogen-specific T cells responding in each of these infectious disease models. Thus, the inability of the Itk-/- or Itk-/- Rlk-/- mice to mount an appropriate CD4+ T cell effector response, or in some cases, their paradoxical ability to mount such a response, cannot be dissected at the cellular or molecular level. For instance, in the cases where protective immunity fails to arise, is this due to impaired T cell activation, impaired T cell expansion, impaired production of effector cytokines, or potentially, impaired migration of effector T cells to the site of infection? Such information will be critical in resolving the interesting and sometimes confusing outcomes from these infectious disease studies.

In contrast to the studies described above, only a single study has so far examined CD8+ T cell responses in Itk-/- mice, and none have examined CD8+ T cell function in Itk-/- Rlk-/- mice. In addition, no studies have yet directly addressed the potential biochemical defects in purified CD8+ T cells lacking Itk or Itk and Rlk. In the one study that did examine the function of Itk-/- CD8+ T cells, mice were infected with three different viruses, lymphocytic choriomeningitis virus (LCMV), vaccinia virus, and vesticular stomatitus virus (105). In this report, the investigators found that Itk-/- mice

were mildly impaired in their ability to generate functional CTL responses to LCMV infection, and although able to clear a vaccinia virus infection, did so with delayed kinetics. While this study provides the only evidence to date that CD8+ T cell function is also affected by the loss of Itk, no evidence was provided to address the mechanism(s) that might have contributed to the impaired CD8+ T cell responses seen in the Itk-/- mice. Further, these data did not address the role of Itk in other aspects of the CD8+ T cell anti-viral response, such as CD8+ T cell expansion and attrition, or the ability to generate an efficient and protective recall response. Finally, as mentioned above, the additional role of Rlk in CD8+ T cell-mediated antiviral immune responses has never been explored.

To address these issues, we first examined both biochemical and functional responses of Itk-/- and Itk-/- Rlk-/- CD8+ T cells in vitro. To assess CD8+ T cell responses to virus infection, we then took advantage of the well-characterized mouse model of LCMV infection. These studies demonstrated that CD8+ T cells from mice lacking Itk or both Itk and Rlk show greatly impaired TCR signalling, resulting in substantial defects in T cell activation and cytokine production in vitro. Furthermore, both Itk-/- and Itk-/- Rlk-/- mice have significantly impaired CD8+ T cell responses to LCMV infection, characterized by substantial reductions in T cell expansion, survival, and IFNγ production. We also show that the impaired expansion of virus-specific CD8+ T cells lacking Itk or Itk and Rlk cannot be rescued by providing WT LCMV-specific CD4+ T cell help, thereby substantiating the important role of Tec kinases in CD8+ T cell signalling.

RESULTS

CD8+ T cells lacking Itk or Itk and Rlk have impaired responses to TCR stimulation

Initial studies characterizing Itk-/- and Itk-/-Rlk-/- mice indicated that the absence of these two Tec kinases did not result in a deficit in peripheral CD8+ T cell numbers. For instance, in contrast to the situation for CD4+ T cells, the CD8+ T cell compartment in Itk-/- and Itk-/- Rlk-/- mice contains a similar fraction of cells in both lymph nodes and spleen, and has a comparable total cellularity, as that found in wild type mice (93). One difference that is observed, however, is that the surface phenotype of CD8+ T cells in Itk-/- and Itk-/-Rlk-/- mice resembles that of memory or activated T cells, rather than naïve cells. For instance, Itk-/- and Itk-/-Rlk-/- CD8+ T cells are predominantly CD44hi, CD62Lhi, Ly6Chi and CD122hi (Figure 2.1). Together with the known signalling defects in Itk-/- and Itk-/-Rlk-/- CD4+ T cells, these observations raised issues concerning the functional competence of Tec kinase-deficient CD8+ T cells.

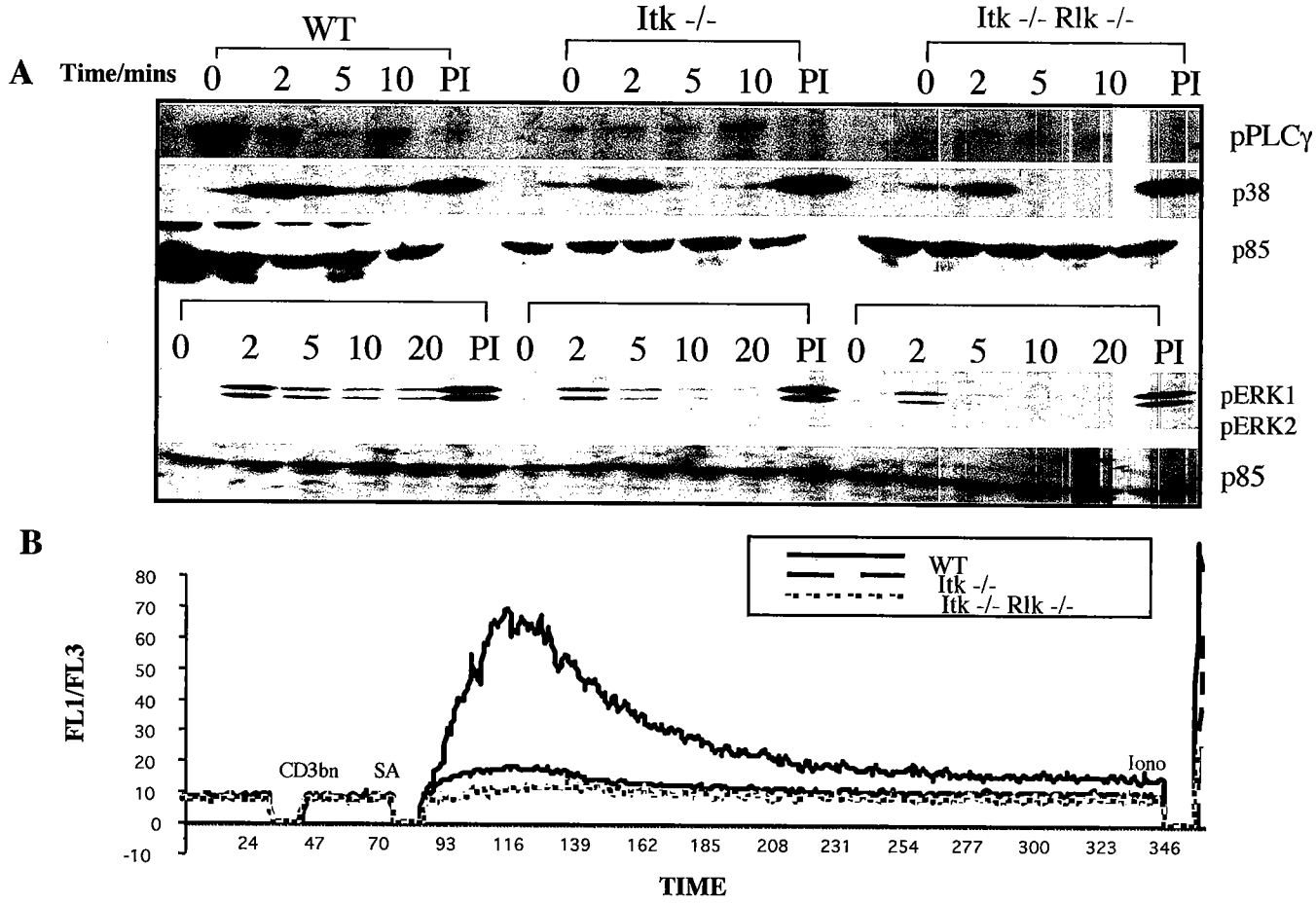
As a first step in addressing this issue, I examined several biochemical responses of CD8+ T cells from Itk-/- and Itk-/-Rlk-/- mice to in vitro stimulation. For these studies, CD8+ T cells were isolated from wild type, Itk-/-, and Itk-/- Rlk-/- mice and stimulated by anti-CD3 antibody crosslinking. Cells were then assessed for calcium mobilization, ERK, JNK and p-38 MAP-kinase activation and PLCγ1 tyrosine phosphorylation. Interestingly, despite their activated/memory phenotype, CD8+ T cells from Itk-/- and Itk-/-Rlk-/- mice are impaired in their response to TCR stimulation. As shown in Figure 3.1A, following anti-CD3 antibody crosslinking, CD8+ T cells lacking

Itk or Itk and Rlk show impaired phosphorylation of the PLCγ. In addition, Itk -/- and Itk -/- Rlk -/- CD8+ T cells are also impaired in their ability to sustain the phosphorylation of the p38 and ERK1 and ERK2 MAP-kinases following TCR stimulation. Surprisingly, the unstimulated WT CD8+ T cell controls had a high basal level of phosphorylation of PLCγ, despite being serum starved for five hours before analysis of PLCγ phosphorylation levels. As this result was seen in all experiments done to look at PLCγ phosphorylation in CD8+ T cells, it is possible that this was a condition of the WT CD8+ T cells having been previously stimulated with PMA and Ionomycin and then expanded in media containing IL-2 before the analysis. These results raise the interesting possibility that CD8+ T cells regulate PLCγ phosphorylation through a variety of different means including serum receptor activation. Interestingly, the unstimulated Itk -/- and Itk -/- Rlk -/- CD8+ T cells responded to the serum starvation more readily than the WT CD8+ T cells and had no detectable levels of PLCγ phosphorylation. This suggests that Itk -/- and Itk -/- Rlk -/- CD8+ T cells may not be as capable of WT CD8+ T cells at maintaining phosphorylation of PLCγ in response to other stimuli such as serum receptor activation. These are interesting possibilities that should be further investigated. As would be expected from these defects, CD8+ T cells lacking Itk and both Itk and Rlk fail to generate a sustained calcium flux following TCR cross-linking (Figure 3.1B). These results demonstrate that in the absence of Itk and both Itk and Rlk, CD8+ T cell fitness – the ability to mount an optimal response to TCR stimulation, is impaired. These data suggested that loss of Itk and both Itk and Rlk would affect the ability of CD8+ T cells to mediate effector functions.

Figure 3.1: Analysis of CD8+ T cell signalling and function.

(A) To assess activation of PLCγ and the MAP-kinase signalling pathways, 5 x 10^6 CD8+ T cells were labelled with 25μg/ml of biotinylated anti-CD3. The CD8+ T cells were then activated for various times by cross-linking with strepavidin. As a positive control, cells were stimulated with PMA at a concentration of 2.5ng/ml and Ionomycin at 375 ng/ml. Lysates were then assessed for activation of PLCγ, p-ERK and p-38 MAP-kinases as described in the Materials and Methods. These experiments are one representative of five for PLCγ phosphorylation, three for p-38 phosphorylation and two for ERK phosphorylation. (B) For calcium flux analysis 1 x 10^7 cells were loaded with the calcium sensitive dyes Fluo-3 and Fura Red for 1hr at 37°C. 100μl was removed to serve as unstimulated controls. The remaining cells were first labelled with biotinylated anti-CD3 for 45s then crosslinked with strepavidin for 6 mins. As a positive control the cells were stimulated with Ionomycin for 2 mins. The calcium flux was calculated by ratioing the intensities of the dyes over time. This experiment is one representative of four similar experiments.

IMPAIRED SIGNALLING DOWNSTREAM OF TCR CROSS-LINKING IN Itk -/- AND Itk -/- Rlk -/- CD8 T CELLS



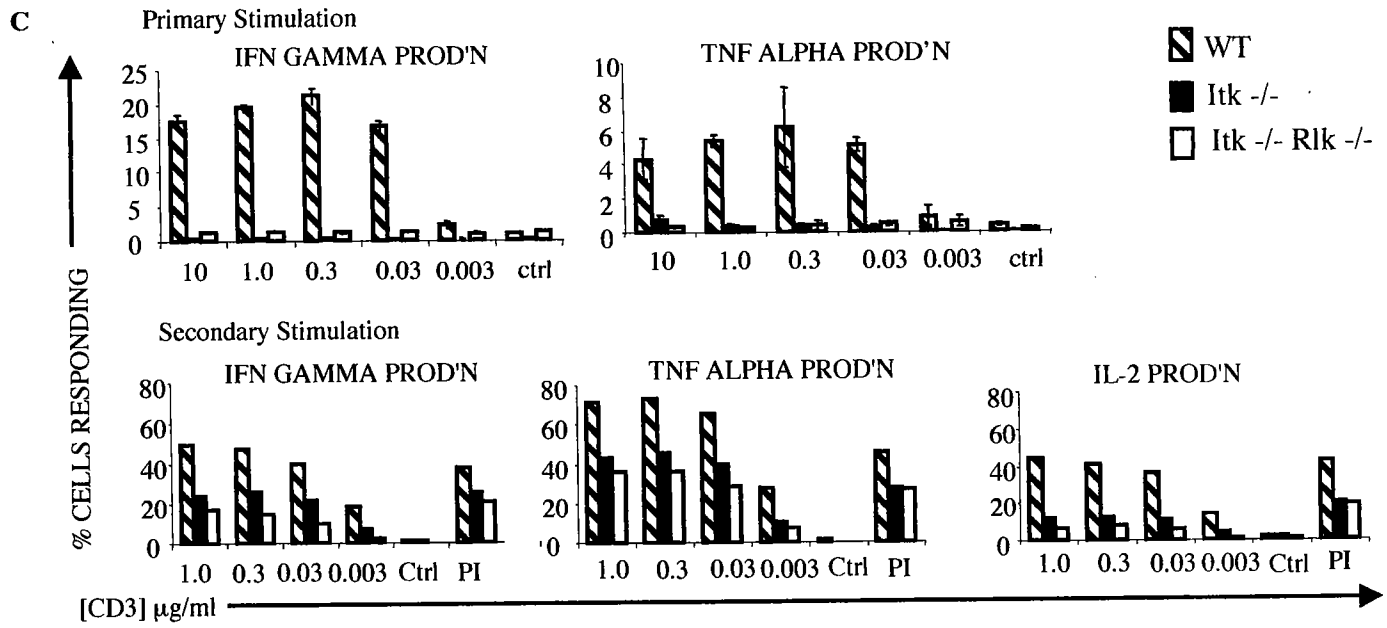
To determine if these signalling deficiencies translate into functional defects, I assessed the ability of CD8+ T cells from Itk-/- or Itk-/- Rlk-/- mice to proliferate and to produce effector cytokines in response to TCR stimulation. Figure 3.1C shows that freshly isolated CD8+ T cells from mice lacking Itk or Itk and Rlk are virtually unable to produce IFNγ or TNFα in response to TCR crosslinking. However, if the cells are stimulated with PMA and ionomycin, and then cultured for three days in excess IL-2, CD8+ T cells from both Itk-/- and Itk-/- Rlk-/- mice regain some functional responsiveness when restimulated. For example, as shown in Figure 3.1C, approximately 1 – 2% of the Itk -/- and Itk -/- Rlk -/- CD8+ T cells stimulated directly following isolation respond by making IFNγ and TNFα. However, following secondary stimulation, approximately 18 – 25 % of the Itk -/- and Itk -/- Rlk -/- CD8+ T cells are capable of making IFNγ and TNFα. Next, I looked at the ability of CD8+ T cells from the Itk -/- and Itk-/- Rlk -/- mice to undergo proliferation. I measured this ability using CFSE labelling which, due to the halving of the dye content of the cells with every round of cell division, allows the proliferation history of the cells to be visualized. As can be seen in Figure 3.1D, the CD8+ CD44hi cells from the Itk -/- and Itk -/- Rlk -/- CD8+ mice are more impaired at proliferation in response to TCR stimulation, as compared to WT CD8+ CD44hi T cells (Figure 3.1D). So, removal of Itk and Rlk from the TCR signalling cascade profoundly impairs CD8+ T cell function.

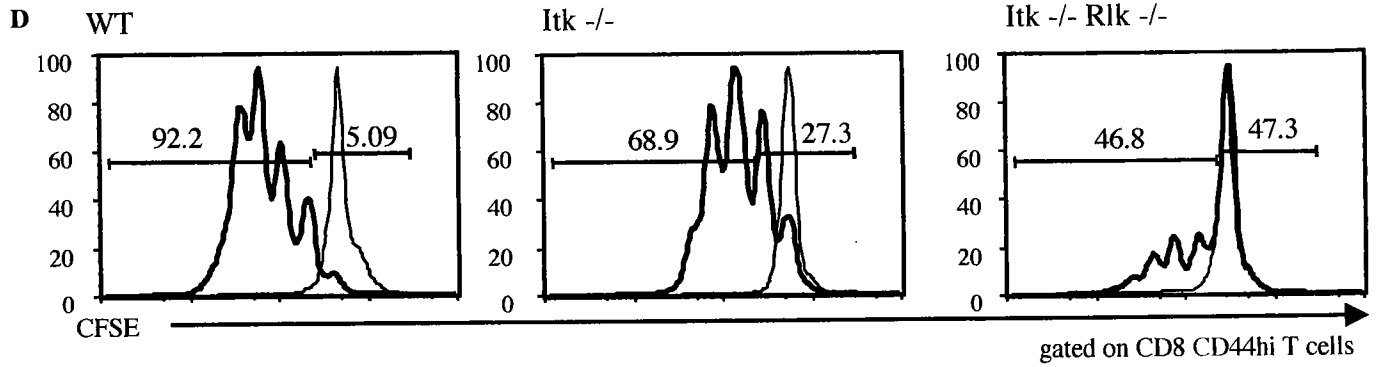
Figure 3.1 2: CD8+ T cell signalling and function.

(C) CD8+ T cells were stimulated with the indicated concentrations of plate-bound anti-CD3 and cytokine secretion analysed by intracellular cytokine staining as detailed in Materials and Methods. For primary stimulation, CD8+ T cells were activated immediately following isolation. For secondary stimulation, the isolated CD8+ T cells were first stimulated with PMA and Ionomycin, and then expanded in RPMI-10 containing IL-2, before being restimulated and assessed for effector function.

(D) Cells were labeled with 1uM CFSE and then stimulated $\pm$ 10ug/ml plate-bound anti-CD3 for 48hrs. The CFSE content of CD8+ T cells was then assessed by FACS Analysis.

IMPAIRED SIGNALLING DOWNSTREAM OF TCR CROSS-LINKING IN Itk -/- and Itk -/- Rlk -/- CD8 T CELLS





The Kinetics of LCMV clearance is delayed in Itk-/- and Itk-/-/Rlk-/- mice

To determine whether loss of Itk and the cumulative loss of both Itk and Rlk would also disrupt CD8+ T cell function in-vivo, I utilized the well-characterized cellular response system of LCMV infection. Viral infection of healthy C57BL/6 mice with LCMV Armstrong results in robust replication of the virus by day 2 post-infection and subsequent clearance of the virus five days hence. The clearance of LCMV is mediated largely by the perforin and granzyme cytolytic mechanisms of virus specific CD8+ T cells (44, 139). The activation of these cytolytic mechanisms is reportedly influenced by the generation of a sustained calcium flux downstream of TCR signalling in CD8+ T cells (139, 140). Since loss of Itk and Rlk has a substantial impact on the generation of a calcium signals in CD8+ T cells in-vitro, I first asked whether loss of these proteins would affect the ability of CD8+ T cells from these mice to mount an antiviral immune response and mediate viral clearance. To do this I infected Itk -/- and Itk-/- Rlk-/- mice intraperitoneally (i.p.) with approximately 4 x 10^4 PFU of LCMV Armstrong, and looked at a time-course of viral replication and clearance by plaque assay analysis of viral supernatants collected from the spleens of infected mice at various days post-infection.

As can be seen in Figure 3.2A, at two days post-infection, viral titres in the spleens of WT mice averaged 5.3 $\pm$ 0.58 log pfu/ml. In comparison, viral titres in the spleens of day 2 infected Itk -/- and Itk-/- Rlk-/- mice are approximately five-fold (4.8 $\pm$ 0.62 log pfu/ml) and ten-fold (4.3 $\pm$ 0.97 log pfu/ml]) lower respectively. However, this difference disappears one day later as viral titres in the spleens of Itk -/- mice are similar to that seen in the spleens of WT mice by day three post-infection and like the titres in

WT mice, peaks at day four post-infection. Viral titres in the spleens of Itk-/- Rlk-/- mice peak one day later, at day five post-infection. In addition to the slight delay in viral replication, Itk -/- and Itk -/- Rlk -/- mice have a slight delay in clearance of LCMV as compared to WT mice. While viral titres in the spleens of WT mice undergo an almost 2 log decline by day six post infection, the viral titres in Itk -/- and Itk -/- Rlk -/- spleens are only ten-fold and five-fold reduced respectively as compared to that of WT mice at this timepoint. Despite these differences in viral replication and clearance, like WT mice, the spleens of Itk -/- mice are completely clear of LCMV by day eight post-infection. However, some virus is still detectable at day eight post infection in the spleens of Itk -/- Rlk -/- mice.

Since the clearance of LCMV is largely perforin dependent, this result suggested that WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells may have similar cytolytic activity. To test this hypothesis a chromium release assay was done using gp 33 (an MHC Class I restricted immunodominant LCMV epitope) loaded RMA target cells and splenocytes from day eight infected mice at various E: T ratios. As shown in Figure 3.2B, splenocytes from day eight infected Itk -/- and Itk -/- Rlk -/- mice are able to clear infected targets as well as WT splenocytes in the timeframe tested. As another approach to this question, I looked at the ability of Itk -/- and Itk -/- Rlk -/- mice to clear antigen specific target cells in-vivo at day eight post-infection. Naïve splenocytes were loaded with gp 33 or left unloaded to serve as controls and were labelled with 0.3 μM and 0.9 μM CFSE respectively. They were then mixed in a 1:1 ratio and adoptively transferred into day eight infected WT, Itk -/- and Itk -/- Rlk -/- and uninfected control mice for five

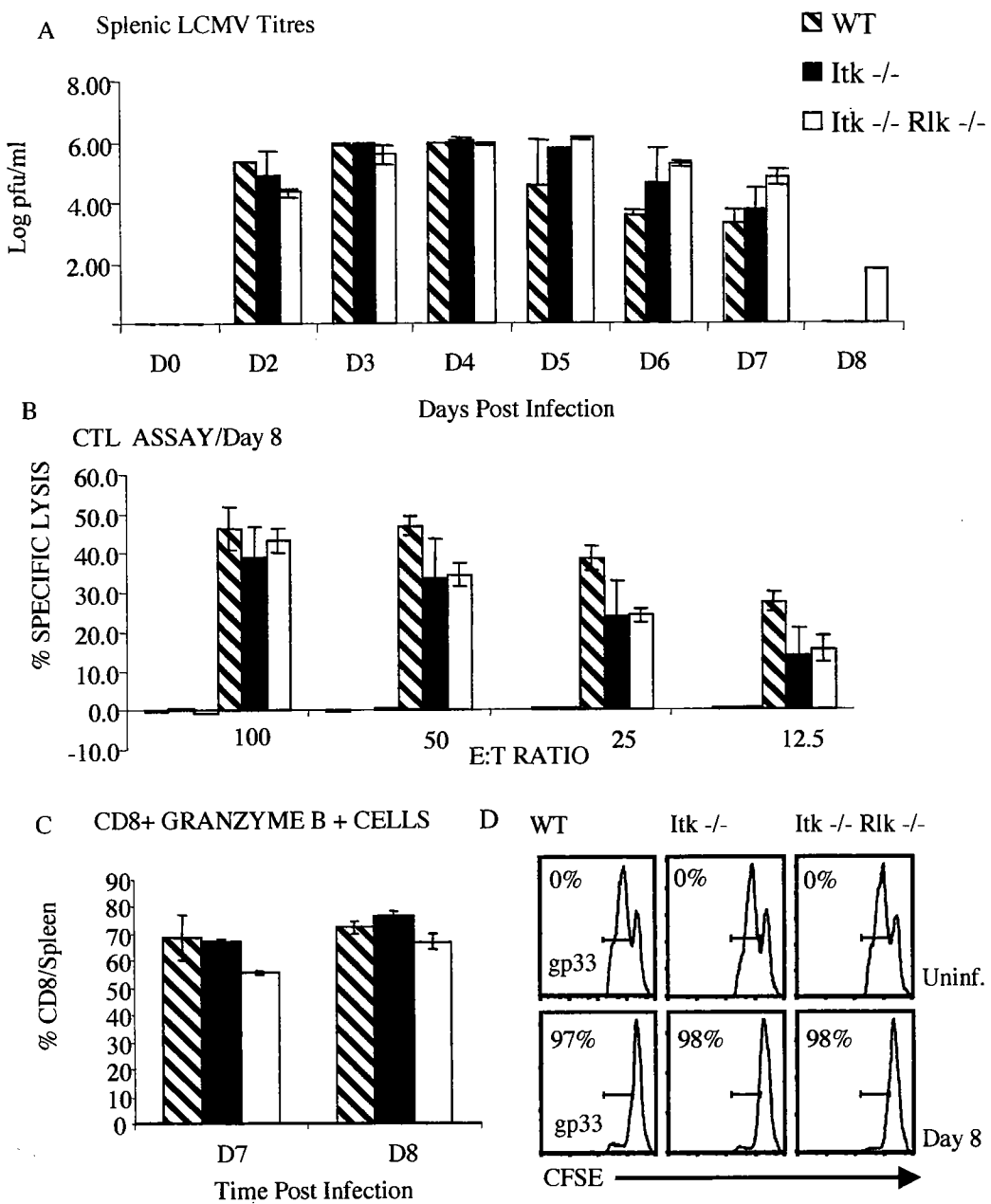
hours. WT, Itk -/- and Itk -/- Rlk -/- splenocytes were able to clear an equal proportion of target cells in an antigen-specific manner (Figure 3.2C).

These results, along with an analysis of the granzyme B content of WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells day eight post-infection, which showed that Itk -/- and Itk -/- Rlk -/- mice generate a similar frequency of granzyme B+ cells as WT LCMV infected mice at the peak of the immune response, suggest that despite the impairment of the calcium signal seen in in-vitro analyses, Itk -/- and Itk -/- Rlk -/- CD8+ T cells no defect in their cytolytic abilities. These results suggested that the delay in viral clearance seen in the spleens of Itk -/- and Itk -/- Rlk -/- mice was not due to an impairment in the cytolytic activity of the CD8+ T cells from these mice, but were potentially due to other defects in the CD8+ T cell response in the absence of Itk and both Itk and Rlk such as an impairment in CD8+ or CD4+ T cell numbers.

Figure 3.2: Viral Replication and Viral Clearance.

(A) For analysis of viral replication, viral supernatants were generated from infected spleens harvested at various times post LCMV infection. For assessment of viral titres, an aliquot of viral supernatant was thawed, serially diluted and titres assessed by plaque assay as described in the Materials and Methods. The results shown are the average of the log $\pm$ SD of titres obtained from the infected spleens of individual mice. 4 mice were used for the D2 timepoint, while 2 mice were used for the remaining timepoints. WT mice are depicted by the striped bars, while Itk-/- and Itk-/- Rlk-/- mice are represented by the solid black and the white bars respectively. (B) 1 x 10^4 gp 33 loaded RMA target cells were incubated with splenocytes from day eight LCMV infected mice at various E: T ratios in a 96 well plate. The cells were incubated for 5 hrs at 37°C and supernatant removed for analysis of ^{51}Cr release. This is one representative experiment of three. (C) The granzyme B content of WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells from day 8 post infection was assessed by intracellular stain analysis. (D) Splenocyte suspensions from WT uninfected animals were labelled with 0.3 μM and 0.9 μM CFSE and then loaded with 1μm of gp 33 or a reference peptide respectively. The labelled cells were mixed at a 1:1 ratio and injected into either WT, Itk -/- or Itk -/- Rlk -/- mice at day eight post-infection or uninfected controls. Spleens were harvested 5 hrs after injection and specific killing assessed. This experiment was done once with two mice per timepoint.

IMPAIRED VIRAL REPLICATION AND CD8 T CELL CYTOTOXICITY IN RESPONSE TO LCMV INFECTION



Impaired Accumulation of CD8 T cells from Itk-/- and Itk-/-Rlk-/- in response to LCMV infection

To investigate the impact of loss of Itk and both Itk and Rlk on the CD8+ T cell response, I followed the T cell response in WT, Itk -/- and Itk -/- Rlk -/- mice over a time course of LCMV infection. Murine LCMV infection causes a characteristic and reproducible expansion of mainly CD8+ T cells to generate a pool of antigen-specific effectors (32, 141). This peak of expansion is followed by an equally characteristic and reproducible decline in effector CD8+ T cell numbers and in the generation of LCMV specific memory CD8+ T cells.

To examine expansion of the CD8+ T cell subset in response to infection, the spleens of WT, Itk -/- and Itk -/- Rlk -/- were harvested at various days post infection. The size of the CD4+ and CD8+ T cell compartments was determined by quantitation of total splenocyte numbers and percentages of CD4+ and CD8+ T cells present at various days post-infection, by staining with anti-CD4 and CD8 antibodies and subsequent FACS analysis. Total cell numbers/spleen was calculated from the product of the percentage and total cell numbers. Interestingly, in Itk -/- and Itk -/- Rlk -/- mice, the magnitude and the kinetic of accumulation of the CD8+ T cell subset was impaired in response to infection as compared to that of WT mice (Figure 3.3). The WT CD8+ T cell response peaks at about d8 post infection, with about 28.7% of the spleen being CD8+. At this time-point, CD8+ T cells make up approximately 18% of the spleen in Itk-/- mice, and 22% of the spleen in Itk-/- Rlk-/- mice. These percentages correspond to approximately 1.0×10^8, 2.9×10^7 and 3.7×10^7 CD8 T cells per spleen respectively. CD8+ T cell

numbers in Itk -/- and Itk-/- Rlk-/- mice peak instead at about day 9 – day 10 post-infection (Figure 3.3). Interestingly, and in keeping with observations previously made by James M. McNally, at day three post-infection, there is a greater reduction in the frequency of the CD8+ T cells in the spleens of the Itk -/- and Itk -/- Rlk -/- mice as compared to that of WT mice. As only CD8+ CD44hi cells are lost at this point, this is likely due to the fact that most of the CD8+ T cells in the spleens of Itk -/- and Itk -/- Rlk -/- mice are CD44hi. Despite the delay in the accumulation of CD8+ T cell numbers in Itk -/- and Itk -/- Rlk -/- mice, attrition of the CD8+ T cell response appears to occur at the same rate in Itk -/- and Itk-/- Rlk-/- mice. In Figure 3.3B, the total spleen cell counts are tabulated, followed by tabulation of the total numbers of CD8+ T cells responding over the course of the infection in WT, Itk -/- and Itk -/- Rlk -/- mice.

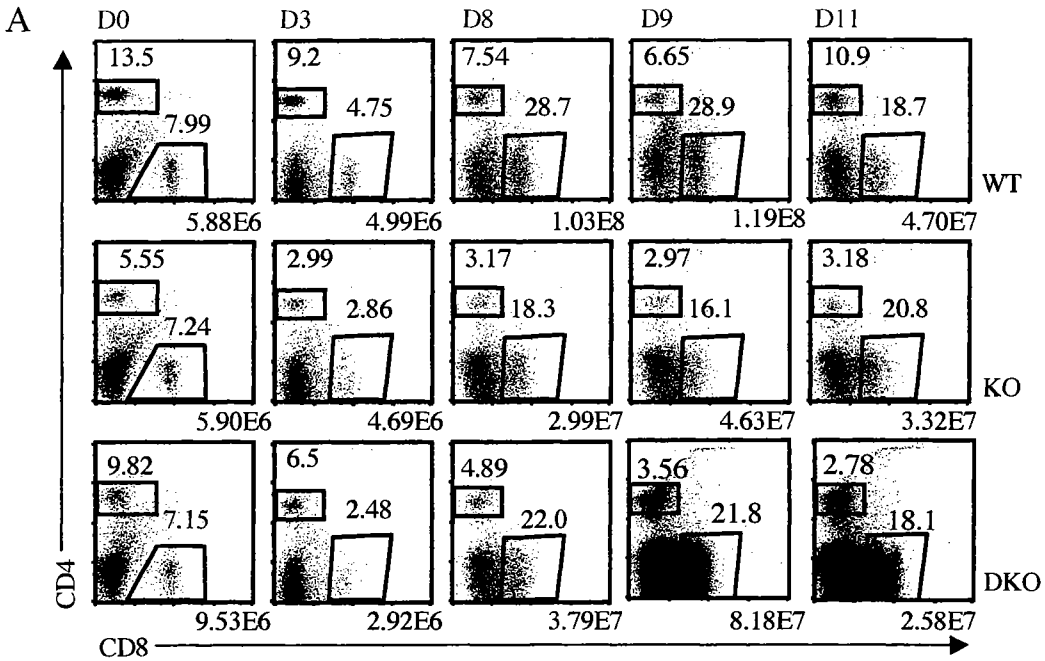
These results showed that the loss of Itk and both Itk and Rlk impaired the ability of CD8+ T cells to mount an optimal response to infection with LCMV, particularly in their ability to accumulate following activation. Given this interesting kinetic delay and decreased magnitude of the CD8+ T cell compartment in Itk -/- and Itk -/- Rlk -/- mice, I next looked at the antigen specific response to LCMV infection.

Figure 3.3: The CD8+ T Cell Response in WT, Itk -/- and Itk -/- Rlk -/- Mice

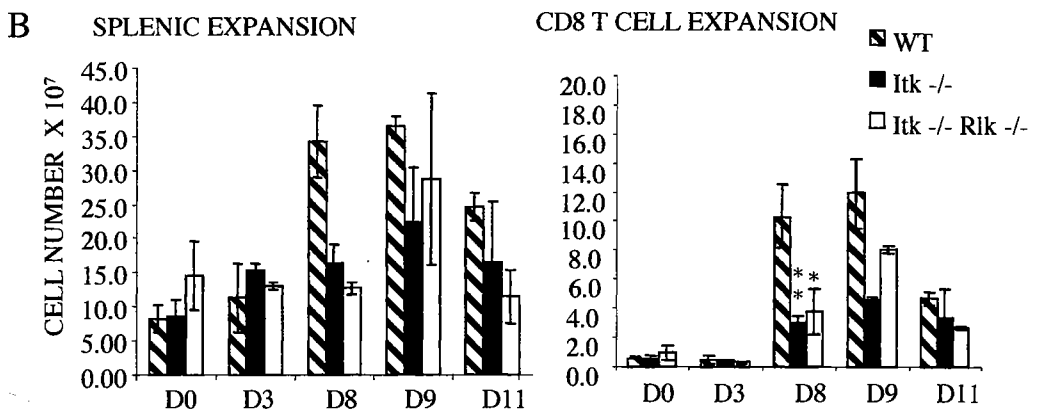
(A) WT, Itk -/- and Itk-/- Rlk-/- mice were infected intraperitoneally (IP) with 4 x 10^4 PFU of LCMV Armstrong. The kinetics of the cellular response was followed by the assessing the percent of CD4+ and CD8+ T cells in the spleens of infected mice over the course of the infection. CD8+ T cell numbers are noted. This experiment is representative of nine such experiments looking at the T cell response to LCMV infection in WT, Itk-/- and Itk-/- Rlk-/- mice.

In (B), the total spleen numbers and the numbers of CD8+ T cells responding are quantitated. Results shown are the averages of the total number of spleen cells and total numbers of CD8+ T cells/spleen +/- standard deviation respectively for one experiment of five. Statistics done using the Kruskal-Wallis and Dunn's Multiple Comparisons Test on data from five separate experiments indicated that the difference in total CD8+ T cell numbers between WT and Itk -/- mice ($P < 0.0001$) and between WT and Itk -/- Rlk -/- mice ($P < 0.05$) were statistically significant at day eight and day nine post-infection.

IMPAIRED CD8+ T CELL RESPONSE TO LCMV INFECTION IN WT, Itk -/- AND Itk -/- Rlk -/- MICE



IMPAIRED WT, Itk -/- AND Itk -/- Rlk -/- CD8 T CELL ACCUMULATION IN RESPONSE TO LCMV INFECTION



The Antigen Specific Response to LCMV is Impaired in Itk -/- and Itk-/- Rlk-/- mice

LCMV infection results in the amplification of CD8+ T cells specific for the MHC 1 restricted immunodominant epitopes gp 33 and np 396. T cells are also generated to the subdominant epitopes gp 276 and np 205 among others (41). The epitope specific response to LCMV has been extensively characterized, and the combination of tetramer staining and intracellular cytokine staining has proven powerful in allowing the dynamics of the LCMV antigen specific response to be followed at a single cell level (32, 141). To look at antigen specific responses in WT, Itk -/- and Itk -/- Rlk -/- mice, splenocytes from infected mice were stimulated with epitope specific peptides and the antigen specific response followed by analysis of the generation of cells producing the effector cytokine IFNγ.

As shown in Figure 3.4A, splenocytes from Itk -/- and Itk-/- Rlk-/- mice generate a slightly smaller frequency of IFNγ+ CD8+ T cells in response to stimulation with the immunodominant peptides np 396 and gp 33. Specifically, in this experiment, approximately 13.6% of CD8+ T cells in the spleen of WT mice make IFNγ in response to gp 33 stimulation while Itk -/- mice have about 10% and Itk-/- Rlk-/- mice about 6% IFNγ+ CD8+ T cells (Figure 3.4). The same is true of the stimulation with the subdominant peptides np 205 and gp 276 (Figure 3.7). The CD8+ T cells from Itk -/- and Itk -/- Rlk -/- mice are also less efficient at making the effector cytokine IFNγ as shown by analysis of the median fluorescent intensity (MFI) of the cytokine producing cells (Figure 3.4A). The impact of the absence of Itk and Itk and Rlk on the antigen-specific response to infection is further illustrated when the reduction in the percentages of

antigen specific cells responding to peptide stimulation is combined with the lowered magnitude of the CD8+ T cell response. Itk -/- and Itk-/- Rlk-/- mice have a significant reduction in their overall antigen-specific response to LCMV infection as compared to WT mice. These data showed, that like the in-vitro analyses, loss of Itk and both Itk and Rlk had a significant impact on the ability of T cells to respond to TCR stimulation, showing that these TEC kinase family members are important for the generation of an optimal CD8+ T cell mediated immune response (Figure 3.4B).

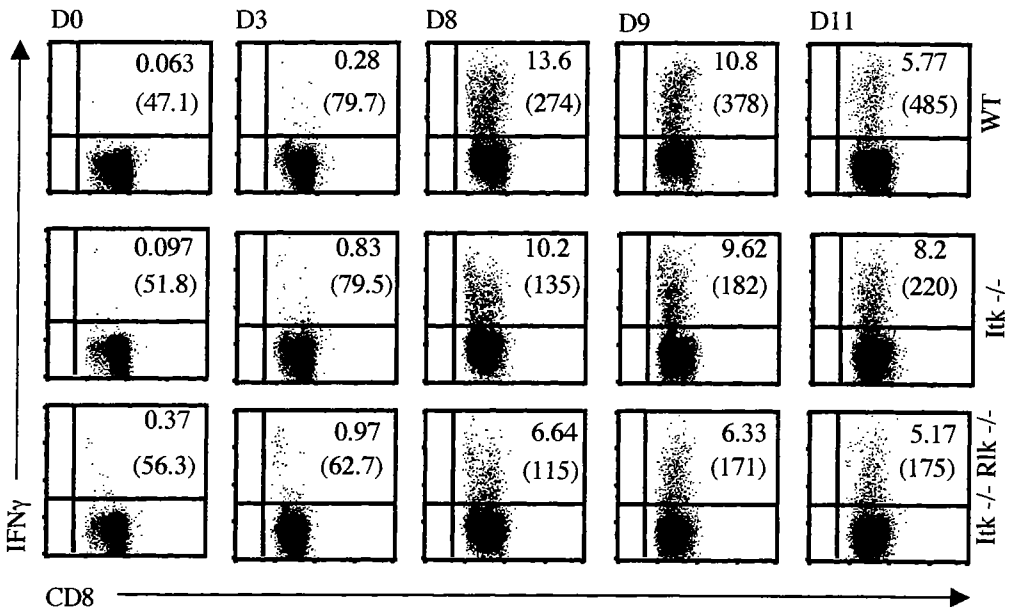
Figure 3.4: The LCMV Antigen Specific Response

(A) The antigen specific response was assessed by intracellular cytokine staining. $2-4 \times 10^6$ splenocytes from WT, Itk-/- and Itk-/- Rlk -/- mice were incubated with the immunodominant LCMV specific epitopes np 396 and gp 33 for 5 hrs at 37°C, in medium containing Brefeldin A and Monensin. Following incubation, cells were spun down and stained first for surface expression of CD4 and CD8 and then intracellularly for IFNγ. The np396 IFNγ response is shown.

(B) Quantitiation of the antigen specific IFNγ response to stimulation with np 396 and gp 33 over a time course of LCMV infection.

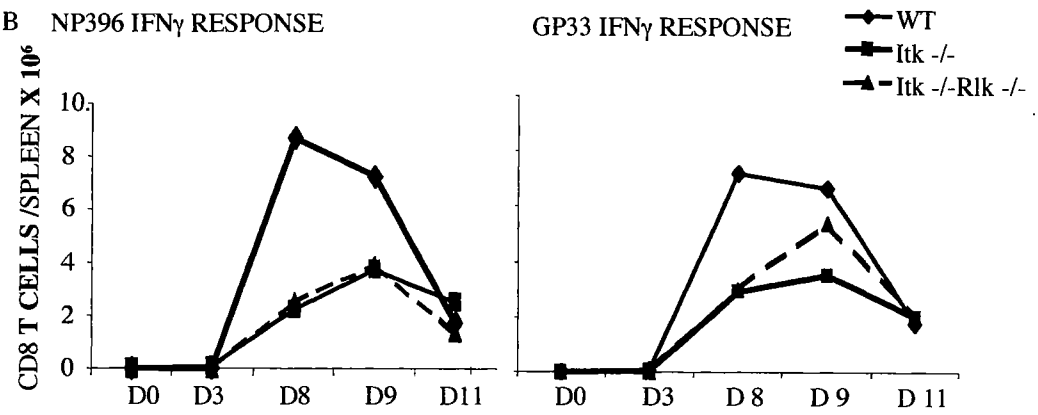
THE ANTIGEN SPECIFIC RESPONSE OF WT. ITK -/- AND ITK -/- RLK -/- CD8 T CELLS TO LCMV INFECTION

A NP 396 PEPTIDE STIMULATION



B NP396 IFNγ RESPONSE

GP33 IFNγ RESPONSE



TIME POST INFECTION

Tetramer positive CD8+ T cells from Itk -/- and Itk-/- Rlk-/- are impaired at production of effector cytokine

The data show that Itk and Rlk are important in the transduction of signals downstream of TCR cross-linking that regulate both T cell responsiveness and the activation of factors needed for the transcription of cytokine genes including IFNγ. This impacted the antigen specific response by impairing the accumulation of CD8+ T cells following infection and the ability of the T cells to make effector cytokines.

The impaired accumulation of Itk -/- and Itk -/- Rlk -/- antigen specific CD8+ T cells, instead of being due to the impaired TCR responsiveness of CD8+ T cells in the absence of both Itk and Rlk, may alternatively be due to a reduction in the precursor frequency of CD8+ T cells capable of responding to LCMV. To distinguish between these possibilities, I analyzed the np 396 and gp 33 antigen specific response by tetramer staining and compared this to the frequencies of IFNγ and TNFα antigen specific cells detected by intracellular cytokine staining following stimulation of the infected splenocytes with the corresponding peptide epitopes. The technology now available for the analyses of the antigen specific response, i.e. tetramer staining and intracellular cytokine staining, has been shown to detect similar frequencies of antigen specific cells (32). As such, these two technologies can be used interchangeably in WT mice to look at the precursor frequencies of T cells responding to an immune stimulus.

The data show that at eight days post-infection, the frequency of gp 33 and np 396 tetramer positive antigen specific CD8+ T responding to LCMV infection in the Itk-/- and Itk-/- Rlk-/- mice are comparable to those seen in WT mice (Figure 3.5). However,

there are fewer total numbers of gp 33 and np 396 specific CD8+ T cells in the spleens of Itk -/- and Itk -/- Rlk -/- mice as compared to WT mice, possibly due to the impaired accumulation of these cells in the Itk -/- and Itk -/- Rlk -/- mice. Furthermore, upon stimulation of the Itk -/- and Itk -/- Rlk -/- CD8+ T cells with the corresponding gp 33 and np 396 peptides respectively, fewer Itk -/- and Itk -/- Rlk -/- CD8+ T cells were capable of making the effector cytokines IFNγ and TNFα than were detected by tetramer stain analysis. Although fewer total effector cytokine producing cells are also generated from stimulation of WT infected splenocytes, the difference is far smaller than in the Tec kinase knockout samples (Figure 3.5).

Extrapolating from these data suggest that the initial repertoire of cells capable of responding to LCMV is similar in the spleens of uninfected Itk -/- and Itk -/- Rlk -/- mice, and that the accumulation of antigen specific CD8+ T cells is impaired in Itk -/- and Itk-/- Rlk-/- mice. In addition, fewer of the antigen specific cells that do accumulate in Itk -/- and Itk -/- Rlk -/- mice are able to be activated to make an effector cytokine response to infection, suggesting that the ability of CD8+ T cells to respond to antigen stimulation is impaired in the absence of Itk and both Itk and Rlk. This experiment also demonstrated that, while WT mice were able to generate a cells making IFNγ (single – producers) as well a fraction of cells making both IFNγ and TNFα (double –producers), very few Itk -/- and Itk -/- Rlk -/- CD8+ T cells could make TNFα during the acute LCMV response.

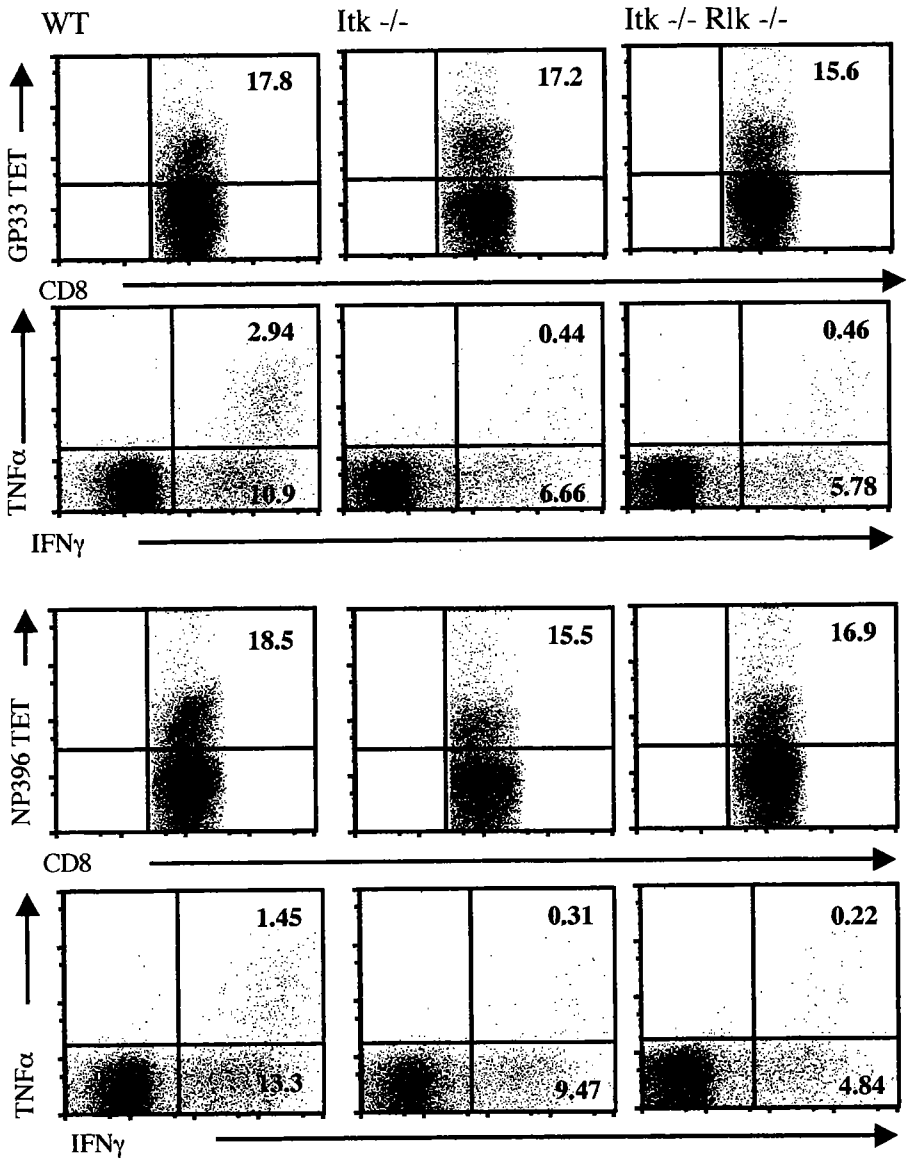
This result led to experiments to determine whether the impaired accumulation of Itk -/- and Itk -/- Rlk -/- CD8+ T cells during the course of LCMV infection might be due

either to defects in CD8+ T cell proliferation and or survival in the absence of Itk and both Itk and Rlk.

Figure 3.5: Impaired Cytokine Response of Antigen Specific CD8+ T Cells

The frequency of the antigen specific response as measured by tetramer staining and intracellular cytokine staining was compared. Tetramer stain analysis was done on splenocytes from day eight infected mice as described in the Materials and Methods. Intracellular cytokine staining was done on separate samples from the same mice. Results shown are the percent of total CD8+T cells that bind the gp 33 and np 396 tetramers and the cytokine profile generated in response to stimulation with the corresponding peptides. Results are one representative experiment of three. There were two mice per group.

IMPAIRED CYTOKINE PRODUCTION BY LCMV SPECIFIC CD8+ T CELLS IN Itk -/- AND Itk -/- Rlk -/- mice



Loss of Itk and Rlk modulate CD8 T Cell Proliferation and Apoptosis during LCMV Infection

During acute LCMV infection, the spleens and CD8+ T cell compartments of Itk -/- and Itk-/- Rlk-/- mice do not expand to the size seen in WT mice (Figure 3.3). This did not seem to be due to a lower precursor frequency of antigen specific cells in the Tec kinase deficient mice (Figure 3.5). Generally, the massive expansion of CD8+ T cells that occurs during the LCMV infection is caused by extensive proliferation of antigen specific CD8+ T cells during the acute response. This proliferative capacity is very characteristic of CD8+T cells, and there is an extensive body of literature showing that CD8+ T cells proliferate profusely in response to stimulation with antigen, and that this initial stimulation is sufficient to sustain an autonomous program of clonal expansion and differentiation (37, 38, 142). We hypothesized that the impaired expansion of the CD8+ T cell compartment in Itk -/- and Itk-/- Rlk-/- mice could be caused by either an impaired proliferation of those cells in response to LCMV infection or by impaired survival of the CD8+ T cells in the Tec deficient mice. To this end, I first looked at CD8+ T cell turnover in-vivo, by assessing the ability of WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells to incorporate BrdU during a time course of LCMV infection

I used the activation marker CD44 to identify the fraction of activated CD8+ T cells proliferating during the acute response. Briefly, cells were gated on the CD8+ CD44hi population and then analyzed for BrdU incorporation (Figure 3.6A). The results show that early in the CD8+ T cell response, at approximately day 5 post infection, a smaller fraction of Itk -/- and Itk -/- Rlk -/- CD8 CD44hi T cells incorporate BrdU as

compared to WT CD8+ CD44hi T cells (Figure 3.6A). However, there was no statistical significance to the difference in BrdU incorporation seen between WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells.

Defects in CD8+ T cell survival during the primary immune response may also affect the overall numbers of CD8+ T cells accumulating at the peak of the response. I then asked whether activated CD8+ T cells in the Itk -/- and Itk-/- Rlk-/- mice were more susceptible to apoptosis during the acute LCMV response. To do this I analyzed the uptake of the vital dye 7-AAD and staining of the activated CD8+ T cell population with the apoptosis marker Annexin-V. To evaluate apoptosis of activated CD8 T cells, I divided ungated cells into 7-AAD+ and 7-AAD- populations. I then quantitated the 7AAD- CD8+ $CD44^{hi}$ Annexin V- T cell populations in WT, Itk -/- and Itk-/- Rlk-/- mice. I found that at the peak of the acute response, around day seven – day eight post infection, there were fewer live (7-AAD- Annexin V-) CD8+ $CD44^{hi}$ T cells in the spleens of Itk -/- and Itk-/- Rlk-/- mice as compared to WT. At day eight post LCMV infection, there was about an 80% /fourfold reduction in the numbers of CD8+ $CD44^{hi}$ Annexin V- 7AAD- T cells in Itk -/- compared to WT mice ($9.3 \times 10^7 \pm 7.0 \times 10^5$ in WT mice to $1.9 \times 10^7 \pm 2.2 \times 10^6$ cells in Itk -/- mice). The reduction in the Itk-/- Rlk-/- CD8+ T cell compartment was about 50%/twofold ($9.3 \times 10^7 \pm 7.0 \times 10^5$ in WT to $3.9 \times 10^7 \pm 5.5 \times 10^6$ in Itk-/- Rlk-/- mice) (Figure 3.6B). As a further determination of whether Itk -/- and Itk-/- Rlk-/- CD8+ $CD44^{hi}$ T cells were impaired in the activation of survival signals during acute LCMV infection, I looked at expression of the anti-apoptotic molecules Bcl-2 and Bcl-XL by flow cytometry in CD8+ T cells from WT, Itk -/- and Itk -/- Rlk -/- mice

at day eight post infection. I could detect no differences in intracellular protein expression of either Bcl-2 or Bcl-XL at day eight post LCMV infection in Itk -/- and Itk-/- Rlk-/- CD8+ CD44hi cells as compared to WT (Figure 3.6C). These data suggest that the defect in accumulation of Itk -/- and Itk -/- Rlk -/- CD8+ T cells at the peak of the LCMV immune response, may result from a combination of impaired proliferation early in the response (between day three and day five post-infection) and a defect in CD8+ T cell survival of cells at the peak of the response. However, since there was no statistical significance to the differences in BrdU incorporation seen between the ET, Itk -/- and Itk -/- Rlk -/- CD8+ T cells, the impaired accumulation of the CD8+ T cells seen in the absence of Itk and Rlk may be due more to differences in survival rather that proliferation. These observations will be the subject of future studies.

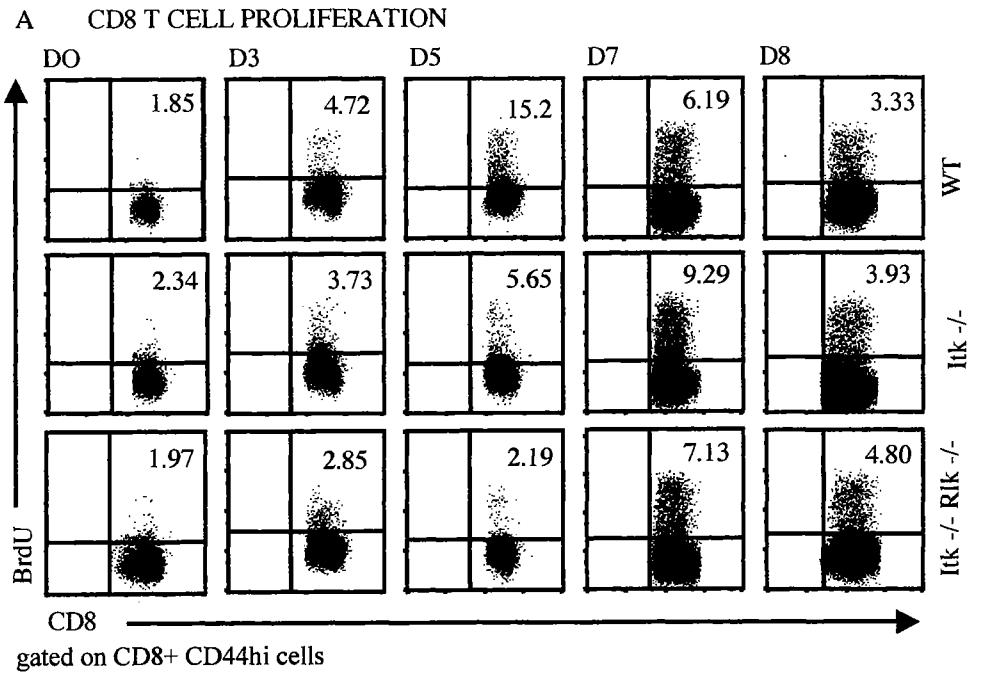
Figure 3.6: CD8+ T Cell Proliferation and Survival During LCMV Infection

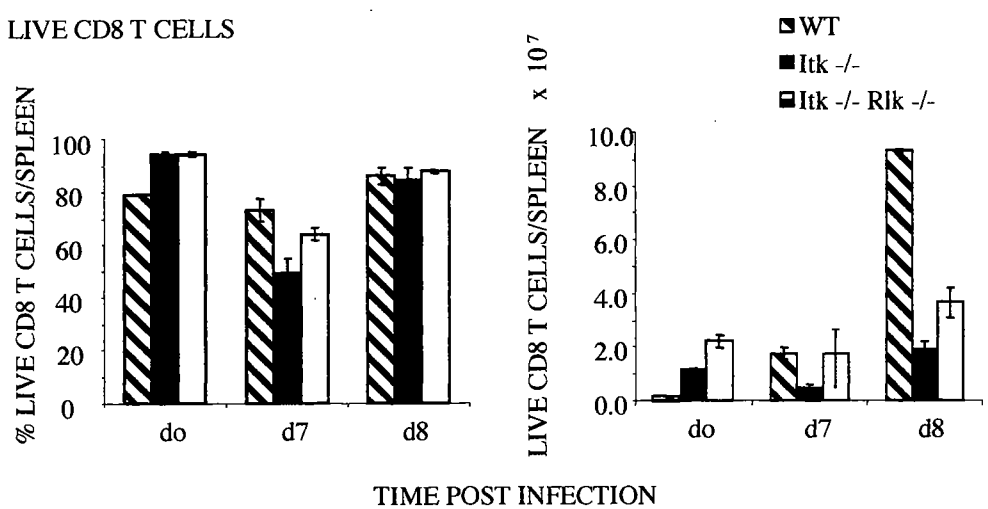
(A) CD8 T cell proliferation was analyzed by BrdU staining during a timecourse of LCMV infection. Mice were all infected at the same time, and infected mice were injected with 100λ of BrdU [15mg/ml in PBS] 12 hours before harvest on the indicated day of infection. 4 x 10^6 cells were stained for surface expression of CD8, CD4 and CD44 and BrdU incorporation was assessed by intracellular staining. This is one experiment of three with two mice per timepoint. There was no statistical difference in BrdU incorporation between WT, Itk -/- and Itk -/- Rlk -/- mice.

(B) To look at cell death during the immune response, splenocytes were stained extracellularly with antibodies to Annexin-V, CD4 or CD8, CD44 and also with the vital dye 7-Amino-actinomycin D (7-AAD). To assess cell survival, the fraction of 7-AAD-AnnexinV- CD8+ CD44hi cells was assessed. The kinetics of cell death during the course of an LCMV infection is shown. This result is representative of three experiments done to look at Annexin-V staining and one experiment looking at the double staining of T cells with Annexin-V and 7-AAD. There were two mice per timepoint.

(C) Splenocytes from uninfected and day eight LCMV infected WT, Itk -/- and Itk -/- Rlk -/- mice were stained extracellularly for expression of CD4, CD8 and CD44 and then intracellularly for expression of Bcl-2 or Bcl-XL.

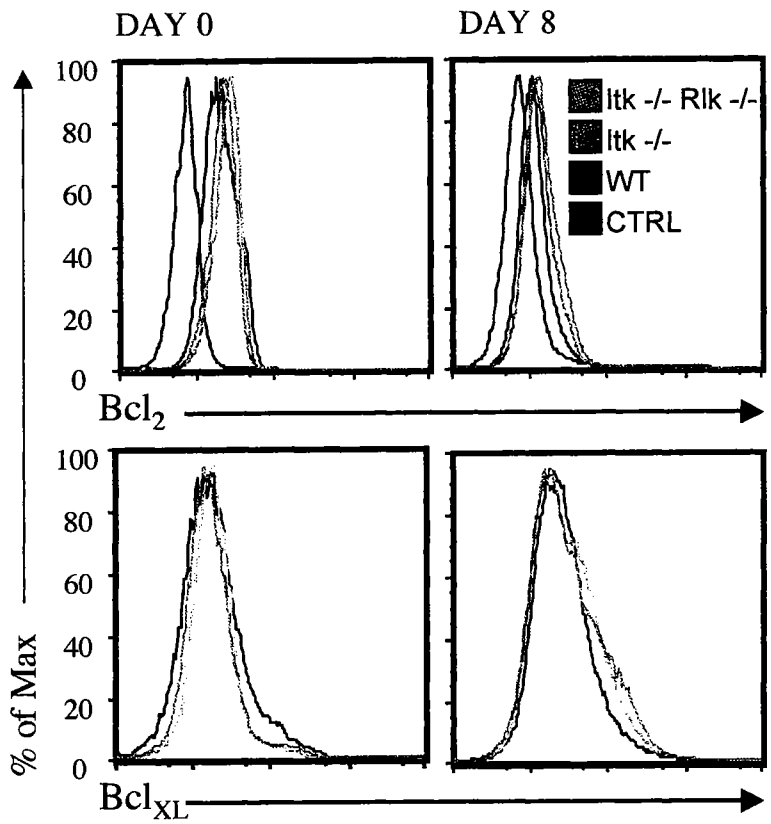
ALTERED CD8+ T CELL PROLIFERATION and CELL DEATH IN Itk -/- AND Itk -/- Rlk -/- CD8+ T CELLS





SIMILAR EXPRESSION OF BCL_2 AND BCL_{XL} IN WT, Itk -/- AND Itk -/- Rlk -/- CD8+ CD44HI T CELLS

C



gated on CD8+ CD44hi cells

Impaired CD4 T cell function in Itk -/- and Itk-/- Rlk-/- mice does not affect CD8 T cell Expansion

In addition to intrinsic defects in T cell fitness, the accumulation of CD8+ T cells following LCMV infection may also be affected by the quality of CD4+ T cell help provided during the acute immune response. The role of CD4+ T cells in the viral immune response in general, and the LCMV acute response in particular, has been a topic of much discussion and dissent. It has been shown that CD4+ T cells are dispensable for generation of an effective acute response to infection with LCMV (143-145), and for infection with vaccinia virus (146). Paradoxically, this is in spite of data demonstrating that significant numbers of CD4+ TH1 (IFNγ+ and IL-2+) type cells are generated in response to LCMV infection (40); that CD4+ T cells are the main producers of IL-2 during the anti-viral immune response (34) and that IL-2 is necessary for efficient expansion of the CD8+ T cell subset during viral infections (35, 147, 148). The CD4+ compartment is however necessary for the maintenance of viral clearance, for CD8+ cytolytic function during a chronic infection (149, 150) and for the generation of an effective memory CD8+ T cell response to secondary infection (145, 150-152).

As shown in Figure 3.7, CD4+ T cells in Itk -/- and Itk-/- Rlk-/- mice are deficient in their production of IL-2 (92, 93), and fewer CD4+ T cells in the spleens of Itk -/- and Itk-/- Rlk-/- mice produce IL-2 in response to LCMV infection. To determine whether a defective CD4+ T cell response in Itk -/- and Itk-/- Rlk-/- mice could account for the impaired expansion of the CD8+ T cell subset during the anti-viral response, I adoptively transferred 1 x 10^7 WT congenic LCMV memory CD4+ T cells into WT, Itk -/- or Itk-/-

Rlk-/- mice, (I used memory CD4+ T cells to ensure a high precursor frequency of LCMV specific CD4+ T cells). The mice were then infected with 4 – 5 x 10^4 pfu of LCMV Armstrong and host CD8+ T cell expansion analyzed eight days post-infection (Figure 3.8). Uninfected mice receiving donor memory CD4+ T cells only and mice infected with LCMV alone were used as controls.

Transfer of LCMV specific memory CD4+ T cells did not rescue the defect in expansion of the CD8 T cell compartment in host Itk -/- and Itk -/- Rlk -/- mice (Figure 3.7A). This was despite the massive expansion of the transferred memory CD4+ T cell population in these mice, and their secretion of wild-type levels of IL-2 in response to infection (Figure 3.7B). This result suggests that the defect in CD8+ T cell accumulation observed in the Itk -/- and Itk-/- Rlk-/- mice are not due to defects in the CD4+ TH mediated response in the Itk -/- and Itk -/- Rlk -/- mice. These data suggest instead that the defect in CD8+ T cell accumulation seen in Itk -/- and Itk -/- Rlk -/- mice are most likely due to defects intrinsic to the CD8+ T cells themselves, most likely defects in CD8+ T cell survival and/or CD8+ T cell proliferation during the primary immune response. However, statistical analyses done on the BrdU incorporation of Itk -/- and Itk -/- Rlk -/- CD8+ T cells showed no significance difference in BrdU incorporation as compared to WT CD8+ T cells, raising the issue that additional mechanisms may be plying a role in the impaired accumulation of CD8+ T ells in the Itk -/- and Itk -/- Rlk -/- mice.

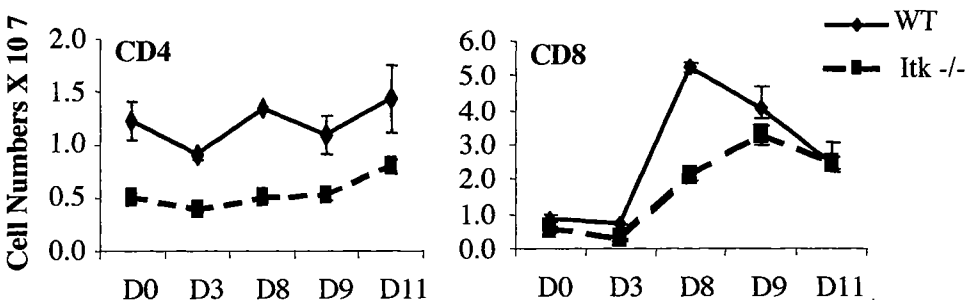
Figure 3.7: The CD4+ T Cell response in WT, and Itk -/- mice and the CD8+ Subdominant T Cell Response

(A) CD4 and CD8 T cell numbers over a timecourse of LCMV infection.

(B) The antigen specific IFNγ and IL-2 cytokine response to the immunodominant CD4+ epitope gp 61 as well as the subdominant epitope np 309. The CD8+ T cell IFNγ response to the subdominant epitopes np 205 and gp 92 are also shown.

CD4+ T_H Response and CD8+ Subdominant Response

A T Cell Response



B Antigen Specific Cytokine Response

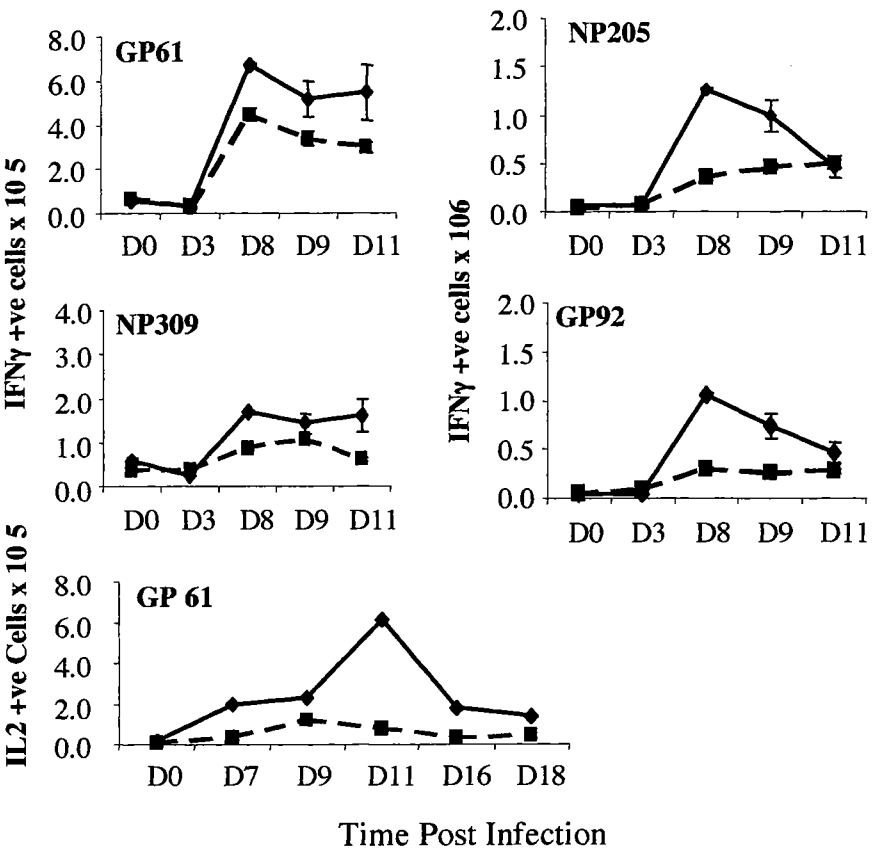


Figure 3.8: Adoptive Transfer of LCMV Memory CD4+ T Cells

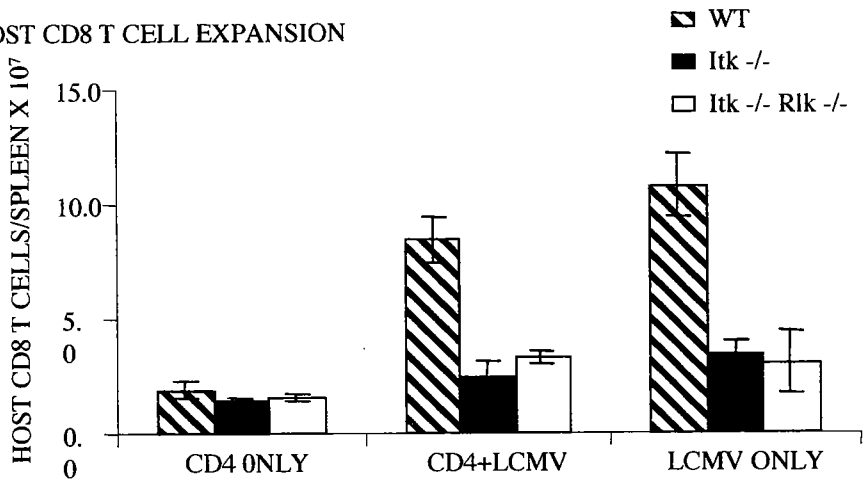
(A) 1 X 10^7 CD45.1 WT CD4+ memory T cells were injected intravenously into WT, Itk-/ or Itk-/- Rlk-/- mice. The host mice were then either infected with 4 - 5 X 10^4 PFU of the LCMV Armstrong strain, or left uninfected to serve as controls for engraftment of donor cells. Single cell suspensions were made from spleens harvested 8d post-infection and stained for CD4, CD8, CD45.1 and CD44. The expansion of the host CD8+ T cells from the kinase deficient mice was then analyzed. This is one of two experiments done with two mice per timepoint.

(B) Host and donor CD4+ cells were stimulated with gp 61 (an immunodominant LCMV CD4+ T cell epitope) to assess IL2 production, and the extent of the TH response supplied.

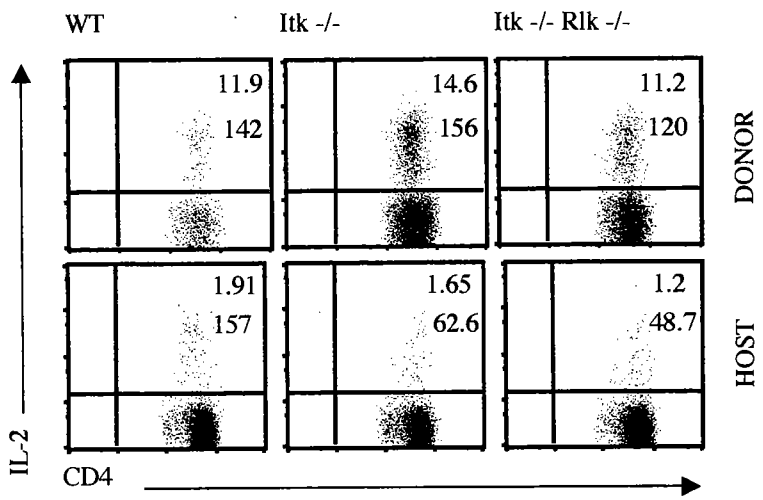
IMPAIRED ACCUMULATION OF CD8+ T CELLS IN Itk -/- AND Itk -/- Rlk -/- MICE IS CD4 TH INDEPENDENT

ADOPTIVE TRANSFER OF CONGENIC WT MEMORY CD4 T CELLS INTO WT, Itk -/- AND Itk -/- Rlk -/- MICE

A HOST CD8 T CELL EXPANSION



B DONOR AND HOST CD4 T CELL IL-2 SECRETION TO GP61 PEPTIDE STIMULATION



Impaired Accumulation of Antigen specific Transgenic Cells in Response to Infection

The data show that removal of Itk and both Itk and Rlk from the TCR signalling cascade diminishes the magnitude of CD8+ T cell accumulation in response to infection. However, given the unique phenotype of the Itk -/- and Itk -/- Rlk -/- CD8+ T cells, there is still potential for factors extrinsic to CD8+ T cell signalling to influence CD8+ T cell accumulation. For example, loss of a significant portion of CD8+ CD44hi T cells early in LCMV infection may affect the precursor frequencies of LCMV responsive cells in the Itk -/- and Itk -/- Rlk -/- repertoire. In addition, the environment of infected Itk -/- and Itk -/- Rlk -/- mice may be substantially different from that of WT mice undergoing a similar immune response, given the poor effector cytokine producing properties of both the CD4+ and CD8+ T cell populations in the Itk -/- and Itk -/- Rlk -/- mice. To address these issues, I decided to analyse the response of Itk -/- CD8+ T cells using the well-characterized OT-I transgenic adoptive transfer system.

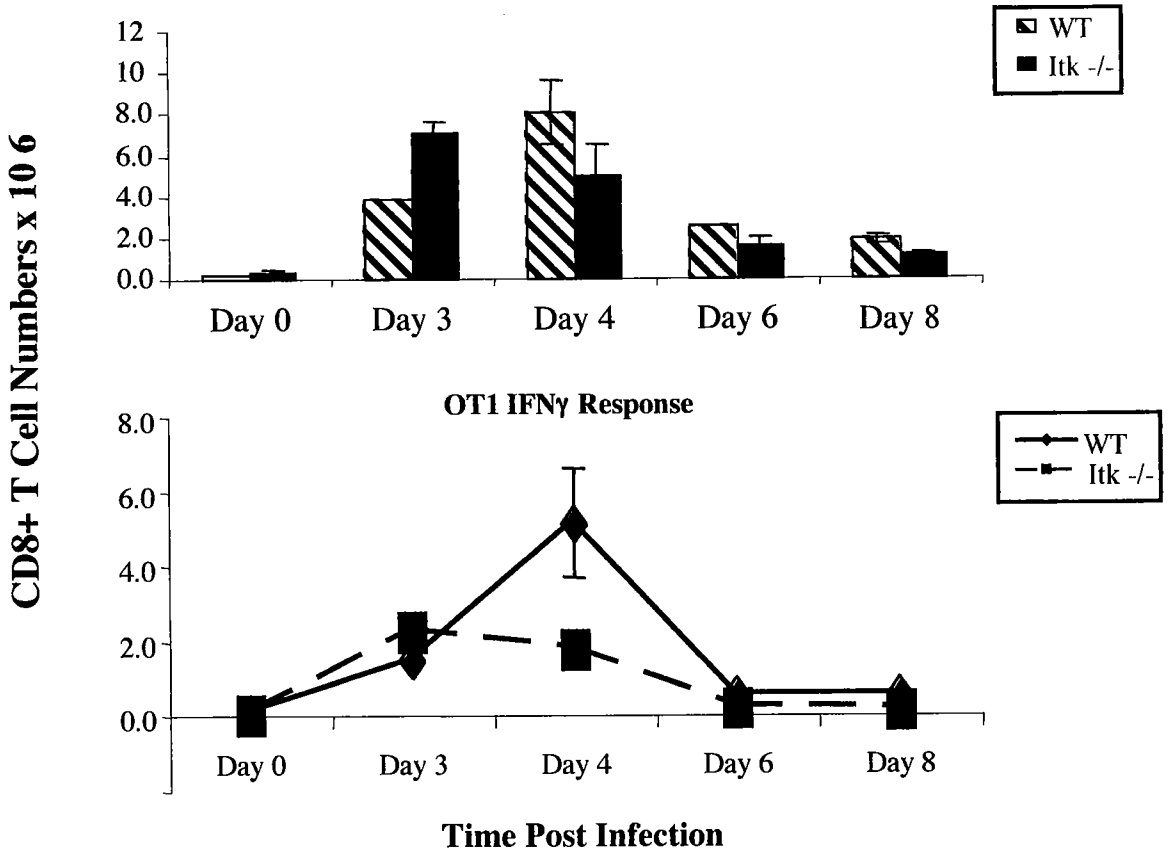
5 x 10^6 OT-1 WT and Itk -/- CD8+ T cells were adoptively transferred into WT CD45.1 congenic mice. The host mice were infected one day post transfer with 1 x 10^7 pfu of the recombinant vaccine construct containing the OT-1+ CD8+ T cell specific epitope SINFEKL (Vaccinia –OVA). The accumulation of CD8+ T cells and the antigen specific effector cytokine responses were assessed at various times post infection. As shown in Figure 3.8, loss of Itk -/- impaired the sustained accumulation of the transgenic CD8+ T cells at the peak of the response, which occurred approximately four days post infection with the Vaccinia-OVA construct. In addition to the defect in CD8+ T cell accumulation, the Itk -/- OT-1+ CD8+ T cells were also impaired in their ability to

produce the effector cytokines IFNγ and TNFα during the course of the response. This result demonstrates that even in a WT environment and given similar precursor frequencies of responding cells, loss of Itk impairs the ability of the CD8+ T cell to mount an efficient immune response to infection. These data show that loss of Itk and both Itk and Rlk impairs the intrinsic ability of CD8+ T cells to mount an optimal antigen specific immune response to infection, demonstrating the importance of these Tec kinases in CD8+ T cell signalling and function.

Figure 3.9: Impaired Accumulation of Itk -/- OT1+ T Cells in Response to Infection

5 X 10^6 WT and Itk -/- CD8+ CFSE labelled T cells were adoptively transferred into WT CD45.1 congenic hosts, and mice infected intraperitoneally with 1 X 10^7 pfu of the recombinant Vaccinia-OVA construct. (A) Accumulation of responding CD8+ T cells and the antigen specific IFNγ response (B) are shown. This is the result of one experiment done with two or three mice per timepoint.

IMPAIRED ACCUMULATION OF OTI+ Itk -/- CD8+ T CELLS IN RESPONSE TO INFECTION WITH VACCINIA-OVA



Itk -/- and Itk-/- Rlk-/- mice have an altered cytokine profile during the Memory Immune Response

The last phase of the anti-viral immune response is the generation of memory CD8+ T cells, and the generation of an efficient memory response has been shown to be affected by the magnitude of the acute response (153). To determine whether a deficiency in Itk or in both Itk and Rlk, and the impaired accumulation of CD8+ T cells seen in the acute response in the absence of these kinases would affect the establishment of effective long-term memory, I looked at the gp 33 and np 396 epitope specific responses of LCMV immune mice or LCMV immune mice that were re-challenged with $4-5 \times 10^4$ pfu of LCMV Armstrong.

In both Itk -/- and Itk -/- Rlk -/- immune mice, and in similarly deficient LCMV immune mice undergoing a secondary challenge with LCMV, similar percentages of CD8+ T cells respond to stimulation with gp 33 (Figure 3.10). The total numbers of antigen-specific responders are also similar during the memory response. Unlike during the primary response, there is no defect in expansion of the CD8+ T cell compartment in LCMV immune Itk -/- and Itk-/- Rlk-/- mice.

Analysis of the IFNγ and TNFα cytokine profiles of the memory response however, reveals a defect in the Itk -/- and Itk-/- Rlk-/- memory CD8+ T cell effector cytokine response as compared to WT CD8+ T cells. In WT LCMV immune mice, peptide stimulation results in mainly a population of IFNγ+ TNFα+ "double-producers". This cytokine profile was shown to be characteristic of CD8+ T cells undergoing a memory response (154). In contrast, Itk -/- and Itk-/- Rlk-/- CD8+ T cells from LCMV

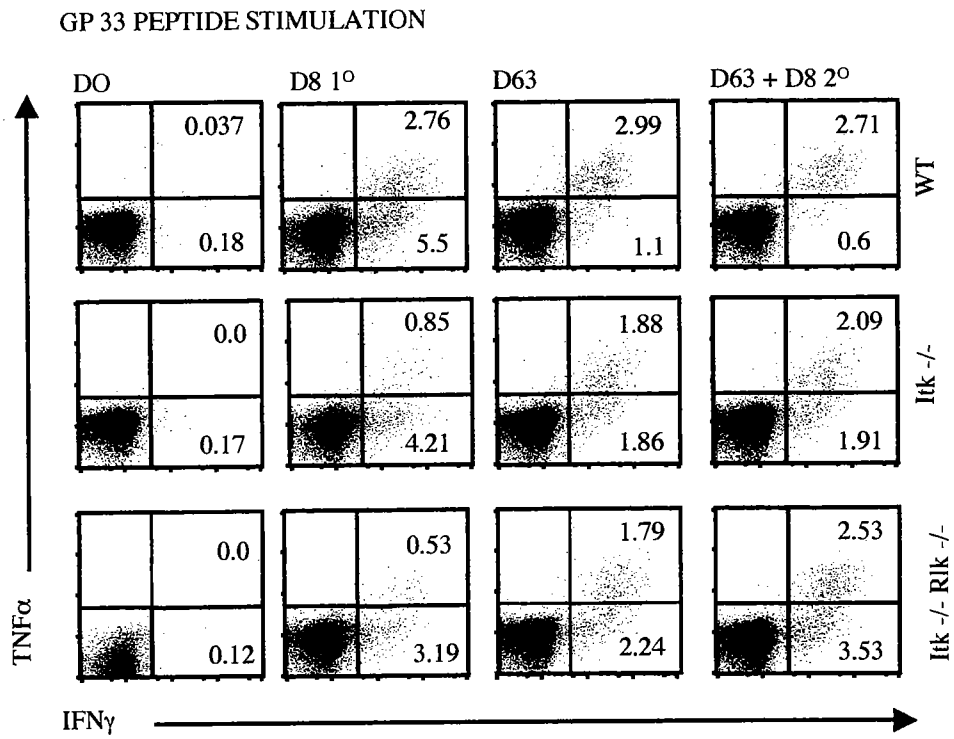
immune mice have an almost 50:50 distribution of CD8+ T cells producing both IFNγ and TNFα (double-producers), and cells producing IFNγ only (single-producers) (Figure 3.10). This result is also true of CD8+ T cells from LCMV immune Itk -/- and Itk-/- Rlk-/- mice undergoing a secondary challenge with LCMV. Paradoxically, unlike during the acute response, when CD8+ T cells from Itk -/- and Itk-/- Rlk-/- mice failed to make much TNFα, they seem to recover this capacity during the memory response. In addition, unlike the cytokine response profile of WT memory CD8+ T cells, the cytokine profile of Itk -/- and Itk -/- Rlk -/- memory CD8+ T cells is more characteristic of that seen during a primary immune response. These data show, that while Itk and Rlk are required for the generation of an optimal antigen specific acute CD8+ T cell immune response, loss of these Tec kinases had no apparent effect on the quantity of the CD8+ T cell memory immune response and only slightly affected the quality of that response, the significance of which is questionable.

The data in this study show that Itk and Rlk are important in the regulation of CD8+ function and provide an important analysis of the ways in which the "programmed" CD8+ T cell response can be modulated. An analysis that is potentially important for our understanding of how this response can be controlled and manipulated in the design of vaccination strategies that seek to optimize the CD8+ T cell response.

Figure 3.10: The LCMV Specific Memory CD8+ T Cell Response

Mice were infected and then rested for at least two months to generate LCMV immune mice. Single cell suspensions were generated from spleens harvested 63 days post infection (memory pool), 8 days post secondary infection (secondary response), or 8 days post primary infection (acute response). 4 X 10^6 cells were then stimulated with the epitope specific peptides gp 33 and np 396, and the antigen specific response analyzed by intracellular cytokine staining for IFNγ and TNFα.

THE CD8 T CELL MEMORY RESPONSE IN WT, Itk -/- AND Itk -/- Rlk -/- MICE



DISCUSSION

The Tec kinase family members Itk and Rlk are known to be important in signalling downstream of the TCR in CD4+ T cells and in the CD4+ T cell response to various pathogens. Our study details a role for the Tec kinase family members Itk and Rlk in CD8+ T cell signalling and function. We demonstrate for the first time the effect of loss of Itk and both Itk and Rlk on the biochemical and functional responses of purified CD8+ T cell in-vitro and on the CD8+ T cell immune response in-vivo.

Removal of Itk and Rlk from the TCR signalling cascade greatly impaired the activation of signalling pathways downstream of CD8+ T cell signalling. As shown in Figure 3.1, in-vitro stimulation of CD8+ T cells lacking Itk and both Itk and Rlk resulted in the diminished phosphorylation and activation of PLCγ1, and the subsequent generation of a sustained calcium flux. Itk -/- and Itk -/- Rlk -/- CD8+ T cells were also impaired in activation of the ERK and p-38 MAP-kinase pathways, resulting in impairment of CD8+ T cell effector function. Loss of Itk and the additive deficiency of Rlk attenuated the proliferative capacity of CD8+ T cells, demonstrating that Itk and Rlk are very important for the fidelity of signalling downstream of CD8+ T cell activation, as CD8+ T cells are normally very responsive to activation signals. In addition, fewer cells were able to respond to TCR stimulation in the absence of Itk and Rlk by making the effector cytokines IL-2, IFNγ and TNFα.

These data reinforce the importance of Itk and Rlk in T cell signalling and function, and are in keeping with previous results showing that CD4+ T cells lacking Itk

and both Itk and Rlk are deficient in activation of PLCγ and consequently the ERK and JNK MAP-kinase pathways (18, 92, 93, 100). Subsequent impairment in activation of latent transcription factors like NFAT and NFkB resulted in diminished production of the effector cytokines IL-2, IL-4 and IFNγ (99).

Loss of Itk and Rlk also negatively impacted the ability of CD8+ T cells to generate an efficient antigen specific immune response. In-vivo, loss of Itk and Rlk resulted in a decline in the frequency of antigen specific cells able to make the effector cytokine IFNγ and loss of the ability to make TNFα (Figure 3.5). In addition, loss of Itk and Rlk resulted in deficient accumulation of CD8+ T cells in response to LCMV infection (Figure 3.3). This diminished accumulation of CD8+ T cells was not a result of the weak CD4+ T cell response in Itk -/- and Itk -/- Rlk -/- mice, as supplementation of the CD4+ TH response in Itk -/- and Itk -/- Rlk -/- mice by adoptive transfer of LCMV specific WT memory CD4+ T cells, did not rescue expansion of the CD8+ T cell compartment in the kinase deficient mice (Figure 3.7).

CD8+ T cells are normally very responsive to antigenic stimuli. Numerous data show that CD8+ T cells undergo a massive and autonomous program of expansion and proliferation in response to antigenic stimuli (32, 141). To explore a mechanism by which removal of Itk and Rlk from the T cell signalling cascade may have attenuated this normally very sensitive CD8+ T cell response to antigenic stimulation, we investigated CD8+ T cell turnover and apoptosis during the anti-LCMV immune response in Itk -/- and Itk -/- Rlk -/- mice. This data showed that loss of Itk and both Itk and Rlk impairs CD8+ T cell proliferation in the early phases of the LCMV immune response (Figure

3.4). In addition, in the absence of Itk and both Itk and Rlk, a smaller fraction of CD8+ T cells survive as compared to WT CD8+ T cells (Figure 3.6). These data suggest that defects in CD8+ T cell proliferation early in the LCMV immune response and later defects in CD8+ T cell survival at the peak of the response synergise to affect the accumulation of CD8+ T cells in response to LCMV infection.

This interpretation of the data is however subject to several potential caveats. For example, an important factor affecting CD8+ T cell expansion is antigen load. It has been demonstrated in several experimental systems that the magnitude of CD8+ T cell expansion is dependent on viral load. The antigen dose affects the recruitment of naïve CD8+ T cells into the immune response, thereby affecting the final magnitude of the response (51). While we did not expressly examine this mechanism, we did observe that early in viral infection, Itk -/- and more so Itk -/- Rlk -/- mice have approximately 0.5 log and 1.0 log lower viral titres than WT mice, suggesting that there is a decreased viral burden in these mice early in infection (Figure 3.2). It is possible that this lowered viral burden and the subsequent lowered strength of antigenic stimulation resulted in the impaired expansion of the CD8+ T cell subset in Itk -/- Rlk -/- mice. Contrary to this observation however, analysis of the recruitment of antigen specific responders in Itk -/- and Itk -/- Rlk -/- mice by tetramer analysis revealed no significant difference in the percentages of np 396^{+} and gp 33^{+} cells recruited into the LCMV response as compared to WT mice (Figure 3.5). Since this analysis was done at later stages of the infection when cells are at the peak of their expansion programs, it is conceivable that Itk -/- and Itk -/- Rlk -/- mice contained fewer LCMV specific precursors initially. Moreover, a

significant fraction of the CD8+ CD4+4hi T cells present in the Itk -/- and Itk -/- Rlk -/- mice are lost day two – day three post-infection as previously reported by James M. McNally (155). This loss resulted in a more substantial reduction in total CD8+ T cell numbers in the Itk -/- and Itk -/- Rlk -/- (whose CD8+ T cells are nearly all CD44hi) than in WT mice at this time (Figure 3.3). As such, it is conceivable that Itk -/- and Itk -/- Rlk -/- mice contain an altered and smaller repertoire of LCMV specific precursors than WT at this timepoint, affecting the final accumulation of CD8+ effectors. It is also possible that Itk -/- and Itk -/- Rlk -/- mice may have a higher innate immune response initially, which may affect the early viral replication of LCMV. Also to be considered is the altered splenic environment of the Itk -/- and Itk -/- Rlk -/- mice that have larger germinal centers than the spleens of WT mice.

To address these caveats, WT and Itk -/- OT-1+ CD8+ T cells (which have similar low levels of CD44) were adoptively transferred into WT congenic mice that were subsequently infected with recombinant Vaccinia-OVA. The results of this experiment demonstrate that even in a WT environment and with similar precursor frequencies of responding cells, loss of Itk affected the accumulation of CD8+ T cells responding to the infection (Figure 3.8).

Mathematical models have long since predicted that factors extrinsic to the CD8+ T cell can affect CD8+ T cell expansion (156, 157). Loss of Itk and both Itk and Rlk had a distinct impact on CD8+ T cell proliferation. As shown in Figure 3.3, CD8+ CD44hi T cells in Itk -/- and Itk -/- Rlk -/- mice incorporated less BrdU than WT CD8+ CD44hi cells in the early stages of the LCMV anti-viral immune response (Figure 3.6). Crude

mathematical calculations on our part suggest that these defects in proliferation, over the course of the exponential response to the viral infection, may be sufficient to result in the defect in CD8+ T cell accumulation seen in Itk -/- and Itk -/- Rlk -/- mice.

However recent data has also shown that CD8+ T cells can undergo an autonomous program of expansion and differentiation (37, 38, 142). In light of these and other data, these models were recently revisited (158). The authors demonstrated mathematically that, in keeping with these current data, factors both extrinsic and intrinsic to the CD8+ T cell affect the program of CD8+ T cell expansion and differentiation. While the influence of extrinsic factors such as cytokines and viral load has been the subject of investigation, factors that affect the intrinsic program of CD8+ T cell expansion remain largely uncharacterized. Our data suggests that signalling molecules such as Itk and Rlk may have a role in maintaining the fidelity of that intrinsic program.

In support of this hypothesis, the signalling molecule JNK-1 has been implicated in CD8+ T cell proliferation and survival. Dietrich Conze from the Rincon Lab showed that JNK-1 is important for CD8+ T cell proliferation, while Natalie Arbour from the Oldstone Lab demonstrated that loss of JNK 1 resulted in the impaired expansion of the CD8+ T cell compartment in response to LCMV due to increased T cell apoptosis (159, 160). Activation of the JNK MAP-kinase signalling pathway is downstream of Itk in the TCR signalling cascade and loss of Itk results in reduced activation of JNK in CD4+ T cells (81, 100). It is a reasonable possibility that loss of Itk would negatively affect

activation of JNK 1 and contribute to the increased cell death observed during the CD8+ T cell immune response in Itk -/- mice.

Loss of Itk has also been shown to affect activation of the NFkB signalling pathway in CD4+ T cells (Micheal Li – unpublished data). In addition to being important for T cell activation and effector functions such as proliferation, expansion and cytokine production, NFkB is also important in T cell survival and maintenance (80, 161). It is conceivable that impairment of NFkB activation in Itk deficient CD8+ T cells could also potentially affect CD8+ T cell survival during the LCMV immune response and consequently impairs expansion of the CD8+ compartment in these mice. Since Rlk is also supposed to be upstream of JNK activation, it is perplexing that the combined loss of Itk and Rlk did not exacerbate the cell survival defect observed. However, it is a formal possibility that Rlk is not as important as Itk for activation of the JNK MAP-kinase signalling pathway.

Another factor contributing to the defective accumulation of Itk -/- and Itk -/- Rlk -/- CD8+ T cells during the immune response is impaired CD8+ T cell survival of the responding populations of Itk -/- and Itk -/- Rlk -/- mice. This was not due to impaired levels of anti-apoptotic molecules such as Bcl-2 and Bcl-XL, as analysis of the protein expression levels in CD8+ T cells at the peak of the immune response, detected no differences between WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells. However, the regulation of cell survival versus cell death is very complex. The survival of CD8+ T cells responding to infection depends not only on the expression of anti-apoptotic, but also on the balance maintained between pro-apoptotic and anti-apoptotic molecules. As such it is

possible that the ratio of pro-apoptotic to anti-apoptotic molecules may be higher in CD8+ T cells lacking Itk and both Itk and Rlk than in WT CD8+ T cells. The regulation of expression of these proteins, in addition to being regulated by the presence or absence of growth factors such as cytokines, may also depend on the fidelity of the signal downstream of the TCR activation (162, 163). This is an area of Itk -/- and Itk -/- Rlk -/- CD8+ T cell biology that remains to be explored.

Loss of Itk and Rlk also affected the CD8+ T cell effector cytokine response. As shown in Figure 3.4, a smaller proportion of Itk -/- and Itk -/- Rlk -/- CD8 T cells respond to np396 peptide stimulation by making IFNγ. In addition, Itk -/- and Itk -/- Rlk -/- CD8 T cells make less of the effector cytokines IFNγ and TNFα, as estimated by analysis of Median Fluorescent Intensity (MFI). Combined with the defect in CD8+ T cell expansion, loss of Itk and Rlk significantly affected the overall magnitude of the LCMV antigen specific response (Figure 3.4). This was also true of the OT-1+ Itk -/- T cell response to infection, where fewer cells were capable of producing effector cytokines in response to antigen specific peptide stimulation, and also produced less cytokine as determined by analysis of the MFI. These data reinforce, once again, the importance of Itk and Rlk in the activation of transcription factors such as NFAT, NFκB and the AP-1 complex that are important for the transcription of cytokine genes such as IFNγ and TNFα.

The impaired accumulation of CD8+ T cells during the primary immune response in Itk -/- and Itk -/- Rlk -/- mice did not affect the expansion of the CD8+ T cell compartment to in response to secondary infection, although the magnitude of the

primary immune response has been shown to affect the burst size of the memory CD8+ T cell response (153). Interestingly however, I observed differences in the cytokine profiles of the memory CD8+ T cell response in the Itk -/- and Itk -/- Rlk -/- mice. Memory CD8+ T cells secrete both IFNγ and TNFα in response to peptide stimulation, while effector CD8+ T cells have a predominant population of cells producing only IFNγ and a minor population producing both IFNγ and TNFα (154). Unlike WT CD8+ T cells, Itk -/- and Itk -/- Rlk -/- CD8+ T cells instead have a mixed population of cells producing only IFNγ and of double producers. This cytokine profile is indicative of WT cells undergoing a primary immune response (Figure 3.8). This data suggests that in addition to impacting the ability of CD8+ T cells to make these cytokines, loss of Itk and Rlk impairs the co-ordinate regulation of IFNγ and TNFα cytokine expression in CD8+ T cells. Cytokine expression is in part governed by the accessibility of the cytokine locus to transcription factors (164, 165). It has been shown that in previously activated cells, cytokine loci are permanently remodelled to permit faster activation of cytokine expression in response to secondary stimulation (166). It is possible that loss of Itk and Rlk affects the program of remodelling that cytokine loci undergo in previously activated CD8+ T cells.

Despite being able to generate a population of IFNγ+ TNFα+ CD8+ T cells during the memory response, Itk -/- and Itk -/- Rlk -/- CD8+ T cells were unable to produce TNFα during the primary immune response. Transcription of TNFα in activated T cells is dependent on an efficient calcium flux and the subsequent activation of NFATp (167, 168). There are also putative NFkB, AP-1 and Egr 1 binding sites in the TNFα

promoter region (169). Given the importance of Itk in the translocation of NFATc to the nucleus, and in the activation of pathways leading to efficient activation of NFkB, AP-1 and Egr transcription factors, it is likely that both Itk and Rlk are involved in regulation of transcription of TNFα. TNFα expression is also post-transcriptionally regulated and activation of p38 MAP-kinase is important for stability of TNFα RNA(170). Since Itk and Rlk are important for activation of the p38 MAP-kinase pathway, it is formally possible that this pathway is affected. Given that true memory cells are able to respond to much lower levels of stimulation with cognate antigen, this may indicate that the TCR induced signal necessary for transcription of the TNFα locus is qualitatively different in memory versus naïve CD8+ T cells. Alternatively, it is possible that the small proportion of Itk -/- and Itk -/- Rlk -/- CD8+ T cells that are capable of making both IFNγ and TNFα in the acute response, although very few, preferentially survive into the memory phase of the response.

These experiments demonstrate the importance of signalling molecules such as Itk and Rlk on the fidelity of the CD8+ T cell immune response, and the influence of the strength of the signal transmitted downstream of the TCR on the fate of the responding CD8+ T cell. These studies are in keeping with experiments showing that the strength of the TCR stimulus sets the threshold of CD8+ T cell responsiveness, and affects the capacity of CD8+ T cells in an immune response to accumulate in-vivo and to survive the cytokine withdrawal of the later phases of the immune response (171).

Itk and Rlk were previously found to be important for CD4+ T cell development, activation and function. These results describe for the first time the effect of loss of Itk

and Rlk specifically on CD8+ T cell signalling and on the CD8+ T cell immune response at the single cell level. These studies extend our understanding of the role of Itk and Rlk in the adaptive immune process, and are also potentially important for furthering our understanding of factors that modulate vaccine based CD8+ T cell immune responses.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION AND FUTURE DIRECTIONS

An effective T cell mediated immune response requires that the T cell be capable of integrating multiple signals from its external environment and generate the appropriate effector functions for the situation at hand. The efficient transduction of signals from the TCR is an integral part of T cell function and the T cell depends on the optimal integration of signals transmitted through the mature TCR αβ complex for the fidelity of the majority of its developmental and effector processes. This dependency begins in late thymocyte development and ends when the mature T cell finally undergoes apoptosis in response to homeostatic stimuli. Signalling molecules downstream of the TCR complex play an important role in maintaining the fidelity of the T cell signalling program following TCR activation. They do this by regulating the complex crosstalk between TCR mediated signals and the signals to other stimuli such as cytokines that together facilitate maximal T cell function. Itk and Rlk are two such important signalling molecules. Itk and Rlk are first important in the later TCR dependent phase of thymocyte development, when immature DP thymocytes undergo positive and negative selection and lineage commitment, as removal of Itk and Rlk from the TCR signalling cascade impairs the positive selection of CD4+ and CD8+ SP thymocytes. The data presented in Chapter II of this thesis shows that in addition to modulating the TCR dependent signalling thresholds that regulate thymocyte development, Itk and Rlk play a role in the regulation of the IL-7 common γc dependent lineage maturation of CD8+ SP thymocytes.

Loss of Itk and Rlk modulates the signalling threshold downstream of the common γc cytokines IL-7 and IL-15 by making CD8+ T cells more sensitive to signals

downstream of the IL7R and the IL15R, such as phosphorylation of STAT-5. These data are a first indication that Tec kinases are important for signalling downstream of cytokine receptors in T cells and introduces the intriguing possibility that Itk and Rlk may either regulate the crosstalk between the TCR dependent and cytokine receptor dependent signals that drive thymocyte development, or are directly involved in the regulation of signals downstream of cytokine receptor activation. These findings open up a potential new area of study in the role of Itk and Rlk in T cell function: the activation and regulation of signalling molecules downstream of cytokine receptor activation.

Itk and Rlk in Cytokine Signalling

As detailed in Chapter II, in the absence of Itk and Rlk, CD8+ SP thymocytes are more sensitive to cytokine stimuli. For example, Itk -/- and Itk -/- Rlk -/- CD8+ SP thymocytes have higher levels of phosphorylated STAT-5 after stimulation with the common γc cytokines IL-7 and IL-15 than WT CD8+ SP thymocytes. These experiments need to be repeated and extended to look at the effects of these cytokines on CD8+ SP thymocyte survival, proliferation and effector cytokine production. There is precedence for the involvement of the Tec kinases in cytokine signalling, as the Tec kinases Tec and Btk have previously been shown to be phosphorylated and activated downstream of IL-3, GM-CSF and IL-6 stimulation in myeloid and pro – B cells lines (132, 172). It is possible, that lowering the strength of the TCR signal during the CD8+ SP lineage maturation process negatively impacts the regulation of cytokine signals during this stage of thymocyte development. The hyperresponse of the Itk -/- and Itk -/- Rlk -/- CD8+ SP

thymocytes to cytokine signals suggests that these proteins are involved in the negative regulation of cytokine signalling. The Suppressors of Cytokine Signalling (SOCS) family of proteins negatively regulates cytokine signalling. SOCS-1, the first member of this family to be identified, is expressed throughout thymocyte development, with the highest expression occurring in DP thymocytes. CD8+ SP thymocytes retain expression of SOCS-1, albeit at much lower levels than DP thymocytes, and SOCS-1 is not expressed in CD4+ SP thymocytes (26). In the absence of SOCS-1, CD8+ SP thymocytes become hypersensitive to cytokine signals, particularly IL-7 and IL-15, and become CD44hi. As such, it would be interesting to look at SOCS-1 expression at both the RNA and protein level in Itk -/- and Itk -/- Rlk -/- thymi to determine whether Itk and Rlk are important either directly or indirectly in the regulation of SOCS-1 expression. Interestingly, SOCS-1 expression is maintained in peripheral CD8+ T cells but not in peripheral CD4+ T cells. As such, these experiments can also be done on peripheral CD8+ T cells, to see whether Itk and Rlk are involved in the regulation of cytokine signalling in peripheral CD8+ T cells, or in the integration of TCR dependent and cytokine dependent pathways in these cells. A potential caveat to these investigations is the CD44hi phenotype of the CD8+ SP thymocytes in Itk -/- and Itk -/- Rlk -/- mice, as a similar population of cells is not present in the thymus of WT mice. As such, it may be useful to do these investigations using a MHC Class I restricted transgenic system. It would also be interesting to use MHC Class I restricted transgenics that have different efficiencies of positive selection, to see whether modulation of the strength of the TCR signal in the absence of Itk and Rlk affects CD44 expression on the Itk -/- and Itk -/- Rlk

-/- thymocytes. One might predict that an MHC Class I transgenic TCR with a high avidity TCR and high selection efficiency would be less affected by the loss of Itk and Rlk and would have WT levels of CD44 expression, while a transgenic with a lower avidity would be more dependent on the presence of Itk and Rlk and may have higher levels of CD44 expression in the absence of these signalling molecules. CD8+ SP thymocytes and peripheral T cells from these different transgenic models can then be used to ask questions about the influence of cytokines such as IL-7 and IL-15 on CD8+ T cell development and function.

Figure 4.1: Model of Itk and Rlk in CD8+ SP Thymocyte Development

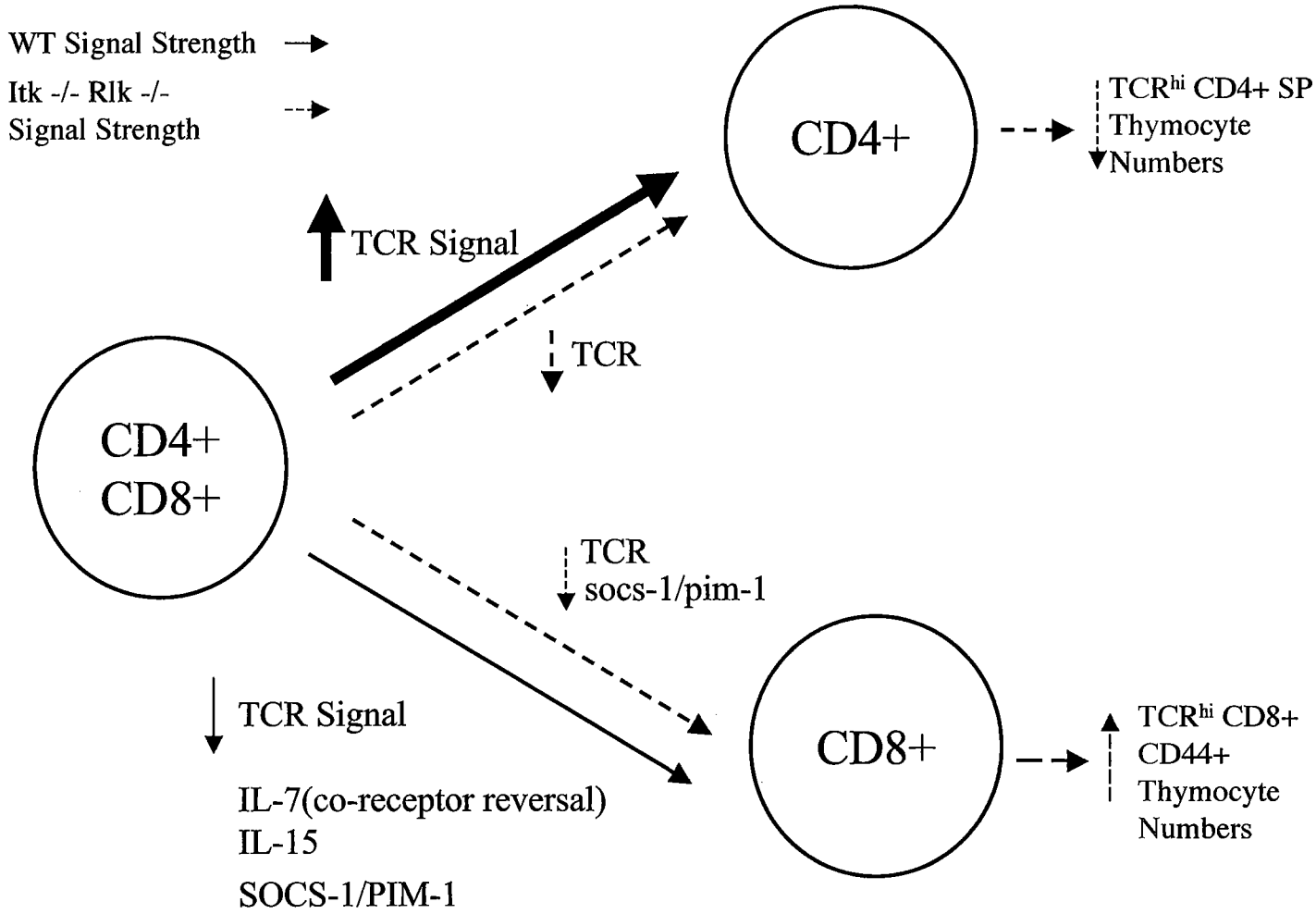
This model illustrates how lowered TCR dependent signals in the absence of Itk and both Itk and Rlk affect CD4+ SP thymocyte positive selection and CD8+ SP thymocyte maturation during lineage commitment.

In the absence of Itk and Rlk, the strength of the TCR signal DP thymocytes receive is lowered. This shifts the signalling threshold for positive selection such that fewer CD4+ SP thymocytes are generated.

WT CD8+ SP thymocyte development depends on a lower strength of signal than WT CD4+ SP thymocyte development. A further lowering of this threshold in the absence of itk and Rlk could affect the regulation of IL-7 and possibly IL-15 cytokine signals upon which CD8+ SP thymocytes maturation seems more dependent. The expression of molecules such as SOCS-1, which negatively regulates cytokine signalling, and Pim –1 which regulates the stability of SOCS-1 protein levels, may be negatively affected by a lowering of the TCR signals developing CD8+ SP thymocytes receive. This would make the Itk -/- and Itk -/- Rlk -/- CD8+ SP thymocytes more sensitive to stumulation with IL-7 and IL-15 and lead to their accumulation and subsequent upregulation of CD44, two of the effects caused by exposure of CD8+ cells to these common γc cytokines.

TCR dependent signals received in the absence of Itk or both Itk and Rlk are indicated by the dashed arrows, while WT TCR dependent signals are illustrated by the filled arrows.

Model: Loss of Itk and Rlk Alters CD8+ SP Thymocyte Maturation



Itk and Rlk in CD8+ T Cell Survival

The in-vivo experiments in Chapter III show that in the absence of Itk and Rlk, CD8+ T cell proliferation and cell survival are diminished, leading to the cumulative loss of T cell numbers at the peak of the LCMV response. In addition, fewer CD8+ T cells make less of the effector cytokine IFNγ, while the ability to make TNFα is lost during the primary immune response. Accumulation of CD8+ T cells at the peak of the response is also impaired in the OT-I TCR transgenic response to infection with a recombinant Vaccinia virus expressing the OVA OT-I epitope. The frequency of cells making IFNγ and the amount of IFNγ made on a per cell basis is also reduced. These data, together with the in-vitro data showing that Itk -/- and Itk -/- Rlk -/- CD8+ T cells are impaired in the activation of signalling pathways downstream of TCR activation show that removal of Itk and Rlk from the TCR signalling cascade impairs CD8+ T cell fitness. This impaired fitness is also demonstrated in-vitro as these cells are also impaired in their ability to mediate effector functions such as CD8+ T cell proliferation and cytokine production. One explanation for the impaired accumulation of CD8+ T cells at the peak of the LCMV immune response is that these cells are impaired either in their ability to proliferate or to survive after activation. Our data in the LCMV system, although indicating that Itk -/- and Itk -/- Rlk -/- CD8+ T cells were indeed impaired both in their ability to proliferate and survive in response to LCMV infection, was subject to several caveats. These included diminished precursor frequencies of Itk -/- and Itk -/- Rlk -/- LCMV responsive cells, as a significant fraction of the CD8+ CD44hi cells from Itk -/- and Itk-/- Rlk -/- mice undergo apoptosis between day two – day three of the LCMV

immune response as has been previously demonstrated. In addition, there seemed to be lower LCMV titres in the spleens of Itk -/- and Itk -/- Rlk -/- mice early in infection, raising the possibility that a differential antigen load may have influenced the CD8+ T cell response in the Tec kinase deficient mice. To circumvent these caveats, I looked at the WT and Itk -/- OT-1 T cell response to Vacc-OVA in an adoptive transfer system. The OT-1 experiment showed that in the absence of Itk, CD8+ T cells are impaired in their ability to accumulate in response to infection and to produce the effector cytokines IFNγ and TNFα, reinforcing the importance of the Tec kinases in modulating T cell fitness. This result once again raised the question of the role of Itk and Rlk in CD8= T cell survival.

The processes regulating T cell survival are complex. Some of the molecules regulating T cell survival include activation of NFκB downstream of TCR activation and modulation of the ratio of pro-apoptotic to anti-apoptotic molecules that is controlled both by TCR dependent processes and by growth factor stimuli such as cytokines. Although I did not observe a difference in Bcl-2 or Bcl-XL expression levels in Itk -/- and Itk -/- Rlk -/- CD8+ T cells in response to LCMV infection, it is possible that while the expression of these particular anti-apoptotic molecules are similar, the expression levels of pro-apoptotic molecules such as Bim may be differentially affected by the loss of Itk and Rlk. This hypothesis can be tested by western blot or FACS analysis of the relative expression levels of these and other pro and anti-apoptotic molecules in activated WT, Itk -/- or Itk -/- Rlk -/- CD8+ T cells. It is intriguing to think that given the apparent sensitivity of Itk -/- and Itk -/- Rlk -/- CD8+ cells to IL-7 and IL-15, that the withdrawal

of one or the other of these cytokines that occurs during the peak phase of the immune response, would affect the regulation of Bcl-2 family members in Itk -/- and Itk -/- Rlk -/- CD8+ T cells. It would be interesting therefore to look at the influence of these cytokines in-vitro on the survival of activated Itk -/- and Itk -/- Rlk -/- CD8+ T cells.

CHAPTER V

MATERIALS AND METHODS

Mice

Itk -/- mice were generated previously in our lab and are backcrossed at least nine times to the C57/Bl10 (H-2^b) background. Itk -/- Rlk -/- deficient mice were a kind gift from Pamela Schwartzberg, and were backcrossed at least 5 times to the H-2^b background. C57/Bl10 non-litter mate mice were used as controls. IL-15 -/- mice on a B6 background were obtained from the Immunex Corporation through Dr. Joonsoo Kang and were crossed to the Itk -/- mice. All mice used were between 6 – 12 weeks of age and were maintained at the University of Massachusetts Medical Center mouse facility where they were housed under pathogen free conditions.

CD8 T Cell Isolation and Stimulation

Single cell suspensions were made from spleens and lymph nodes of WT, Itk -/- and Itk -/- Rlk -/- mice. After RBC lysis and washing, the cells were spun down and resuspended in 90λ Macs Bfr [1X PBS/2mmEDTA/0.5%BSA] per $1x10^7$ cells and then labelled with 10λ of CD8 Macs Beads/ 1 x 10^7 cells. The cells were then incubated in the refrigerator for 12 - 15 mins and washed before being run over the AutoMacs columns and positively selected. After selection, the CD8 T cells were stimulated with PMA (1.25 - 2.5ng/ml) and Ionomycin (187.5 - 375 ng/ml) at a concentration of 5 x 10^5 cells/ml for 36 – 48 hrs. At the end of this time period, the cells were spun down and washed with RPMI-10 to remove the PMA/Ionomycin and then expanded in RPMI-10 containing 20% IL-2 supernatant. These cultured CD8+ T cells were then used for various in-vitro analyses.

3H Proliferation

1 – 2 x 10^5 purified and cultured CD8 T cells were incubated with a titration of platebound anti-CD3 and anti-CD28 at 37^{0}C in duplicate or triplicate in a 96 well plate. To coat plates, wells were initially incubated with 50λ of 5 μg/ml goat anti-hamster antibody in carbonate buffer pH 9.5 for 1 – 2 hrs at 37°C. Following incubation, the wells were washed with 1X PBS and then incubated with 75λ of a titration of anti-CD3 (2C11 clone) and anti-CD28 for 1 – 2 hrs at 37°C. 48 hrs after stimulation, the cells were pulsed with 3H at 1μCi/well. The cells were harvested 20 hrs later using a TOMTEC harvester and proliferation assessed by measuring 3H incorporation using a Microbeta scintillation counter.

CFSE Labelling

Whole lymph node suspensions, cultured CD8+ T cells or CD8+ T cells isolated directly ex-vivo were labelled with 1μM CFSE in 1X serum free PBS at a concentration of 2.5 x 10^7 cells/ml for 20 mins at 37°C. The cells were then spun down and resuspended in media containing 10% serum, and were ready for use in multiple assays.

Intracellular Cytokine Staining

Whole lymph node suspensions, cultured CD8+ T cells or CD8+ T cells isolated directly ex-vivo were stimulated with a titration of plate-bound antibodies in media containing Brefeldin A and Monensin (golgi-plug and golgi-stop). The incubations were done for 5hrs at 37°C. Following incubation, the cells were spun down and stained first for

surface antigens and then for the cytokines IFNγ, TNFα and IL-2 using the intracellular stain Cyto-fix/Cyto-perm kit from Pharmingen.

Calcium Flux Analysis

For calcium flux analysis 1 x 10^7 isolated WT, Itk -/- and Itk -/- Rlk -/- CD8 T cells were removed from culture and resuspended in 1ml RPMI-0. The cells were loaded with the calcium sensitive dyes Fluo-3 and Fura Red for 1hr at 37°C. 1 x 10^6 cells were removed to serve as unstimulated controls. The remaining cells were first labelled with 25 μg of biotinylated anti-CD3 for 45s then crosslinked with 40 μg of strepavidin for 5 mins. As a positive control the cells were stimulated with Ionomycin for 6 mins. The calcium flux was calculated by ratioing the intensities of the fluo-3 and Fura-Red dyes over time using the Facs Assistant Software (BD Biosciences).

PLCγ AND MAPK Phosphorylation

To assess activation of MAP-kinase signalling pathways, 2.5 - 5 x 10^6 WT, Itk -/- and Itk -/- Rlk -/- CD8+ T cells were labelled with 25μg/ml of biotinylated anti-CD3. The CD8+ T cells were then activated for various times by cross-linking with strepavidin. As a positive control, cells were stimulated with PMA at a concentration of 2.5ng/ml and Ionomycin at 375 ng/ml. The reactions were quenched by addition of 1ml of ice-cold stop solution (1X PBS containing 20mM NaF and 1mM Na_3VO_4. The cells were spun down, the supernatants were removed and the cell pellets lysed for 15 mins on ice using 50λ of protein lysis buffer containing 25mM HEPES (pH 7.5), 150mM NaCl, 1mM

EDTA, 1mM EGTA, 1% Triton-X, 1mM PMSF, 1mM Na_3V0_4 and 10μg/ml leupeptin. 50λ of 2X protein loading buffer was then added to each cell lysate. 25λ of total cell lysate was resolved on a 10% SDS-page gel, transferred to Immobilon-P membrane blocked and then blotted for phosphorylated PLCγ, p42/44 MAPK (ERK), p-38 and p-JNK MAP-kinases. Membranes were then either blotted for the PI3K subunit p85 or stripped and reprobed for total PLCy1, p42/44 ERK, p38 or JNK as protein loading controls.

Antibodies, Other Reagents and FACS Analysis

CD8-fitc, CD4-pe, CD44-cy, TNFα-apc, IFNγ-pe, Granzyme B-pe and CD3-bn were all purchased from BD Pharmingen. Immobilon-P membrane was purchased from Millipore (Bedford, MA). Antibodies to phospho PLCγ-1783, ERK, p38 and SAPK/JNK were all purchased from Cell Signalling Technologies, Beverly, MA. BrdU was purchased from Sigma Aldrich (St. Louis, MO. Anti-BrdU-fitc Ab was obtained from BD Biosciences. CFSE was purchased from Molecular Probes (Eugene, OR). For FACS Analysis, cells were as indicated, stained at 4 – 12^{0}C in the refrigerator, washed and resuspended in Facs Buffer (1X HBSS, 2% FCS). Antibody staining was analysed using a FACSCalibur (BD Biosciences) and data analyzed using Flowjo software (Treestar).

LCMV Infection

To generate acutely infected mice, WT, Itk -/- and Itk -/- Rlk -/- mice were infected intraperitoneally with 100λ of LCMV Armstrong at 2 – 5 x 10^5 pfu/ml. Spleens were

harvested on various days post infection (d2 – d11 for acute infection and d63 for analysis of the memory response) and single cell suspensions generated. The red blood cells were lysed by incubation in buffered ammonium chloride for two to five minutes. Cells were then washed and resuspended in RPMI-10 for further analysis.

Plaque Assay

Viral supernatants were generated from infected spleens harvested at various times post LCMV infection. The spleens were cut in half and ground in 1ml of media. The resulting tissue suspension was spun at 1500 rpm for 15 mins and supernatants removed and frozen in 500 ml aliquots. For assessment of viral titres, an aliquot of viral supernatant was thawed and then serially diluted. Briefly, 100λ of each serial dilution was added to one well of a six-well plate containing an approximately 70% confluent monolayer of Vero cells. The plates were incubated at 37° for 90 mins and then each well was overlaid with a 1:1 mixture of agarose and EMEM complete [1 part 0.8% Seakem agarose: 1 part EMEM complete - 2X EMEM, 6 mls FCS, 5 mls Penn-Strep Glutamine]. 4 days later, the plaques were visualised by overlaying the current agarose layer, with 2mls of the above-mentioned agarose mix containing 1% neutral red.

CTL Assay

RMA target cells were incubated with 10uM gp 33 or np 396 peptide and 51Cr for 1hr at 37°C. After extensive washing, 1 x 10^4 targets were incubated with splenocytes from d7, d8, d9 and d11 LCMV infected mice at various E: T ratios in a 96 well plate. The cells

were incubated for 5 hrs at 37°C. At the end of the incubation period, the plate was spun at 200g for 5 mins. and 70λ of supernatant removed for analysis of ^{51}Cr release.

In-Vivo Cytotoxicity Assay

Splenocyte suspensions from WT uninfected animals were labelled with 0.9 μM and 0.3 μM CFSE and then loaded with 1μM of gp 33 or a reference peptide respectively. The labelled cells were then counted resuspended at 2 x 10^8/ml and mixed at a 1:1 ratio. 200λ were then injected into either WT, Itk -/- or Itk -/- Rlk -/- mice at d8 post-infection or uninfected controls. Spleens were harvested 5 hrs after injection and specific killing assessed by calculating the percent loss of the peptide specific population by Facs Analysis of the relevant CFSE labeled populations.

Peptide Stimulations and ICS analysis/Analysis of Epitope Specific Response

Intracellular cytokine staining was used to quantitate LCMV specific gamma interferon (IFNγ), (TNFα) and IL-2 producing T cells. 2 – 4 x 10^6 splenocytes from WT, Itk-/- and Itk-/- Rlk -/- mice were incubated with LCMV specific peptides (2μg/ml; gp 33, np 396, gp 276, np 205 [CD8 epitopes] and gp 61, np 309 [CD4+ epitopes]) in – vitro for 5 hrs at 37°C, in medium containing Brefeldin A and Monensin [golgi-plug and golgi-stop]. After incubation, cells were spun down and stained first for surface expression of CD4 and CD8 and then intracellularly for IFNγ, TNFα and IL-2, using the Cytofix/Cytoperm Kit from Pharmingen. Intracellular staining was also used to analyze Granzyme B expression in CD8 T cells during LCMV infection.

Tetramer Analysis

Tetramer stain analysis was done on splenocytes from d8 and d9 infected mice. 2 x 10^6 cells were incubated in Fc-block (1/200) plus strepavidin for 20 mins. Cells were washed twice and then 4λ of tetramer in 100λ of FACS buffer containing 0.01% azide was added to each sample. The samples were then incubated for 1hr at 4 – 12°C, washed twice, fixed for 5 mins in 100λ Cytofix reagent from Pharmingen, washed and then resuspended in 200λ of buffer for FACS analysis. Tetramers used, gp 33-pe H2b and np 396-pe H2b were a kind gift from R. Welsh (UMass).

BrdU Labelling

LCMV infected mice were injected intraperitoneally with 100λ of BrdU [15mg/ml in PBS] 12 hours before harvest. 4 x 10^6 splenocytes were then added to the wells of a 96 well plate, washed and then stained for surface antigens such as CD4, CD8 and CD44. The cells were then washed once in PBS and then fixed in Cytofix/Cytoperm [Pharmingen] for 20 mins at 4 – 12°C. The cells were washed by spinning at 3000rpm for 5 mins, and then fixed again with a freshly made solution of 1% formaldehyde containing 1% Tween-20. Cells were usually left at this stage until the last time-point was harvested. To stain for BrdU, cells were spun down and washed twice in PBS at room temperature. All remaining steps were done at room temperature. The cells were resuspended in 200λ of Dnase Solution (750λ 5M NaCl, 105λ 1M MgCl2, 250λ 1mM HCl, 24mg Dnase, 23.9 mls dH2O), and incubated at RT for 10 – 30 mins. The cells were then washed once and incubated in 50λ of a 1:50 dilution of anti-BrdU for 30 mins.

At the end of the incubation period the cells were washed twice and then analyzed using the FACSCalibur BD Bioscicences and Flowjo software.

Annexin-V and 7-AAD Staining

2 – 4 x 10^6 splenocytes from LCMV infected mice were stained extracellularly for CD4 or CD8, CD44 and Annexin-V fitc in 100λ of 1X Annexin-V buffer [Pharmingen]. To these samples was added 5λ of the vital dye 7-amino actinomycin D [7-AAD]. The cells were incubated for 20 mins at 4°C, washed twice with 200λ of Annexin-V buffer and then resuspended in 200λ of Annexin-V buffer for FACS analysis. Cells were not fixed for extended periods as this resulted in lower levels of Annexin-V staining. If cells were fixed, 5λ of Cytofix (Pharmingen) was added per 100λ of Annexin-V buffer.

Adoptive Transfer of Memory LCMV-specific CD4 T Cells

WT CD45.1 congenic mice were infected with LCMV and rested for 2 months. CD4+ T cells were then isolated using CD4+ beads and the Automacs. 1 x 10^7 donor CD45.1 CD4 T cells were injected into WT, Itk -/- and Itk -/- Rlk -/- CD45.2 host mice. Host mice were then infected with LCMV. Host mice receiving only CD4+ cells or host mice only infected with LCMV were used as controls. Mice were harvested eight days post-infection and WT, Itk -/- or Itk -/- Rlk -/- host CD8 T cell expansion assessed.

Adoptive Transfer of OT-1 WT and Itk -/- CD8 T cells

CD8+ T cells were isolated from pooled single cell suspensions of OT-1 WT and Itk -/- spleens and lymph nodes. The cells were than labelled with CFSE and 5 x 106 cells injected intravenously into WT CD45.1 congenic hosts. The hosts were infected 24 hours later with 1 x 10 7 pfu of the recombinant Vaccinia – OVA construct. Staining for expression of CD45.2, CD8 and Va2 identified cells responding to infection. The antigen specific response was analysed by assessing the effector cytokine response to stimulation with the OT-1 specific epitope SINFEKL.

Cytokine Stimulations and Analysis of STAT-5 phosphorylation

To look at the effect of cytokines on T cell effector function and Stat –5 phosphorylation, total thymocytes and total lymph node cells ($1 - 20 \times 10^5$) were incubated with a 1:20 dilution of IL-2 and IL-7 supernatant and 40ng/ml of murine IL-15 in media containing 1mg/ml BSA and 0.5% FCS, for 48 – 72 hours at 37°C in 48 or 96 well culture plates. Cytokine induced Stat-5 phosphorylation was examined by intracellular staining. Cells were stimulated for 30 mins to allow for maximal Stat-5 phosphorylation. Immediately following cytokine stimulation, an aliquot of cells were fixed in 2% PFA for 10 mins at room temperature (RT) to serve as positive controls. The remaining cells were washed and returned to culture without cytokine. Aliquots were drawn at different timepoints and fixed. The fixed cells were then washed in 1X PBS and incubated in methanol:acetone (1:1 vol/vol) for 30 mins on ice. Cells were rehydrated by washing in 1X PBS/2% FCS. They were then incubated with anti-phospho-Stat-5 antibody for 30

mins in 1X PBS/2% FCS, washed and then incubated with anti-rabbit IgG Oregon green 488 for 30 mins. To stain for expression of surface markers the cells were blocked in Fc block and then incubated with CD4, CD8 and CD44 antibodies in 1X PBS/2% FCS.

Fetal Thymic Organ Culture (FTOC)

Thymi isolated from d15 – d17 fetal mice were cultured on round nitrocellulose membranes (Millipore Corp. Bedford, MA) placed on inserts made of stainless steel mesh (Small Parts Inc. Miami, FL). Thymi were cultured for 7 – 14 days at 37°C, and then analysed for distribution of CD4, CD8 and CD44.

Re-aggregate Thymic Organ Culture (RTOC)

For RTOC, thymi isolated from d14 – d15 mice were pooled and then digested for 4 – 6 mls of a pre-warmed enzyme mixture containing dispase (0.8U/ml), collagenase (0.1U/ml) and DNAse (150U/ml) in serum free 1X HBSS. The cells were incubated for 10 mins at 37°C, then cells were spun down and the pellet further resuspended in 3mls enzyme mixture for another 10 mins. 1 ml FCS was added to stop the digestion. The cells were then sedimented, the supernatant aspirated and the cells resuspended by lightly tapping tube. The cells were then pipetted unto a dry filter to form a standing drop using a fine tipped glass capillary pipette. The filter was then placed on mesh inserts in 24 well plates at the interface of media containing 10% FCS.

CHAPTER VI

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