DEFINING THE ROLE OF CD3 ζ ITAMS IN T CELL SIGNALING AND DEVELOPMENT

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DEDICATION

To my husband, my son, and my parents for their unyielding support

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αβ T cells express a T cell receptor (TCR) composed of an αβ heterodimer that is responsible for ligand recognition, which is associated with six invariant signaling chains, termed the CD3 subunits. These are organized into two independent signaling modules, the CD3 γε/δε heterodimer and the CD3 ζζ homodimer. In their cytoplasmic tail, the CD3 chains contain multiple copies of a motif termed an ITAM. The TCR contains 10 ITAMs, one in each CD3 γ, δ

and ε chain, and three in each CD3 ζ chain. The TCR complex, when isolated from thymocytes and peripheral T cells, contains a constitutively tyrosine-phosphorylated CD3 ζ molecule, p21, which results from ongoing interactions between the TCR and peptide/MHC molecules.

The magnitude, extent, and duration of the CD3 ITAM phosphorylations are critical for the proper development of several subsets of T cells, including conventional T cells and invariant natural killer T (iNKT) cells. The mechanism by which these phosphorylations are regulated is not fully resolved. Herein, we report that p21 results from TCR interactions with MHC molecules prior to selection, supporting a model that the TCR has an inherent avidity for MHC molecules. Biochemical analyses of the TCR complex before and after TCR stimulation suggested that p21, which exists complexed to ZAP-70, is excluded from new TCR-driven signals. Conventional T cell development proceeds with as few as 4/10 functional CD3 ITAMs; however, iNKT cell development was significantly reduced when one CD3 ζ ITAM was rendered non-functional. This was due to an early block in development, at least in part attributable to an increase in cell death. iNKT cells are thought to be critical for the prevention of inflammatory arthritis following infection with the spirochete, *Borrelia burgdorferi*. Despite significantly reduced iNKT cells in the CD3 ζ ITAM mutant mice, there was no difference in the severity of Lyme arthritis in these mice compared to wild type controls following *B. burgdorferi* infection. These

data suggest that CD3 ITAMs provide important signals during the selection and development of conventional and iNKT cells. Furthermore, these data suggest that iNKT cells are not the primary cell type responsible for preventing Lyme arthritis.

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ABBRIVIATIONS

Ab: antibody

APC: antigen presenting cell

APC: allophycoerythrin

Bb: *Borrelia burgdorferi*

BCR: B cell antigen receptor

CFU: colony forming unit

CD: cluster of differentiation

Cy: cyanine

CPM: counts per minute

DAG: diacylglycerol

DC: dendritic cell

DN: double negative

DNA: deoxyribonucleic acid

DP: double positive

DPBS: Dulbecco's phosphate buffered saline

ERK: extracellular regulated MAP kinase

FACS: fluorescence activated cell sorter

FcR: Fc receptor

FITC: fluorescein isothiocyanate

Grb2: growth factor binding protein 2

HBSS: Hanks Balanced Salt Solution

HSC: hematopoietic stem cell

IHL: intrahepatic lymphocytes

Ig: immunoglobulin

IFN-γ: interferon gamma

i.d.: intradermal

IL: interleukin

i.p.: intraperitoneal

i.v.: intravenous

kDa: kilodalton

LAT: linker of activated T cells

LM: *Listeria monocytogenes*

rLM-Ova: recombinant *Listeria monocytogenes*-Ova albumin

MAPK: mitogen activated protein kinase

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

NK: natural killer

qPCR: quantitative polymerase chain reaction

Pac: pacific

PE: phycoerythrin

PI3K: Phosphoinositide-3 kinase

pMHC: peptide/MHC

PTK: protein tyrosine kinase

PTPase: protein tyrosine phosphatase

RAG: recombinase activating gene

RNA: ribonucleic acid

SDS-PAGE: SDS polyacrylamide gel electrophoresis

SH2: Src homology 2

SH3: Src homology 3

SLP-76: SH2 domain containing leukocyte protein of 76 kDa

TCR: T cell receptor

Tg: transgene

YF: tyrosine to phenylalanine

ZAP-70: zeta associated protein of 70 kDa

CHAPTER I

INTRODUCTION

As a part of the adaptive immune system, T cells respond to foreign antigens by either the directly killing infected cells or releasing cytokines that regulate other cells. T cells recognize self- and foreign-peptides that are embedded in self-MHC proteins via the T cell receptor (TCR). αβ T cells express a TCR composed of the ligand binding αβ heterodimer that is non-covalently associated with six invariant signaling chains, including one CD3 γ and δ chain and two CD3 ε and ζ chains (1, 2) (Figure 1). The CD3 proteins contain at least one copy of a conserved signaling motif, termed an immunoreceptor tyrosine-based activation motif (ITAM), in their cytoplasmic tail (3). ITAMs have the conserved sequence D/E(x_2)YxxL/I (x_{6-8}) YxxL/I, and following TCR engagement by self- or foreign-ligand/MHC complexes, the CD3 ITAMs become bi-phosphorylated on critically spaced tyrosine residues. This initiates a signaling cascade that results in T cell development, differentiation or effector function.

Several distinct lineages of T cells develop in the thymus, including αβ and natural killer T cells (NKT) (4, 5). Both of these subsets are derived from a common $CD4^+CD8^+$ (DP) precursor. T cell development involves a rigorous selection process, whereby only cells with appropriate reactivity to self-ligand/self-MHC molecules survive (4). This process generates a pool of peripheral T cells that remain tolerant to self-peptide/MHC complexes, but react strongly to foreign-antigen/MHC complexes. Work

in this thesis will focus on elucidating the role of phosphorylated CD3 ITAMs in T cell receptor signaling and the development of effector T cell populations.

The T cell receptor complex: An overview of TCR structure and assembly

T cells bind self- and foreign-peptides embedded in self-MHC molecules via the T cell receptor (Figure 1) (6). While the αβ TCR heterodimer is responsible for recognition of peptide/MHC molecules, these subunits are unable to transmit intracellular signals (7-10). These subunits have short cytoplasmic tails of 9-12 amino acids (aa) that do not have intrinsic enzymatic functions. Instead, intracellular TCR signaling is processed by six invariant signaling chains, CD3 ε, γ, δ, and ζ chains. These subunits are non-covalently associated with αβ subunits (1). *In vitro* translation and assembly studies show that the TCR complex contains eight components, a single clonotypic TCR α and β subunit, one CD3 γ and δ chain, and two CD3 ε and ζ chains (2).

All of the TCR subunits are synthesized in the endoplasmic reticulum (ER). Assembly of the TCR αβ with the CD3 γε/δε signaling subunits occurs in the endoplasmic reticulum, while CD3 ζζ is added in the Golgi apparatus (2, 11-15). TCR assembly occurs in a stepwise fashion. First, CD3 γε and δε heterodimers are formed, and paired with the TCR α and β subunits, with association between TCRα/CD3 δε occurring first (11, 16). The αβ subunits are then disulfide linked. The final step of TCR assembly involves the association of the CD3 ζζ homodimer (17). Partially assembled TCRαβ/ CD3γε/δε complexes are produced in excess of CD3 ζζ. The complexes that do not pair with CD3 ζζ are retained in the Golgi and targeted to

lysosomes for degradation (18). The pairing of CD3 ζζ is the rate limiting step in TCR assembly and provides a mechanism by which TCR surface expression is controlled (15). Although CD3 ζζ regulates TCR expression, all of the CD3 subunits are required for efficient TCR assembly as elimination of any one subunit results in the partial or complete loss of surface TCR expression (17, 19, 20).

The pairing of the different TCR/CD3 subunits is mediated by non-covalent interactions between positively and negatively charged residues in the membrane spanning portions of the various TCR/CD3 subunits. Specifically, the lysine residue in the TCR α chain pairs with the two glutamic acid residues found within each chain of the δε heterodimer. The arginine residue in the transmembrane region of TCR α pairs with each of the aspartic acid residues present in the CD3 ζζ molecules (21-23). TCR β complexes with the γε chains via a lysine residue that associates with the glutamic and aspartic acid residues of the γ and ε chain, respectively (21, 22).

ITAMs: discovery and function

The CD3 γ, δ, ε, and ζ subunits of the TCR complex contain a conserved ITAM signaling motif in their cytoplasmic tails (Figure 1) (3, 24). ITAM sequences are not limited to the CD3 subunits, as components of the B cell receptor, Fcγ (mast cells), and some NK receptors are now known to use ITAMs to propagate intracellular signals (3, 25, 26).

Interestingly, the ITAM signaling system is not restricted to the immune system, as reformation of neuronal networks in the lateral geniculate nucleus also requires this

conserved pathway (27, 28). ITAMs have also been shown to be incorporated into the signaling machinery of several viruses, including Epstein Barr Virus, Herpes simplex virus 8, bovine leukemia virus, a mutant Simian Immunodeficiency virus, and hantavirus pulmonary syndrome-associated strains of Hantavirus (29-34). These ITAMs are believed to be used as a form of molecular mimicry to subvert host cell defenses by interrupting cell signaling pathways. For example, LMP2A, the EBV ITAM containing protein, sequesters Syk in B cells, thereby blocking BCR signal transmission (30). Similar effects were noted in EBV infected T cells, where LMP2A binds ZAP-70 (35).

The first description of an ITAM signaling pathway in invertebrates (*Drosophila melanogaster*) was recently reported (36). This signaling pathway involves Draper, and ancient immunoreceptor on glial cells, that recognizes "modified self" presented by target cells to initiate phagocytosis of dead or dying cells. These interactions are translated into intracellular signals using a conserved ITAM signaling domain in the cytoplasmic tail of Draper. Like other ITAMs signaling pathways in T and B cells, these signaling events rely on ITAM phosphorylation by a Src kinase and signal propagation by the Syk PTK homolog, Shark. Such data suggest that the Draper pathway could have been the evolutionary precursor to mammalian ITAM signaling pathways. Furthermore, given the high rate of necrosis and apoptosis of developing thymocytes, this ancient system could also provide the early basis for the thymic education of T cells whereby the TCR complex evolved in order to recognize modified self-proteins and translate this into intracellular signals that would promote self tolerance.

All of the ITAMs defined to date are twenty six amino acids in length, and contain the evolutionary conserved sequence of D/ExxYxxL /I (x_{6-8}) YxxL/I, (where x denotes any amino acid) (3, 24). Following receptor triggering, ITAMs become bi-phosphorylated on the two critically spaced tyrosine residues by Src protein tyrosine kinases (PTK). This results in recruitment and activation of signaling proteins that initiate intracellular signaling cascades that can either support activation or inhibition of receptor signaling (25, 37, 38). Signaling through ITAMs is typically terminated following dephosphorylation by protein tyrosine phosphatases and/or ubiquitin mediated degradation pathways (39-42).

ITAMs, as their name implies, typically activate cell signaling pathways. Surprisingly, ITAMs have also been shown to inhibit cell signaling (43). For example, in a model of receptor desensitization, FcαRI, which is associated with the Fcγ ITAM containing subunit, can inhibit mast cell degranulation by cross regulating the activity of FcεR1 following binding to monomeric IgA (44). This inhibition was ITAM dependent, and required the recruitment of the protein tyrosine phosphatase SHP-1 to the Fcγ ITAM. Inhibition of FcεRI activation was mediated by SHP-1 targeted dephosphorylation of the downstream FcεRI signaling molecules, Syk, LAT, and Erk. Alternatively, aggregation of FcαRI molecules by IgA immune complexes produces activating signals via recruitment of Syk kinase. This provides a mechanism by which low affinity interactions between immunorecptors and their target complexes could generate inhibitory signals though ITAMs by recruiting proteins that could actively dampen signals through heterologous receptors.

The CD3 ζ Chain

The CD3 γ, δ and ε chains share a high degree of sequence homology, are all members of the immunoglobulin (Ig) superfamily, and are all clustered on chromosome 9 (11 in humans). Contrasting this, CD3 ζ is present on chromosome 1 and contains only a short extracellular domain of 9 aa, followed by a transmembrane region of 21 aa, and a long intracellular tail of 113 aa (45).

As an unphosphorylated protein, CD3 ζ has a molecular weight of 16 kDa (p16) (Figure 2) (45). CD3 ζ ITAM phosphorylation occurs in a stepwise fashion, proceeding from the carboxy terminal to the juxtamembrane tyrosine residues (46). Phosphorylation of CD3 ζ ITAMs results in the formation of two phosphorylated derivatives with a distinct molecular masses of 21 (p21) or 23 kDa (p23). These forms arise from the bi-phosphorylation of the two membrane distal or all three ITAMs, respectively (46, 47). Contrasting all other CD3 subunits, CD3 ζ appears as a constitutively phosphorylated protein (p21) in thymocytes and peripheral T cells and is complexed to an inactive pool of ZAP-70 (47-51). However, p23 is only detected following TCR crosslinking or strong agonist stimulation. In only one other circumstance involving the EBV ITAM containing protein LMP2A is an ITAM present in a constitutively phosphorylated state (30).

Proximal T cell receptor signaling

T cell receptor engagement of peptide/MHC complexes on target cells results in the generation of intracellular signals (Figure 3) (25, 37). Following receptor ligation, the Src family kinases, Lck and Fyn, are catalytically activated and bi-phosphorylate the CD3 ITAMs (52). Following ITAM phosphorylation by Src kinases, the Syk family of PTKs,

Zeta associated protein of 70 kDa (ZAP-70) and Spleen tyrosine kinase (Syk), are recruited to and bind with high affinity to the different phospho-ITAMs (52). Syk and ZAP-70 are structurally related, with each protein containing tandem SH2 domains (2SH2) that are linked by an interdomain A (53). The SH2 domains mediate binding to the bi-phosphorylated ITAMs. These domains are connected to the kinase domain via an SH2-kinase linker (interdomain B). While both proteins are able to bind bi-phosphorylated CD3 ζ ITAMs with high affinity, Syk can also bind mono-phosphorylated ITAMs and its kinase domain is more enzymatically active (54-56).

ZAP-70 activation is regulated by both structural and post-translational modifications (57-59). Prior to binding phospho-ITAMs, ZAP-70 is in an inactive conformation in which portions of interdomain A are bound to the kinase domain. Binding occurs such that the SH2 domains face outward, allowing for phospho-ITAM binding (60). Once the two SH2 domains bind the phospho-ITAMs, they are brought into close proximity to one another. This causes a change in the conformation of ZAP-70, simultaneously destabilizing binding of the interdomain A to the kinase domain and stabilizing the two SH2 / phospho-ITAM interactions (60, 61). The conformational change also exposes the regulatory tyrosine (Y) residues in the interdomain B (Y292, Y315 and Y319) and the kinase domain (Y492 and Y493). Y292 has been shown to negatively regulate the activity of ZAP-70 as a phenylalanine substitution at this location results in hyper signaling through the TCR (62-64). Y315 and Y319 are phosphorylated by Lck and phosphorylation of these residues results in an increase in ZAP-70 activity. As phosphotyrosines, these sites are necessary for recruiting other proteins and are

thought to stabilize ZAP-70 in an active confirmation (65). Y319 is a binding site for the SH2 domain of Lck, and a mutation at this site results in decreased PLC-γ activation (66). Y315 is critical for Vav association with ZAP-70; however, mutating this residue does not limit Vav activation (64, 67). The catalytic activity of ZAP-70 is regulated by the phosphorylation of tyrosine residues within the kinase domain of ZAP-70. Y492 and Y493 are either activated by Lck or trans-autophosphorylated by ZAP-70 following activation through the TCR (68-70). Y492 negatively regulates ZAP-70 activation, whileY493 is required for full ZAP-70 activation (69, 70). The importance of ZAP-70 in TCR signaling is reflected by the numerous mechanisms that have evolved in order to regulate its activity. These mechanisms ensure that ZAP-70 activation only occurs at appropriate times. Furthermore, the multiple phosphorylations sites on ZAP-70 that are protein binding sites potentially provide a system by which distinct signaling pathways could be differentially regulated and/or integrated. Furthermore, regulatory phosphorylation sites provide the means whereby ZAP-70 could bind to phospho-ITAMs in an inactive state.

Once activated, ZAP-70 phosphorylates a number of downstream proteins, including the adaptor proteins LAT and SLP-76 and effector proteins Vav, Itk and PLCγ1 (71-73). LAT is a transmembrane adaptor protein that contains nine conserved tyrosine residues in its cytoplasmic tail. LAT is central to the organization of multiple signaling protein complexes including Gads/SLP76 and PLCγ1 (74, 75). PLC-γ1 is recruited to LAT via its SH2 domain where it is phosphorylated and subsequently activated by ZAP-70 (74) . PLC-γ1 hydrolyzes phosphatidyl 4,5 bisphosphate (PIP_2) into the second

messengers IP3 and DAG, leading to the release of intracellular calcium, or induction of the Ras/MAPK signaling pathways, respectively (76). Gads, a Grb-2 adaptor protein, is constitutively bound to SLP-76 and the Gads/SLP76 complex is recruited to phospho-LAT via the SH2 domain of Gads (77). Vav is recruited to tyrosine phosphorylated SLP-76 via its SH2 domain (78). Vav, a guanine nucleotide exchange factor, is essential for the activation of the Rho proteins Rac and CDC42, which have been shown to induce actin cytoskeletal rearrangements (79-81).

Itk is a member of the Tec family of PTKs and is induced downstream of TCR signaling (82). Itk is recruited to the plasma membrane via its pleckstrin homology domain, which has a high affinity to phosphatidyl inositol triphosphate. Itk can also bind LAT, SLP-76 and PLC-γ1 (78, 83, 84). Itk augments TCR signaling by phosphorylating and activating PLC-γ1 (82). Thus, Itk plays an important role in regulating the magnitude and duration of intracellular calcium elevations in activated T cells.

Calcium signaling in T cells is critical for development, differentiation and effector function. Increases in intracellular calcium levels are mediated by two mechanisms, the release of intracellular Ca^{++} from the ER and the influx of Ca^{++} from outside of the cell (85). The former pathway results in transient increases in calcium levels, while the latter supports sustained increases. Following activation through the TCR, intracellular calcium is released from the ER. This process requires the binding of IP3 to IP3 receptors. This results in the activation of STIM1, an ER transmembrane protein that acts as a sensor for ER calcium levels. STIM1 binds calcium via its EF-hand domain that faces the lumen of the ER and binds Ca^{++}, and a decrease in calcium levels

result in its activation (86). STIM1 activation leads to store operated calcium entry (SOCE) which is regulated by calcium-release activated calcium (CRAC) channels in the plasma membrane (87, 88). STIM1 is thought activate Orai1, a tetraspanning plasma membrane protein that is either the pore forming unit in CRAC channels or may in fact be a CRAC channel (89-93). Following Orai1 activation, extracellular Ca^{++} is allowed to flow into the cells, resulting in sustained increases in intracellular calcium. The precise mechanism by which STIM1 and Orai1 regulate Ca^{++} signaling is poorly understood; however, deletion of either of these proteins results in profound deficits Ca^{++} signaling (94, 95).

There are several key transcription factors activated as a consequence of TCR signaling. Nuclear factor of activated T cells (NFAT) transcription factors are a family of 5 related proteins (NFAT1-5), with conserved DNA binding domains (96). All but one NFAT family member (NFAT5) is activated downstream of calcium signaling. NFAT is localized to the cytoplasm prior to its activation, and is maintained in an inactive state via the phosphorylation of multiple serine residues. An increase in intracellular calcium levels results in the activation of the phosphatase, calcineurin. This serine/threonine phosphatase dephosphorylates NFAT, thereby exposing its nuclear localization signal. This event permits NFAT translocation into the nucleus where it can regulates the transcription of several target genes. Subsequent phosphorylation on serine residue by nuclear serine/threonine kinases results in the rapid export of NFAT back to the cytoplasm.

NFκB is another transcription factor that is localized in the cytoplasm, and it is found in a complex with IκB (inhibitor of NFκB), masking its nuclear translocation signal (97, 98). PKCθ is activated following T cell activation and is required for NFκB activation (99). The mechanism by which PKCθ is activated is unclear; however, recruitment to Vav and phosphorylation by Lck are proposed necessary (100). PKCθ phosphorylates the adaptor protein CARMA1 on three serine residues. CARMA1 then forms a complex with Bcl10 and MALT1 (CMB complex) (101, 102). This complex is responsible for the recruitment and activation of the IKK complex, which is made up of three proteins, IKKα and IKKβ catalytic subunits and the IKKγ (NEMO) regulatory protein. Activation of the IKK complex requires serine phosphorylation of the IKKα and IKKβ subunits by TAK1, a serine/threonine kinase (103). Following activation, IKK phosphorylates IκB, resulting in the subsequent ubiquitination and degradation of IκB, thereby releasing NFκB into the nucleus (104). Following the activation of multiple transcription factors in the nucleus, numerous genes are transcribed that are required for various aspects of T cell biology, including T cell development, differentiation, and effector function.

The Unique Features of the TCR Complex

There are a number of interesting features that distinguish the TCR from other ITAM containing receptors. First, the TCR is the most ITAM rich receptor to date with a total of 10 ITAMs in the TCR complex, three from each CD3 ζ chain, and one in each of the CD3 γ, δ, and ε chains. The reason for the complexity of this receptor is unknown; however, this suggests that signal amplification and/or discrimination are important for T

cell development and function. Several studies support the role of the TCR ITAMs in signal amplification. As such, CD3 ITAMs function in a quantitative fashion during the development of T cells bearing low affinity T cell receptors (105-109). Recent data also suggests that there are a minimal number of CD3 ITAMs that are required to prevent autoimmunity, such that mice with 2-6 functional CD3 ITAMs in the TCR developed severe autoimmunity, with 4 or less resulting in rapid death of the mice (110). A second feature is that TCR signal transduction is thought to be organized into two, independent signaling modules, the CD3 ζζ homodimer and gε/δε heterodimers (25, 52, 111). This might enable T cells to activate distinct pathways involved in T cell development, selection, and function.

CD3 ζ is detected as a constitutively phosphorylated protein (p21) in thymocytes and peripheral T cells (49-51). The formation of p21 *in vivo* requires ongoing TCR interactions with peptide/MHC complexes, as the constitutively phosphorylated p21 is absent in MHC class I $^{-/-}$ II $^{-/-}$ thymocytes and TCR $\alpha^{-/-}$ thymocytes (52). Lck is the primary kinase responsible for phosphorylating and maintaining the constitutive phosphorylation of CD3 ζ, as phospho-ζ levels in unstimulated thymocytes and peripheral T cells from the Lck$^{-/-}$ mice are significantly reduced (52, 58). ZAP-70 is necessary for stabilization of phospho-ζ, as p21 is undetectable in thymocytes from ZAP-70 deficient mice (52, 58). This is likely due to protection conferred by the binding of the two SH2 domains of ZAP-70 to phospho-ITAMs (112). The reason for the constitutive phosphorylation of p21 is not known. One proposed mechanism is the

passive or active attenuation of TCR signaling by sequestering inactive ZAP-70 and/or recruiting proteins, such as phosphatases, that would negatively regulate signaling.

αβ T cell development

The development of αβ T cells begins after bone marrow-derived lymphoid progenitor cells home to the thymus (Figure 4) (113). Once in the thymic milieu, cells progress through a series of developmental stages that are identified by the differential regulation of CD4, CD8, CD25, and CD44. During the initial phases of development, T cells are $CD4^-CD8^-$ (DN) and modulate CD25 and CD44 in four distinct stages, from $CD25^-CD44^+$ to $CD25^+CD44^+$ followed by $CD25^+CD44^-$, and finally $CD25^-CD44^-$ (DN1, 2, 3, and 4, respectively). Progression from the DN1 stage to the DN2 stage is dependent on IL-7, which induces the expansion of progenitor cells, the upregulation of CD25, and promotes the expression of recombinase activating genes (RAG) that initiate TCR β chain rearrangements (113, 114). Rearrangement of the TCRβ chain continues through the DN3 stage. At this stage, TCR β forms a complex with the pre-TCR α chain and the CD3 signaling chains (113, 115). Following surface expression of a functional pre-TCR complex, productive signals result in decreased RAG expression and the termination of TCR β chain rearrangement (114). The DN4 stage is marked by a robust expansion and upregulation of the co-receptors CD4 and CD8 (DP). During the DN to DP differentiation, the immature thymocytes re-express the RAG proteins and rearrange the TCR α locus (114). The αβ TCR is then expressed at low levels on the cell surface (αβ TCR, CD3 γε/δε and ζζ). RAG expression is downmodulated following the positive selection of DP thymocytes into $CD4^+$, $CD8^+$, or natural killer T (NKT) cell lineage.

The DP thymocytes undergo a rigorous selection process, the outcome of which is determined by the affinity of the TCR for selecting peptide/MHC complexes on thymic epithelial cells (4). T cells expressing TCRs with a low to negligible affinity for selecting self-peptide/MHC complexes die by neglect. On the other hand, T cells bearing high affinity TCRs undergo negative selection, which results in programmed cell death. Only T cells with an intermediate affinity for the selecting peptide/MHC complexes are positively selected, and proceed to the $CD4^+CD8^-$ (CD4 SP) or $CD4^-CD8^-$ (CD8 SP) stage. Selection into these subsets is determined by the specificity of the TCR for such that T cells with TCRs that recognize MHC class I, MHC class II, or CD1d are directed into the corresponding CD8 SP, CD4 SP, or NKT lineage.

The CD3 signaling chains are required for proper TCR surface expression and T cell development. This is evidenced by the lack of mature peripheral T cells in CD3 γ, δ, ε , or ζ knockout mice (19, 20, 106, 116-119) (Table 1). CD3 $\gamma^{-/-}$ and CD3 $\varepsilon^{-/-}$ mice are blocked at the DN3 stage. CD3 ζ deficient mice are blocked at the DP stage, however, some CD4 SP and CD8 SP cells are found in the periphery of these mice. Surface TCR expression is extremely low on these cells. Interestingly, in HY TCR Tg/CD3$\zeta^{-/-}$ male mice, a decrease in the efficiency of negative selection was observed as autoreactive HY specific T cells were detected in the periphery (120). CD3 δ deficient mice have normal thymic cellularity; however, T cell development is blocked at the DP stage.

Proximal T cell signaling is also critical for $\alpha\beta$ T cell development. $Lck^{-/-}$ mice have a reduction in thymic cellularity and a decrease in the $CD4^+CD8^+$ T cells in the thymus, whereas $Fyn^{-/-}$ thymocytes develop normally (58, 121-123). While Lck is the

primary kinase necessary for T cell development, Fyn can act in a partially redundant manner as $Fyn^{-/-}Lck^{-/-}$ doubly deficient have a more profound block in T cell development, and are arrested in the DN stage (124). Fyn is also critical for mediating intracellular calcium levels in developing thymocytes. Moreover, $Fyn^{-/-}$ mice showed impaired deletion of some T cell clones responsive to superantigen, suggesting a role for Fyn in selection of a subset of conventional T cells (121, 123). Fyn has also recently been shown to be critical for the development of NKT cells, which will be discussed in more detail below (125).

ZAP-70 and Syk are also necessary for T cell development, with the most profound phenotypes noted in ZAP-70 deficient mice compared to Syk-null animals (48, 55, 115, 126, 127). $ZAP\text{-}70^{-/-}$ T cells are blocked at the DP stage, while $ZAP70^{-/-}$ $Syk^{-/-}$ cells are blocked in the DN stage of development (128, 129). This could be due to the fact that Syk was recently it was shown to be required for pre-TCR signaling at the DN3 stage of T cell development (130, 131). Furthermore, fewer $Syk^{-/-}$ DN4 cells were detected in the thymus compared to $ZAP\text{-}70^{-/-}$ cells in competitive repopulation assays in which ZAP-70 and Syk deficient bone marrow was transferred into irradiated wild type mice (130, 131). While $Syk^{-/-}$ T cells have relatively normal development, one study noted a slight reduction in thymocyte cellularity in mice reconstituted with $Syk^{-/-}$ compared to $Syk^{+/-}$ cells (132, 133).

Several mouse and human immunodeficiencies are caused by mutations in ZAP-70 that negatively impact its function. A severe combined immunodeficiency in patients has been identified resulting from mutations in ZAP-70 (134-136). In humans, CD4 but

not CD8 T cell development can occur in the absence of functional ZAP-70, partly due to increased Syk expression in these cells compared to normal T cells (137). However, as Syk expression is reduced in mature peripheral T cells, the CD4 SP T cells in these SCID patients were not functional.

Two ZAP-70 mutations have also been characterized in mice that do not lead to a primary immunodeficiency but result in autoimmunity (138, 139). These mutations are a result of single nucleotide substitutions in ZAP-70, resulting in decreased function and TCR signaling and lead to a reduction in the efficiency of negative selection, allowing autoreactive cells to populate the periphery of affected mice. The SKG mutation (ZAP-70^{skg}) in BABL/c mice results in the onset of and inflammatory arthritis that resembles rheumatoid arthritis in humans (138). This results from a spontaneous mutation in the C-terminal SH2 domain of ZAP-70. The ZAP-$70^{mrd/mrd}$ and ZAP-$70^{mrt/mrt}$ mutations were a result of N-ethyl-N-nitrosourea (ENU) mutagenesis in C57BL/6 mice (139). ZAP-$70^{mrd/mrd}$ arose due to a mutation in the catalytic domain which is thought to alter the dimensions of the ATP-binding pocket. This mutation results in a mild phonotype with slight alterations in T cell development, increasing the $CD44^{hi}$ CD4 and CD8 T cell populations. ZAP-$70^{mrt/mrt}$ was due to a mutation in the activation loop and causes a complete block in T cell development at the DP stage. Interestingly, ZAP-$70^{mrd/mrt}$, which contains one allele from each mutant, results in severe autoimmunity characterized by increased production in autoantibodies and IgE compared to wild type mice or mice that are homozygous for each mutation. ZAP-$70^{mrd/mrt}$ T cells have an intermediate level of TCR signaling, thus allowing for the selection of autoreactive T cells, and their

subsequent activation in the periphery. In all of these ZAP-70 mutations, a partial impairment in ZAP-70 function results in attenuation of TCR signaling. This provides enough signaling in order to allow for the positive selection of autoreactive T cells. While TCR signaling in mature T cells is dampened, the signals that are generated are enough to promote the activation of autoreactive T cells in the periphery resulting in autoimmune syndromes in affected mice.

CD3 ζ transgenic mice

We previously generated CD3 ζ transgenic (TCR ζ transgenic) mice that contain phenylalanine substitutions on select tyrosine residues in the different ITAMs of CD3 ζ (Figure 5) (46). These mutations were based on data previously generated in which the ITAMs responsible for the specific patterns of phosphorylation were mapped (46). Substitutions were made in the first (YF1,2), third (YF5,6), or all three (YF1-6) CD3 ζ ITAMS. The YF1,2 line was characterized as retaining p21 in the absence of and inducible p23. Contrasting this, the YF5,6 lines only contained weak phosphorylated derivatives of 19 and 20 kDa, and only following agonist stimulation. Of importance, the YF1,2 and YF5,6 transgenic lines were matched for the number of ITAMs available for signaling (8 of 10) in the TCR complex and TCR surface density. The YF1-6 mice contain 4/10 functional CD3 ITAMs. We have used these mice in wild type and TCR transgenic backgrounds in order to study the role of CD3 ζ ITAMs in T cell development, signaling, and function. Briefly, we and others have found differences in the ability of T cells bearing low affinity TCRs undergo proper positive and negative selection when some or all CD3 ζ ITAMs were rendered non-functional (19, 107-109, 140, 141). This

difference was directly related to the total number of CD3 ITAMs available, as a higher number resulted in improved selection. Furthermore, male HY TCR/YF1,2 mice had an increase in the development of potentially autoreactive cells (140). These results will be described in the next section.

The function of the phosphorylated derivatives of CD3 ζ in Thymocyte Development

Due to its ITAM rich nature, CD3 ζ is assumed to be the predominant signaling module of the TCR complex. Interestingly, most T cells appear to develop and function normally in the absence of functional CD3 ζ ITAMs (Table I) (105, 107-109, 116, 141-143). For example, T cells from mice bearing the high affinity MHC-class I P14 TCR are not dependent on the CD3 ζ ITAMs, as development, signaling and function are normal in the absence of all functional CD3 ζ ITAMs. In T cells bearing low affinity TCRs, such as those found in the HY, 2C, OTII, and F5 TCR transgenic mice, there is a direct correlation between the efficiency of selection and functional ITAM numbers (107). A specific role for CD3 ζ ITAMs was not observed as mutating any of the CD3 ITAMs (γ, ε or ζ) results in inefficient positive and/or negative selection (107, 140, 141, 144, 145).

The need for a full complement of CD3 ITAMs during positive selection in T cells expressing low affinity TCRs could promote efficient calcium responses during selection. An increase in ITAM numbers directly corresponds to the efficiency of Ca^{++} signaling in Jurkat T cells (146). This is necessary for NFAT localization to and

Table I: *A summary of T cell signaling proteins in T cell development and selection*

TCR Tg Line	Mutation	MHC Restriction	TCR affinity	Block	Thymic Cellularity	SP	TCR Surface Expression	Positive selection	Negative selection
N/A	γ-/-	Class I and Class II	N/A	DN3	↓↓↓	↓↓	Very Low	ND	ND
N/A	ε -/-	Class I and Class II	N/A	DN3	↓↓↓	↓↓↓	Very low	ND	ND
N/A	ζ-/-	Class I and Class II	N/A	DP	↓↓↓	↓	Very low	ND	ND
N/A	δ-/-	Class I and Class II	N/A	DP	Normal	↓↓	Very low	ND	ND
P14	CD3 ζ ITAM	Class I	High						
	3 ITAMs			None	ND	Normal	Normal	Normal	ND
	0 ITAMs			None	ND	Normal	Normal	Normal	ND
	CD3 ζ YF Series								
	WT			None	Normal	Normal	Normal	Normal	ND
	YF1,2			None	Normal	Normal	Normal	Normal	ND
	YF5,6			None	Normal	Normal	Normal	Normal	ND
	YF1-6			None	Normal	Normal	High DP/ Normal SP	Normal	ND
	CD3 ε ITAM			None	ND	Normal	Normal	Normal	Normal
	WT			None	ND	Normal	Normal	Normal	Normal
	YF								

2C	CD3 ζ ITAM	Class I	Intermediate						
	3			None	ND	Normal	Normal	Normal	ND
	2			DP to SP	ND	Normal	Normal	Normal	ND
	1			DP to SP	ND	Normal	Normal	Normal	ND
	0			DP to SP	ND	↓	Normal	Normal	ND
HY	CD3 ζ ITAM	Class I	Low						
	3			None	ND	Normal	Normal	Normal	Normal
	1			DP to SP	ND	↓	Normal	↓↓	↓↓
	0			DP to SP	ND	↓↓↓	Normal	↓↓↓	↓↓↓
	YF Series								
	WT			None	Normal	Normal	Normal	Normal	Normal
	YF1,2			DP to SP	Normal	↓	Normal	↓	↓
	YF5,6			DP to SP	Normal	↓	Normal	↓	↓↓
	YF1-6			DP to SP	Normal	↓↓↓	High DP/ Normal SP	↓↓↓	↓
	CD3 ε ITAM YF								
	WT			None	ND	Normal	Normal	Normal	ND
	YF			DP to SP	ND	↓	Normal	↓	ND
DO11.10	CD3 ζ ITAM	Class II	Low						
	3			None	ND	Normal	Normal	Normal	Normal
	1			DP to SP	ND	↓↓	Normal	↓	ND
	0			DP to SP	ND	↓↓↓	Normal	↓↓↓	↓↓↓

retention in the nucleus of developing T cells, since this is necessary for positive but not negative selection (147-149).

The need for a minimal total number of CD3 ITAMs for appropriate negative selection is noted by an increase in the incidence of multi-organ autoimmunity in mice in which T cell development occurred with reduced CD3 ITAM numbers (110). Bone marrow cells from CD3ε/ζ$^{-/-}$, which lack all CD3 subunits, were retrovirally transduced with constructs encoding various combinations of wild type and YF substituted CD3 ITAMs. These cells were then transferred into a RAG$^{-/-}$ host. The expression of between 2 and 6 wild type ITAMs resulted in severe autoimmunity, with 4 or less resulting in death. There was a negative correlation between the time to onset of disease and total ITAM number. Interestingly, there were 14 conditions in which 2 or all 3 CD3 ζ ITAMs were mutated either alone or in combination with other CD3 ITAMs, and all of these mutations prevented the expression of p21. In all of these mice there was a significant reduction in thymic cellularity, and in all but one case, autoimmunity ensued. Yet, when CD3 γ, δ, ε were mutated in the absence of CD3 ζ, either alone or in combination, disease was not detected. There are several important difference in this model compared to previous models studying the role of CD3 ITAMs in T cell selection and function. First, bone marrow cells were reconstituted with the CD3 ITAM mutants, and therefore these constructs could have been expressed in all cells. Furthermore, these cells were then injected into a lymphopenic host, which might have allowed autoreactive cells to undergo homeostatic proliferation in the periphery, increasing their number, and thus, the severity of the disease.

We analyzed the efficiency of negative selection in male HY TCR/CD3 ζ ITAM transgenic mice, and found that the selective expression of p21 (HY/YF1,2) resulted in the emergence of $CD8^{int}T3.70^{+}$ T cells that exhibited high levels of CD5. HY/CD3 ζ male mice expressing wild type, YF5,6 or YF1-6 CD3 ζ have a peripheral population of $T3.70^{+}$ cells that are $CD8^{lo}$, rendering them unresponsive to peptide/MHC complexes (140). Taken together, these data suggest that p21 could have reduced signaling in HY/YF1,2 mice, preventing the negative selection of autoreactive T cells. No overt autoimmunity was observed in these mice, however, p21 continues to be constitutively phosphorylated in peripheral T cells (50). Furthermore, signaling through the TCR was crippled due to the fact that two ITAMs of the TCR complex contained YF substitutions. Thus, autoimmunity could have been prevented due to reduced signaling through the TCR as a result of p21 expression and/or a reduction in ITAM numbers.

Phosphorylated CD3 ζ is also involved in regulating the level of TCR surface expression on immature DP thymocytes (41, 42, 150, 151). The adaptor protein, SLAP, binds with phospho-ζ with its SH2 domain (42). SLAP then recruits the E3 ligase, c-Cbl, resulting in the ubiquitination and subsequent degradation of CD3 ζ (41). This eventually leads to the internalization and degradation of the remaining TCR subunits. This data is supported by the fact that there is an increase in TCR expression on DP cells in $SLAP^{-/-}$ T cells compared to wild type cells. This is consistent with higher TCR surface expression in the DP but not SP thymocytes in our CD3 ζ transgenic mice that contain tyrosine to phenylalanine substitutions in all CD3 ζ ITAMs (YF1-6) compared to wild type mice or mice with mutations in only one CD3 ζ ITAM (YF1,2 and YF5,6).

Although a difference in the transgene integration site could account for some differences in TCR surface expression, multiple YF1-6 founders exhibited a similar phenotype suggesting that this does not account for the higher TCR expression levels in DP YF1-6 T cells. Reduced TCR signaling likely dampens TCR signaling in developing DP cells, which might be important in regulating signaling the thresholds necessary for efficient positive and negative selection

The importance of CD3 ζ ITAMs in thymocyte development is highlighted in a child with a severe combined immunodeficiency. This patient was susceptible to recurring infections with Pseudomonas aeruginosa, herpes simplex virus, Candida albicans, and Streptococcus pneumoniea (152). The immunodeficiency was attributed to a nonsense mutation (Q70X), resulting in a premature stop codon within the first ITAM preceding the first consensus sequence. Ninety percent of the peripheral T cells in this patient contained a homozygous Q70X mutation in CD3 ζ, resulting in low TCR expression and poor proliferative responses to various stimulants including, antibodies, mitogens and superantigens. The remaining 10% of T cells contained a heterozygous Q70X mutation, with one allele containing the inherited stop mutation, and the others containing mutations additional mutations that restored some CD3 ζ functionality.

CD3 ζ and Peripheral T cell Function

The chemokine receptor CXCR4 has been shown to physically associate with the TCR (153). Chemokine receptors are G-protein coupled receptors that mediate the migration of immune cells into lymphoid and peripheral tissues (154). CXCR4 has been shown to be critical for B cell development; however, T cell development proceeds

relatively normal in CXCR4$^{-/-}$ mice (155-158). CXCR4 is also a co-receptor for HIV (159). CD3 ζ and CXCR4 interact, and this is important for CXCR4 function. CD3 ζ and CXCR4 co-localize following stimulation through CXCR4 with its ligand, SDF-1α (153). Furthermore, TCR β deficient Jurkat T cells are only able to traffic to SDF-1α following expression of chimeric CD8/CD3 ζ ITAM constructs that contain at least one functional ITAM. CXCR4 mediated cell migration has been shown to be dependent on p52 Shc and ZAP-70, both of which are able to bind phospho-ζ (160-162). The TCR and CXCR4 can also cross-regulate one another, where signaling through the TCR, inhibits CXCR4 signaling and migration (163). Moreover, stimulation of Jurkat T cells with SDF-1α reduces TCR surface expression. It remains unclear; however, whether CD3 ζ ITAMs are specifically required for CXCR4 signaling and migration, or whether other ITAMs within the TCR complex are able to mediate this function as data that implicate CD3 ζ do so using chimeric receptors (CD8/CD3ζ) in systems which lack other functional CD3 ITAMs due to a lack of TCR surface expression (TCRβ$^{-/-}$) (153).

Ongoing interactions between the TCR and self-peptide/MHC complexes are necessary to prolong the survival of peripheral T cells (164-167). Because these signals are also necessary to drive the constitutive phosphorylation of CD3 ζ (p21), it was postulated that p21 was specifically required to maintain peripheral T cell survival. Two studies, however, have generated conflicting results. One group showed that peripheral CD4^{+} T cells show a higher rate of decay when MHC class II is selectively eliminated compared to controls in which MHC class II expression was maintained (168). Another group showed that the absence of p21 did not have an effect on peripheral T cell survival,

as adoptively transferred $CD4^+$ T cells in to a C57BL/6 or MHC class II deficient host exhibited a similar rate of decay despite a decrease in p21 expression (169). As discussed herein, we have expanded on these studies and found that the total number of CD3 ITAMs, rather than the expression of p21, mediates T cell survival.

The necessity of CD3 ζ ITAMs for proper T cell development and function has been revealed in patients with autoimmune diseases and immunodeficiencies. Some patients with systemic lupus erythematosus (SLE), express lower levels of CD3 ζ compared to healthy controls (170). Single nucleotide polymorphism (SNP) analysis was carried out SLE patients, and two SNPs within the 3'UTR region of CD3 ζ correlated with decreased CD3 ζ expression levels. Other studies have found that SLE is associated with this alternatively spliced 3' UTR of CD3 ζ which results in a decrease in the stability and expression of CD3 ζ (171-173). Whether these mutations occur prior to or following disease onset is unclear, therefore, future studies will be needed in order to characterize this mutation in individuals with SLE.

A specific role for CD3 ζ in peripheral T cell function has not been clearly demonstrated. While some studies suggest that CD3 ζ is critical for chemokine signaling and maintaining peripheral tolerance, data have not been generated that unequivocally find a role for CD3 ζ. As such, it is unclear whether total ITAMs numbers or whether specific phosphorylated derivatives of CD3 ζ are important maintaining peripheral tolerance and regulating chemokine receptor signaling. Thus, further experiments will be needed in order to clarify some of these questions.

Invariant natural killer T cell: TCR and antigen recognition

Another subset of αβ T cells, called NKT cells, regulate immune responses to various bacterial and viral challenges (174-176). In addition, these cells have been shown to reduce or prevent the development of diabetes in NOD mice in an IL-4 or IL-10 dependent manner (5). Also, some studies show that injection of an NKT cells agonist leads to a decrease in tumor size and metastasis (177). While they are for the most part beneficial to the host, $CD4^+$ NKT cells have recently been implicated in airway hyper-reactivity in people suffering from asthma due to their ability to secrete high levels of IL-4 in the lungs (178).

iNKT cells express surface receptors that are found on both T and NK cells, such as the αβ TCR and NK1.1, respectively. There are 4 types of NKT cells (179). Type I cells represent the predominant subset of NKT cells and express a semi-invariant TCR, composed of a TCR α chain encoded by the Vα14Jα18 rearrangement in mice (Vα24Jα18 in humans). The TCR Vβ repertoire in Type I NKT cells is restricted to Vβ8.2, 7, and 2 in mice (Vβ11 in humans). (180-185). Contrasting Type I iNKT cells, Type II NKT cells have a slightly broader TCR repertoire. A major difference between these NKT cell subsets and conventional T cells, is that Type I and type II NKT cells recognize glycolipids embedded in the non-classical MHC class I molecule, CD1d. These cells are also either $CD4^+CD8^-$ or $CD4^-CD8^-$ (186, 187). Type III and type IV NKT cells are not CD1d restricted, have a diverse TCR repertoire, recognize MHC class I and/or class II molecules, and can be $CD4^+$ $CD8^-$ or $CD4^-CD8^+$. The work in this thesis will focus on Type I NKT cells, hereon referred to as invariant NKT cells, or iNKT cells.

The first true ligand that was found for iNKT cells was α-galactosylceramide (α-GalCer), a derivative of a non-mammalian glycolipid found in a marine sponge, first identified as a novel cancer therapeutic (177). α-GalCer is an extremely potent iNKT cell agonist. When used as a ligand loaded onto fluorochrome-conjugated CD1d tetramers, it can be used to specifically identify iNKT cells by flow cytometry (188, 189). Recently, a derivative of α-GalCer was engineered, called PBS57. This ligand retains the binding and stimulatory properties of α-GalCer but has increased solubility due to the substitution of a hydroxyl with an amide group on the galactose ring and a *cis*-double bond in the acyl chain. (190).

Only one endogenous glycolipid, iGb3, or isoglobotrihexosylceramide, has been identified in mammals. iNKT cells can be stimulated by iGb3 both *in vitro* and *in vivo*, but much higher concentrations are required compared to α-GalCer (191). The role of iGb3 in iNKT cell development is controversial. Mice that are deficient in β-hexosaminidase B ($HexB^{-/-}$), an enzyme important for the production of iGb3 in lysosomes, were devoid of iNKT cells (191). However, the $HexB^{-/-}$ deficiency also results in lysosomal storage disease, and this could have disrupted the production of unidentified glycolipids other than iGb3 that might be involved in the selection of iNKT cells (192, 193). Since iGb3 cannot be isolated from human or mouse thymuses, and iNKT cell development was normal in iGb3 synthase deficient mice, there are likely multiple endogenous glycolipids are necessary for iNKT cell development (194, 195).

iNKT cell development and selection

The development of glycolipid-loaded CD1d tetramers allows for the direct identification of iNKT cells (188, 189). iNKT cell development occurs in the thymus with precursor cells originating from the same $CD4^+CD8^+$ population as conventional T cells (Figure 4) (196-198). Like conventional T cells, iNKT cells undergo positive selection in the thymus. However, while conventional T cells are selected by MHC class I or class II expressing epithelial cells in the thymic cortex, iNKT cells are selected following interactions between the invariant TCR and glycolipid/CD1d complexes that are expressed on other immature $CD4^+CD8^+$ cortical thymocytes. The necessity of CD1d is confirmed by the lack of iNKT cells in $CD1d^{-/-}$ mice despite the fact that $V\alpha14J\alpha18$ rearrangements can be detected in the thymus by PCR, albeit at reduced levels (199).

While CD1d is expressed on numerous cell types in the thymus, several studies have shown that it must be expressed on DP thymocytes for the proper selection of iNKT cells (200). SCID mice, which lack T cells but express comparable MHC class I and class II levels as wild type mice, failed to reconstitute the iNKT cell compartment following the transfer of fetal liver cells from $\beta2m^{-/-}$ mice. These mice were; however, able to generate $NK1.1^+$ T cells when receiving $\beta2m^{+/+}$ cells (201). Furthermore, when CD1d expression is driven under the MHC class II promoter, CD1d expression is excluded from DP thymocytes as this promoter is not active in DP cells. iNKT cells do not develop in these mice (202). Fetal liver chimera experiments in which irradiated $\beta2m$-deficient mice are reconstituted with fetal liver cells from $\beta2m^{-/-}$ and $TCR\alpha^{-/-}$ mice

are able to generate iNKT cells (203). In such a system, the $\beta 2m^{-/-}$ cells are the iNKT cell precursors, while the $TCR\alpha^{-/-}$ cells contain the CD1d expressing DP thymocytes. iNKT cells do not develop in chimeras generated with $\beta 2m^{-/-}$ / $TCR\beta^{-/-}$ or $\beta 2m^{-/-}$ / $RAG^{-/-}$ liver cells, as T cell development is blocked at the DN stage in the TCRβ and RAG deficient animals. B cells also express CD1d but are not involved in iNKT cell development since $C\mu^{-/-}$ mice, which lack B cells, have normal $NK1.1^{+}$ T cell development (201). Mature SP T cells in the thymus were also ruled out since iNKT cells were detected in the thymus of $\beta 2M^{-/-}$MHC $II^{-/-}$ mice that were reconstituted with MHC class $II^{-/-}$ fetal liver cells (201). Thus, glycolipid/CD1d expressing immature DP thymocytes have a unique role in mediating the selection of iNKT cells.

iNKT cells have a skewed Vβ repertoire that is limited to Vβ8.2, 7, and 2. This is because these cells are preferentially selected during development rather than due to preferential pairing of the Vα14Jα18 TCR with limited Vβ chains (204). The preselection repertoire of iNKT from Vα14Jα18 TCR/$CD1d^{-/-}$ mice contain cells expressing the canonical Vβ8.2, 7, and 2 subunits and Vβ3, 6, 9,1 0, and 14, as evidenced by CD1d tetramer staining. Upon stimulation with iGb3, however, only the iNKT cells expressing the Vβ8.2, 7, and 2 chains expand. In contrast, stimulation of the cells with α-GalCer, results in the expansion of iNKT cells bearing numerous Vβ chains. Because α-GalCer is a much more potent agonist than iGb3, this suggests that the Vβ8.2, 7, and 2 iNKT cells are preferentially selected due to the fact that they can overcome signaling thresholds necessary for positive selection.

Conventional T cells that express autoreactive TCRs are eliminated in the thymus by a process called negative selection. Several lines of evidence support the premise that iNKT cells are also subject to negative selection. First, iNKT cell numbers are drastically reduced in fetal thymic organ cultures stimulated with the agonist α-GalCer but not vehicle or β-GalCer (205). Consistent with these findings, thymocytes treated with α-GalCer exhibited a marked reduction in iNKT cell compartment, as did mice overexpressing CD1d (205). iNKT cells do not express the CD8 co-receptor. This is likely due to the fact that $CD8^+$ iNKT cells are deleted due to the fact that CD8 binding to CD1d induces such strong signals that negative selection occurs. This is supported by the fact that $NK1.1^+$ T cells are drastically reduced in transgenic mice in which the CD2 promoter drives CD8.1 expression, thereby allowing its expression all T cells (206). The reduction is likely due to the fact that most of the iNKT cells in CD8.1 mice are deleted. Taken together, these data suggest that high signaling potentials decrease iNKT cells in a manner consistent with negative selection. The cells that mediate negative selection have not been identified, but some studies suggest dendritic cells are reasonable candidates (205, 207).

iNKT cell development: differentiation and maturation in the thymus and periphery

Following their selection, iNKT cells undergo a programmed differentiation and maturation sequence that can be tracked by the modulation of HSA, CD44 and NK1.1 surface expression (Figure 6). The earliest iNKT cell precursors are found in the thymus as HSA^{hi} $CD69^+$ $CD4^+$ $CD8^-$ CD1d-$tetramer^+$ cells (208). These cells are absent in CD1d deficient mice, suggesting that they arise during or following positive selection. After

their selection, iNKT cells downmodulate HSA expression (HSA^{lo}) and then upregulate CD44 and NK1.1 in three defined stages (208, 209). Stage I is characterized as $CD44^{lo}NK1.1^{-}$. These iNKT cells are able to secrete copious amounts of IL-4 upon stimulation and begin a robust expansion in the thymus. The Stage II cells, $CD44^{+}NK1.1^{-}$, secrete both IFN-γ and IL-4. It is at Stage II that most iNKT cells exit the thymus and undergo terminal maturation in the periphery. There are; however, a small subset of cells that remain in the thymus in order to complete the final stages of maturation. Stage III of iNKT cell development is characterized by the upregulation of NK1.1. These $CD44^{+}NK1.1^{+}$ stop proliferating and secrete high levels of IFN-γ following agonist stimulation.

Signaling requirements for iNKT cell development

Because conventional and iNKT cells express an αβ TCR, some signaling pathways necessary for their development are expected to be conserved. Indeed, like conventional αβ T cells, iNKT cell development is blocked in CD3 $\zeta/\eta^{-/-}$, ZAP-70 $^{-/-}$, and Lck $^{-/-}$ mice (116, 122, 129, 210, 211). In each of these knockout lines, DP precursor cells are present. Thus, iNKT cell development is likely arrested in these mice due to inefficient TCR signaling (116, 124, 129). Pre-TCRα (PTα) signaling is also necessary for iNKT cell development. PTα deficient mice lack iNKT cells, but also lack the necessary DP precursor cells necessary for iNKT cell development (212). Irradiated $J\alpha18^{-/-}$ mice were reconstituted with congenic mixed bone marrow chimeras using wild type and $pT\alpha^{-/-}$ mice. iNKT cells did not develop from $pT\alpha^{-/-}$ donor cells but did develop from the wild type cells. $PT\alpha^{-/-}$ cells did not produce iNKT cells, despite the presence of

CD1d expressing DP T cells from the wild type cells, suggesting that PTα signaling is required for iNKT cell development (213).

Despite these similarities, iNKT cells require other signals that are not necessary for the development of conventional T cells. For example, homotypic interactions between SLAM (Slamf1) or LY108 (Slamf6) are critical for normal iNKT cell development, but are dispensable for conventional T cell development (214). Mixed bone marrow chimeras of 1:1 Slamf1$^{-/-}$CD1d$^{-/-}$ and Slamf6$^{-/-}$ or Slamf6$^{-/-}$CD1d$^{-/-}$ and Slamf1$^{-/-}$ produce very few iNKT cells when transferred into Jα18$^{-/-}$ hosts. In this system, each of the donor populations lacks one of the SLAM receptors. Thus, homotypic SLAM interactions are abrogated in each chimera. However, heterotypic interactions between Slamf1 and Slamf6 can occur. Analysis of Slamf6/Slamf1 double mutant mice will be necessary to determine whether loss both receptors will completely block iNKT cell development. Along these lines, the adaptor protein, SAP, or SLAM-associated protein, is also critical for iNKT cell development. This is evident by a lack of iNKT cells in SAP$^{-/-}$ mice and individuals with X-linked lymphoproliferative due to a SAP deficiency (215-217). SAP is necessary for SLAM receptor signaling (218, 219). Furthermore, FynT is coupled to SLAM signaling pathways via SAP. As a result, iNKT cells are reduced in the thymus, spleen and liver mice with a SAP mutation (R78A) that disrupts FynT association (220).

iNKT cells appear to rely on strong signals through the TCR for their development. This is supported by the fact that Itk$^{-/-}$ mice have a significant reduction in the number of iNKT cells in the thymus and periphery (221-223). Itk augments TCR

signaling by increasing intracellular calcium levels via PLC-γ activation. The iNKT cells that do develop in these mice produced less cytokine and have an immature phenotype as evidenced by a decrease in the percentage of cells that expressed HSA, CD44, and NK1.1. $Itk^{-/-}$ iNKT cells had an increase in cell death due to a decrease in T-bet and CD122 expression (221, 223).

Interestingly, early growth response 2 (Egr2) is specifically required for iNKT, but not conventional T cell development. $Rag^{-/-}$ mice reconstituted with $Egr2^{-/-}$ fetal liver cells have a profound reduction in the percentages and numbers of iNKT cells compared to wild type mice, while the DN, DP and mature conventional SP T cells populations are normal (224). The lack of iNKT cells in $Egr2^{-/-}$ mice was also shown to be a result of an increase in cell death compared to wild type mice.

Egr2 is a transcription factor that is a target gene of NFAT. Calcium signaling is critical for Egr2 expression as calcineurin knockout mice have reduced Egr2 expression and iNKT cell development (224, 225). Egr2 is a member of a family of three transcription factors, (Egr1,2, and 3). iNKT cells from the thymus were stimulated with PMA and ionomycin. The expression Egr2 was only weakly induced compared to Egr1 and Egr3 when analyzed by quantitative real-time PCR (224). This suggests that robust calcium signaling is required for Egr2 expression. Thus, it seems reasonable that Itk could be required for sustained increases in intracellular calcium. This would increase NFAT activation and thus Egr2 expression. The exact mechanism by which Egr2 regulates cell survival is unclear, however, it likely regulates developmental pathways prior to the induction of T-bet, as Egr2 is necessary for early whereas T-bet is required

for late stages of development. This might explain why the phenotype in Egr2 and Itk deficient mice are so similar.

T-bet is required for survival and terminal maturation of iNKT cells. T-bet$^{-/-}$ mice have a significant decrease in the percentage of iNKT cells in the thymus, spleen, liver, bone marrow and peripheral blood (226). T-bet is upregulated at each stage of iNKT cell development, reaching its peak levels at the CD44^{+}NK1.1^{+} stage (226, 227). iNKT cell development is halted at the CD44^{+}NK1.1^{-} stage in the absence of T-bet. T-bet is necessary for CD122 upregulation which is critical for controlling T cell homeostasis and survival. T-bet also regulates iNKT cell migration and effector function, as T-bet$^{-/-}$ mice have significantly reduced IFN-γ, Granzyme B, FasL, and CXCR3 mRNA levels. As a result, these iNKT cells are unable to produce IFN-γ in response to α-GalCer stimulation and show limited cytotoxicity. Furthermore, restoration of T-bet expression in immature thymic iNKT cells is sufficient to restore development and effector function of these cells.

Two additional transcription factors are necessary for early iNKT cell development. Rorγt deficiency leads to a reduction in the iNKT cell compartment (198). Rorγt increases the survival of DP thymocytes by upregulating Bcl-xl. This provides a necessary window of survival whereby T cells are able to rearrange the TCR α chain. Rearrangement of the Vα and Jα segments occur in a 5' to 3' direction, with Vα14 and Jα18 segments located at the 3' of the genes. Rorγt knockout mice contain T cells with proximal, or more 5' Vα and Jα rearrangements (228, 229). Thus, the reduction in iNKT

cells in Rorγt deficient mice is due to the fact that iNKT cells do not survive long enough to produce the canonical Vα14Jα18 TCR α rearrangement. iNKT cell development is rescued by ectopic expression of Bcl-xl in Rorγt deficient mice (198).

PLZF, or promyelocytic leukemia zinc finger protein, a member of the BTB-POZ-ZF transcription factor family, also regulates iNKT cell development. PLZF is downregulated over the course of iNKT cell development, with the highest levels expressed at the $CD44^-$ $NK1.1^-$ stage (230, 231). Furthermore, PLZF deficient mice show a significant reduction in the percentage and absolute number iNKT cells in the thymus and peripheral lymphoid organs. These iNKT cells have an immature phenotype and $CD4^+$ iNKT cells predominate (230, 231).

iNKT cells in bacterial infections

iNKT cells are important in the immunoregulation of several bacterial infections. In some cases, this is likely due to the fact that iNKT cells directly respond to infection following engagement of their TCR by foreign-glycolipid/CD1d complexes. This is supported by the fact that several bacterial antigens have been identified that directly stimulate iNKT cells. The first bacterial lipids to be identified were α-glycuronylceramide antigens in the Gram negative, LPS negative α-proteobacteria *Sphingomonas* (232-235). Diacylglyceride antigens derived from *Borrelia burgdorferi* can stimulate iNKT cells both *in vivo* and *in vitro* (236). Other lipid antigens, such as the phosphatidylinositol mannoside, PIM4, from mycobacteria can stimulate iNKT cells *in vitro*, however, a synthetic version of PIM4 did not elicit a response (232, 237). This data suggest that iNKT cells are capable of directly recognizing glycolipid antigens

derived from a variety of bacteria, suggesting that they could play a direct role in regulating the immune response to such pathogens (5, 175).

Outstanding Questions

One of the first steps in TCR signal transduction involves the phosphorylation of the CD3 ITAMs. These signals initiate the induction of multiple intracellular signaling cascades which lead to T cell development, proliferation and effector functions. How these signals are integrated in order to produce an appropriate functional outcome is unclear. The TCR contains six signaling subunits which encode a total of 10 ITAMs, suggesting that these outcomes could be regulated, at least in part, by the CD3 ITAMs.

Signaling through ten ITAMs in multiple subunits could be useful in signal amplification and/or discrimination. A role for signal amplification is evidenced by the high number of ITAMs associated with the TCR. Alternatively, TCR signaling is organized into two distinct signaling modules, the CD3 γε/δε heterodimer and the CD3 ζζ homodimer, could provide a mechanism whereby signal discrimination could be imparted.

Roles for each of these signaling outcomes could be important during thymocyte development and peripheral T cell functions. T cell development occurs under conditions in which low levels as TCR surface expression are actively maintained. Furthermore, the outcome of selection is determined by the magnitude of the signals generated following TCR recognition of low affinity self-ligand/self-MHC complexes. Furthermore, distinct signaling pathways are necessary to support positive and negative selection. Thus, signal

amplification or discrimination could be necessary in order to overcome signaling thresholds necessary for or to induce the distinct signaling pathways that support positive or negative selection.

In mature single positive T cells, TCR levels are markedly increased compared to DP cells. This is likely due to the fact that T cells must vigorously respond to infections with pathogens. Thus, high TCR surface expression and additive ITAM signaling might be required such that sufficiently large signals are generated at times when the abundance of foreign antigen and the number of antigen specific T cells are limiting. Alternatively, signal discrimination might be important in organizing an appropriate immune response to dangerous versus innocuous foreign antigens. These are not an exhaustive list of possibilities. However, these offer viable reasons whereby a complex, multifaceted T cell receptor could have evolved to shape the immune response by generating a pool self-tolerant T cells.

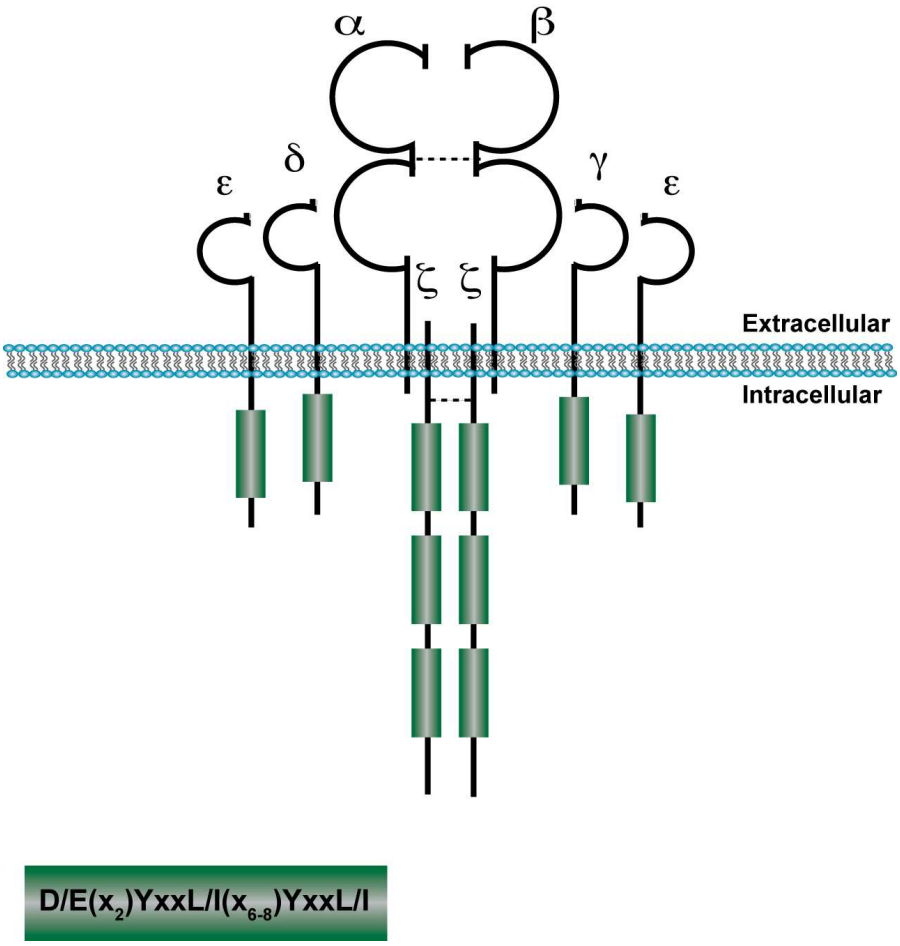


Figure 1: The T cell Receptor Complex. The TCR is a multisubunit complex consisting of a clonotypic αβ heterodimer that is responsible for ligand recognition, and the non-covalently associated intracellular signaling chains CD3 γ, δ, ε and ζ. The stochiometry is shown above. Each CD3 chain contains at least one copy of a signaling motif termed an ITAM with the conserved sequence shown in the green box. There are 10 ITAMs in the TCR complex (denoted by green boxes). CD3 γ, δ and ε have one ITAM per chain while each CD3 ζ molecule has 3 ITAMs per chain**.**

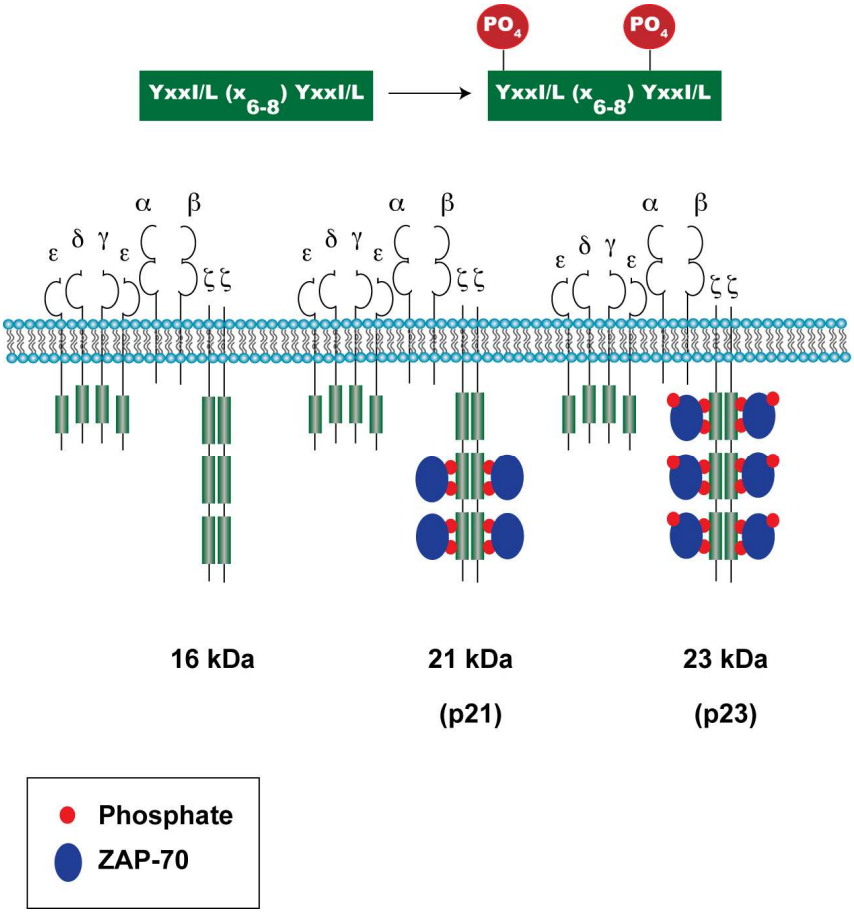


Figure 2: The Phosphorylated Derivatives of CD3 ζ. As an unphosphorylated protein, CD3 ζ has a molecular weight of 16 kDa (left). Following receptor engagement, the CD3 ζ ITAMs are phosphorylated on the two critically spaced tyrosine residues. Phosphorylation of the two membrane distal ITAMs yields a protein of 21 kDa, or p21, (center). When all three ITAMs are fully phosphorylated, this yields the 23 kDa, or p23, form (right) of CD3 ζ. P21 is constitutively associated with inactive ZAP-70, while p23 is complexed to activated ZAP-70.

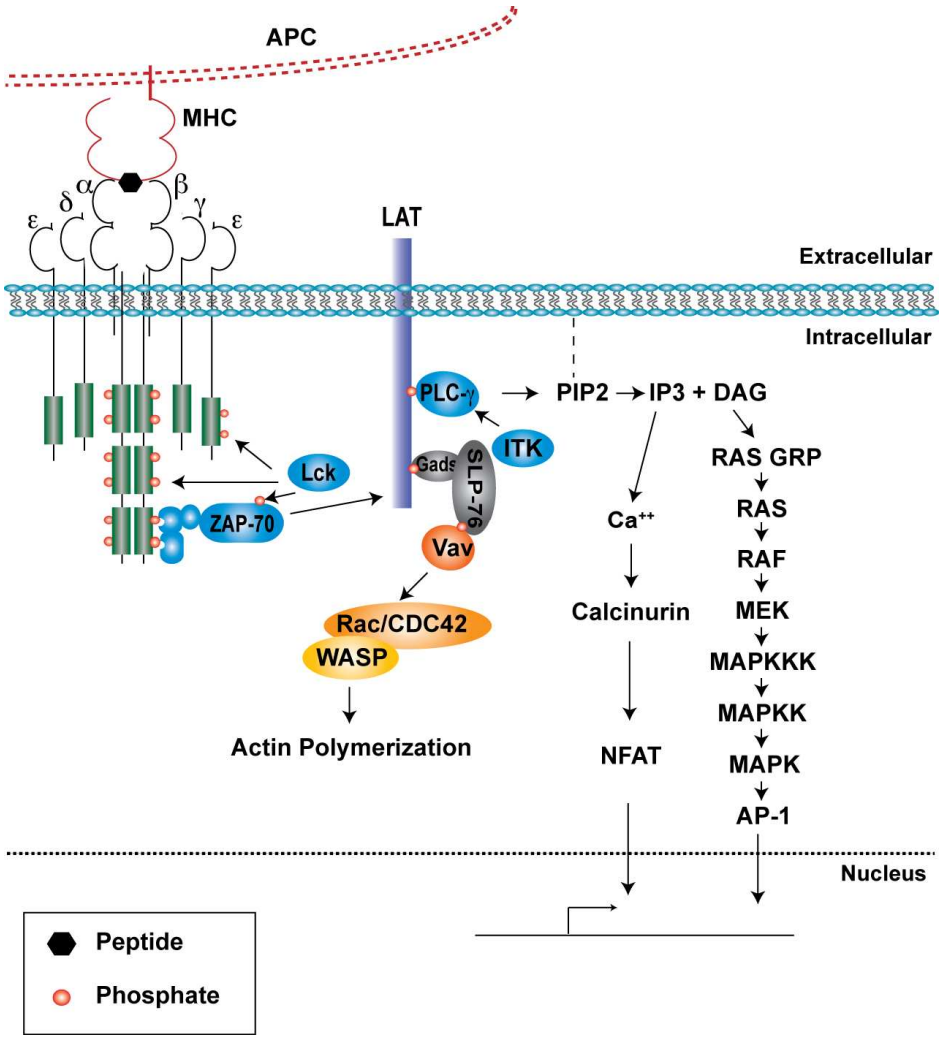


Figure 3: TCR signaling pathways. TCR signaling is initiated following TCR interactions with peptide/MHC complexes. The CD3 ITAMs are biphosphorylated on tyrosine residues by the Src kinases, Lck and/or Fyn. This results in the recruitment of ZAP-70 via its tandem SH2 domains. ZAP-70 is then activated, and phosphorylates and activates a number of downstream adapter and effector molecules, including LAT, SLP-76, Vav, Itk, and PLC-γ. This contributes to transcription factor translocation into the nucleus, where genes important for T cell development, differentiation and effector function are transcribed.

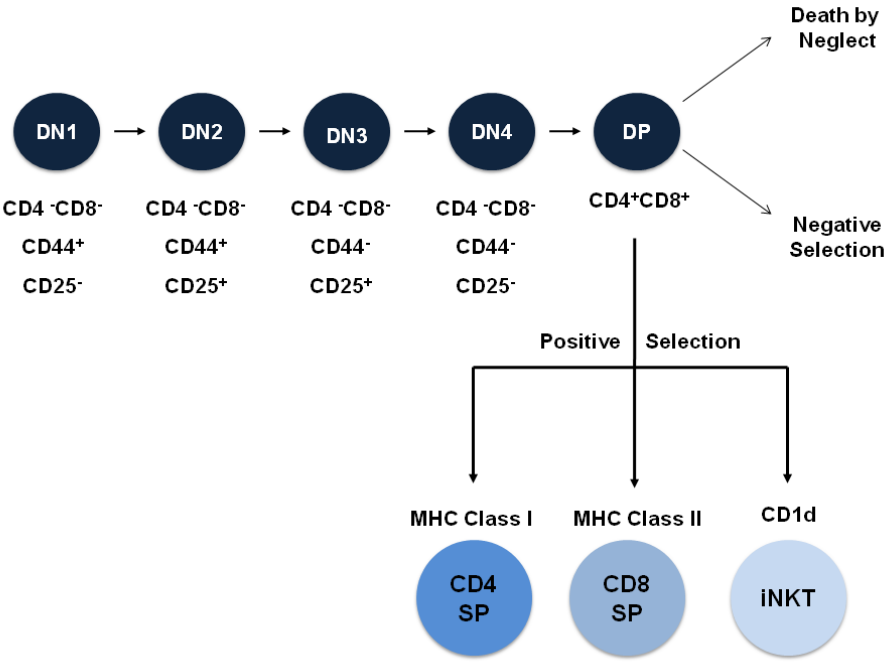


Figure 4: αβ T cell Development in the thymus. αβ T cell development is initiated once bone-marrow derived progenitors migrate into the thymus. Distinct stages of development are identified by the modulation of the cell surface proteins CD4, CD8, CD44, and CD25. During the first stages of development, T cells are $CD4^{-}CD8^{-}$ (DN). DN T cells progress through four stages of development, $CD44^{+}CD25^{-}$ to $CD44^{+}CD25^{+}$ followed by $CD44^{-}CD25^{+}$, and finally $CD44^{-}CD25^{-}$ (DN1, 2, 3, 4). DN4 cells upregulate the co-receptor molecules CD4 and CD8 (DP). DP cell undergo selection. MHC class I, class II, and CD1d restricted TCRs yield CD8, CD4, and iNKT cells respectively.

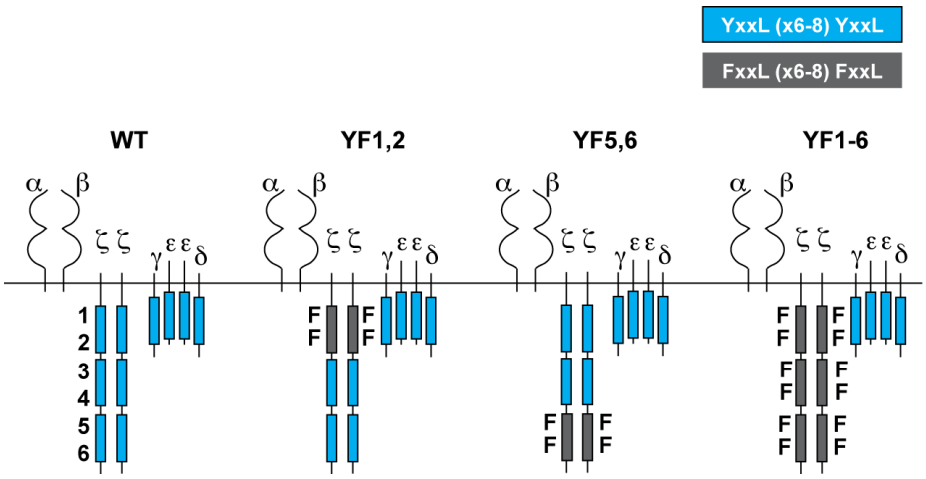


Figure 5: CD3 ζ ITAM composition of CD3 ζ transgenic mice. CD3 ζ transgenic mice contain tyrosine to phenylalanine substitutions in selected ITAMs are shown. Phenylalanine residues cannot be phosphorylated thereby blocking function. Blue boxes represent wild type ITAMs. Grey boxes represent mutated ITAMs. Y = tyrosine, F = phenylalanine, x = any amino acid, YF = tyrosine to phenylalanine substitution. Numbers represent the 6 tyrosine residues from the amino to carboxy terminus.

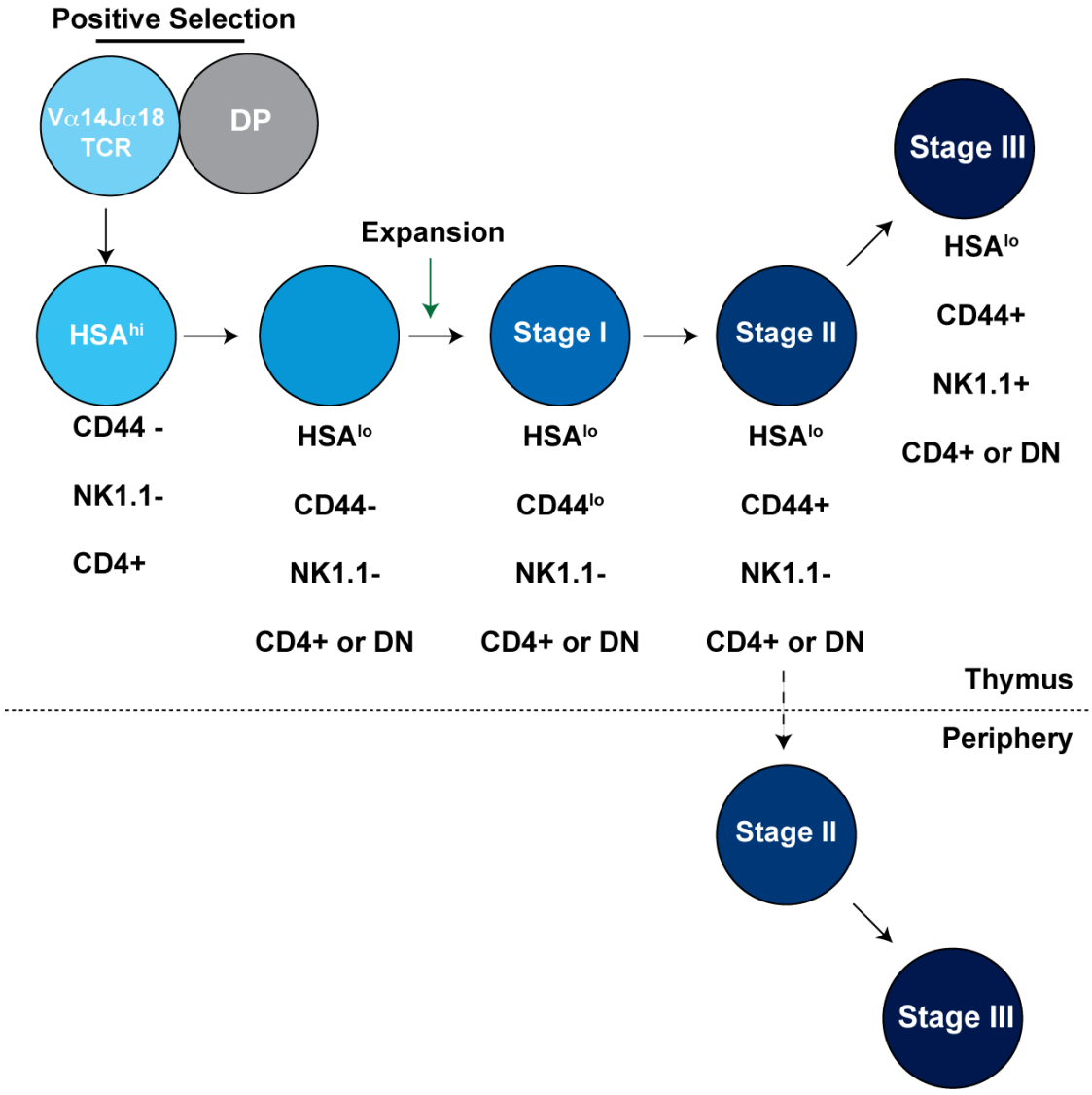


Figure 6: iNKT cell development and differentiation. iNKT cells develop in the thymus from $CD4^+CD8^+$ (DP) precursor cells. iNKT cells are positively selected following TCR interactions with glycolipid/CD1d complexes present on immature $CD4^+CD8^+$ (DP) thymocytes. Following selection, iNKT cells undergo maturation in the thymus and periphery with HSA, CD44, and NK1.1 differentially modulated in distinct stages as shown above.

CHAPTER II

MATERIALS AND METHODS

Transgenic Mice

CD3 ζ transgenic mice (TCR ζ transgenic) were previously described (52). Briefly, the CD3 ζ transgenic mice contain tyrosine to phenylalanine substitutions on the first, third or all three CD3 ζ ITAMs. The CD3 ζ transgene is driven by VA-CD2 transgenic cassette (46, 238). All of the mice were generated on a C57BL/6 background. Mice were termed YF1,2, YF5,6, and YF1-6 denoting mutations on the membrane proximal, distal, or all three CD3 ζ ITAMs, respectively. YF1,2 and YF5,6 have equivalent TCR density and contain equivalent numbers of available ITAMs (8/10) in the TCR complex, however, the YF1,2 lines retains the ability for form the phosphorylated 21 kDa form of CD3 ζ (p21).

MHC class-I-restricted αβ TCR transgenic lines included HY (H-2^b), P14 (H-2^b), OT-I (H-2^b), and C10.4 (MHC class Ib molecule, H2-M3), whereas the MHC class II-restricted mice consisted of OT-II (H-2^b), D011.10 (H-2^d), and 5C.C7 (H-2^k). HY/Rag on H-2^b and H-2^d backgrounds, 5CC7/Rag on H-2^k and H-2^b backgrounds were obtained either from Taconic Farms or through the National Institute of Allergy and Infectious Diseases/Taconic Farms emerging models program.

All mice were housed in a Specific Pathogen Free facility on the North Campus of UT Southwestern Medical Center or the South Campus Barrier Facility (Dallas, TX).

All mouse procedures were carried out in accordance with Institutional Animal Care and Use Committee approved protocols.

Antibodies and CD1d Tetramers

The following antibodies from BD Biosciences (San Jose, CA) were used for flow cytometry analysis: anti-mouse fluorescein isothiocyanate (FITC)-conjugated CD3 ε, CD8, HSA (CD24); phycoerytherin (PE)-conjugated CD1d, CD45 (B220), CD122, LY108, NK1.1; phycoerytherin-Cy5 (PE-Cy5) CD3 ε; CD8; phycoerytherin-Cy7 (PE-Cy7) CD8, allophycocyanin (APC) CD4, CD44, NK1.1; APC-Cy7 conjugated CD45 (B220), CD11b; Peridinin-chlorophyll-Cy5.5 (PerCpCy5.5) CD4; and AlexaFluor647-CD3 ζ (CD247, pY142, fifth of six tyrosines in ζ). Antibodies from eBiosciences (San Diego, CA) included: Pacific Blue-conjugated CD3 ε, IgG2b; CD44 PeCy7, PE-Texas Red CD62L, and CD69. PE and APC labeled murine PBS57-loaded CD1d tetramer and unloaded control tetramer were obtained from the NIAID Allergy and Infectious Diseases core tetramer facility (http://tetramer,yerkes.emory.edu). A mAb recognizing the HY TCR (T3.70) (provided by Dr. Hung-Sia The, University of British Columbia, Vancouver, Canada) was purified from hybirdoma culture supernatants and labeled with FITC. For blocking Fc recpetors, culture supernatants from a 2.4G2 (anti-FcRII) hybridoma cell lines were used.

The following antibodies were used for western blot analyses: purified α-CD3 ζ (CD247, pY142, fifth of six tyrosines in ζ, BD Biosciences, San Jose, CA), mAbs and/or polyclonal antisera to the TCR ζ subunit (6B10.2) and Zap-70 (1E7.2 mAb or 1222-12,

1225-14, or 1600 polyclonal antisera) (115, 239), and anti-phospho-Zap-70 antisera (Y493 and Y319, Cell Signaling Technologies, Danvers, MA).

Cell isolation

Intrahepatic lymphocytes (IHL) were isolated following liver profusions. In brief, mice were anesthetized with Avertin, the peritoneal cavity was exposed to reveal the inferior vena cava. Blood flow through the inferior vena cava was restricted by tying off the vein above the kidney with surgical thread. The portal vein was cut in order to allow fluid to flow out of the liver. A 22 gauge needle was inserted into the right atrium of the heart, and 10 ml ice-cold DPBS was profused though the liver. The liver was harvested into cold Hanks Balanced Salt Solution (HBSS, Mediatech, Inc., Herndon, VA), and crushed though wire mesh. The liver extract was subsequently washed in HBSS and centrifuged at 1200 rpm for 10 minutes at 4°C. The pellet was resuspended in a 35% Percoll (Amersham Biosciences, Piscataway, NJ) in HBSS supplemented with 200 U/ml of Heparin (Sigma-Aldrich, St. Louis, MO). The resuspended cells were then overlayed onto a 67.5% Percoll/HBSS solution. Cells were centrifuged through this gradient at 600 x *g* for 20 minutes. The IHL were removed and washed thoroughly in 40 ml HBSS (containing 2% FBS) to remove all of the remaining Percoll.

Single cell suspensions of lymphocytes were isolated from the thymus, lymph node, and spleen. The cell suspensions were washed in cold PBS and resuspended in appropriate buffers. Red blood cells (RBC) were lysed using an RBC lysis buffer (0.1 mM EDTA, 155 mM ammonium chloride, 10 mM potassium bicarbonate, pH 7.2-7.4). The remaining cells ells were washed twice in DPBS.

In order to isolate bone marrow, the spine, femur and tibia were isolated and debrided of muscle. The remaining material was ground in 20 ml of HBSS using a mortar and pestle. The cells ells were then washed one time in HBSS.

Flow cytometry for surface and intracellular proteins

For the analysis of cell surface proteins, single cell suspensions from various tissues were first generated as described above. Five x 10^5 to 2.5 x 10^6 cells were added to each well of a Costar 96 well U-bottom plate (Corning, Inc., Corning, NY) or into a 5 ml polystyrene round bottom tube (Becton Dickenson, San Diego, CA). Cells were pretreated with 150 - 300 µl of culture supernatant containing a monoclonal antibody specific for the Fc receptor (2.4G2). After a 10 - 30 minute incubation at 4°C, Fc block was removed by centrifugation. Then, 50-200 µl of fluorochrome-conjugated monoclonal antibodies for an additional 30 minutes at 4°C. When PBS-loaded CD1d tetramers were used (at a dilution of 1/600 - 1/1600), and cells were stained for 1 hour at 4°C. Cells were washed twice with FACS buffer. FACS buffer consisted of 1% FBS, 0.05% Sodium Azide (Sigma-Aldrich, St. Louis, MO) in Dulbecco's PBS with Ca^{++}/Mg^{++} (Mediatech, Inc., Herndon, VA). Data was acquired on the FACSCalibur (BD Biosciences, San Diego, CA) or the LSR II (BD Biosciences, San Diego, CA). Data was analyzed using FlowJo software (Tree Star, Inc, Ashland, OR).

For intracellular cytokine staining, the lymphocytes were surface stained as described above. The cells washed and then fixed and permeablized with the BD Cytofix/Cytoperm Plus Fixation/Permeablization Kit per manufacturer's instructions (BD Biosciences, San Diego, CA).

For intracellular phosphoprotein analysis, 1.5 x 10^6 unstimulated or stimulated cells were resuspended in 300µl DPBS (Mediatech, Inc., Herndon, VA) and were added to 5 ml polystyrene round bottom tubes (Becton Dickenson, San Diego, CA). Then, 300 µl of 8% formalin/PBS was added to the cells then left at RT for 15 minutes at room temperature. Cells were washed once in DPBS, and resuspended in 3.0 ml ice-cold 100% Methanol (Fisher Scientific, Pittsburg, PA) for 10 minutes at room temperature. Cells were washed twice with 1 ml phospho-staining buffer (DPBS/0.5%BSA/0.5% saponin/1mM sodium orthovanadate). Cells were then resuspended in 100 µl of fluorochrome-conjugated antibodies diluted in phospho-staining buffer with antibody, and incubated for 30 minutes at room temperature. The cells were then washed twice in 1 ml phospho-staining buffer. The cells were then washed once in 1 ml of wash buffer (DBPS/0.5%BSA/1mM sodium orthovanadate) and then resuspended in 200 µl of wash buffer for flow cytometric analysis. The cells were acquired on the BD FACSCalibur (BD Biosciences, San Diego, CA).

IFN-γproduction by $CD8^+$ T cells

Splenocytes were isolated as previously described. For IFN-γ production following anti-CD3 ε stimulation, 4x10^6 splenocytes were added to each well of a 24 well plate. Cells were stimulated with 10µg/ml anti-CD3 ε for 24 hours at 37°C. During the final 4 hours of stimulation, Brefeldin A (Epicentre Biotechnologies, Madison, WI) was added to cultures. Cells were then harvested, stained for surface CD4 and CD8. Cells were washed and permeablized, and stained for intracellular IFN-γ using the BD Cytofix/Cytoperm kit per manufacturer's instructions (BD Biosciences, San Diego, CA).

For innate IFN-γ production by $CD8^+$ T cells, splenocytes were cultured in recombinant IL-2 (130 U/ml) alone or rIL-2, rIL-12 (5 ng/ml) and rIL-18 (10ng/ml) for 24 hours at 37°C. Cells were harvested and stained for intracellular IFN-γ as described above.

iNKT Cell Proliferation Assays

Splenocytes were isolated as described above. B cells were depleted using anti-mouse B220 magnetic Dynabeads (Invitrogen Life Sciences, Carlsbad, CA). The B cell depleted splenocytes were then stained with CFSE (0.0313 μM) for 10 minutes at room temperature. After labeling, the cells were washed thoroughly with 10% FBS in Iscove's Modified Dulbecco's Media (IMDM, Mediatech, Inc., Manassas, VA) supplemented with penicillin, streptomycin, L-glutamine, and β-mercaptoethanol. Five hundred thousand cells per well were plated in a Costar 96 well U-bottom plate (Corning Life Sciences, Lowell, MA). Cells were incubated with 100 ng/ml α-galactosylceramide (KRN7000, U.S. Biologicals, Swampscott, MA). The cells were harvested at 24 and 96 hours post-stimulation. The cells were then stained and analyzed by flow cytometry as described above.

Immunoprecipitation and Western Blot Analysis

Cells were lysed in Triton-X lysis buffer containing 20 mM Tris-Cl pH 7.6, 150 mM NaCl, 1.0 mM EDTA, and 1% Triton-X 100. The buffer was supplemented with protease inhibitors (10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μm Benzamidine, 10 μg/ml Pepstatin and 0.5 μm PMSF) and phosphatase inhibitors (0.5 μM sodium

orthovanadate, 1.0 μM sodium fluoride, 0.5 μM sodium molybdate) when appropriate. Cells were lysed at 1 x 10^8 cells/ml for 30 minutes on ice. Lysates were cleared of particulate debris by centrifugation for 15 minutes at 14,000 rpm at 4°C. The supernatants were transferred to a new 1.5 mL microcentrifuge tube and used for immunoprecipitation or analysis of whole cell lysates (WCL).

For immunoprecipitations, 2 - 5 μg of the appropriate antibody and 25 μl Protein A or Protein G sepharose beads were added to each sample. Cells were gently mixed by inversion for 2 hours or overnight at 4°C. Precipitates were then washed in lysis buffer, and boiled in 1 x SDS sample buffer for 5 minutes.

For immunoblot analysis, proteins from immunoprecipitations or in whole cell lysates were resolved by 10 - 12.5 % SDS-PAGE. The proteins were then transferred to polyvinylidene fluoride membranes (PVDF, Millipore Ltd., Bedford, MA) for Western blot analysis. The buffer for the Western blots consisted of a Tris-buffered saline solution containing 0.5% Tween-20 (TBST). After transfer, the PVDF membranes were blocked in TBST supplemented with 4% bovine serum albumin (BSA, Fisher, Pittsburg, PA) for 1 hour at RT. The blocking buffer was then removed, and TBST containing the appropriate primary antibody (supplemented with phosphatase inhibitors when necessary). The membranes were incubated at RT for 1 hr or at 4°C overnight. The primary antibody was removed, and the membranes were washed with TBST three times for 5 minutes at RT. Next, TBST containing the appropriate secondary antibody was added, and the membranes were incubated at room temperature for 1 hour. The membranes were then washed three times in TBST, and analyzed by enhanced

chemiluminescence reactions per manufacturer's instructions (Pierce Biotechnology, Inc. Rockford, IL).

For analysis of total CD3 ζ or ZAP-70 following phosphoprotein analysis, membranes were stripped (62.5 mM Tris-Cl, pH 6.7, 100mM b-ME, 2% SDS) for 30 minutes at 50°C. The membranes were then washed thoroughly and immunoblotted using an anti-CD3 ζ or –ZAP-70 monoclonal antibody.

Thymectomy and Peripheral T cell Survival Assays

Four-week-old mice were thymectomized under Institutional Animal Care and Use Committee-approved procedures. Nine weeks post-thymectomy, the mice were euthanized. Lymph node and spleen were obtained from these mice and analyzed by flow cytometry. Littermates that had been mock thymectomized and/or mice that still retained one of two thymic lobes after the thymectomy served as controls.

Listeria monocytogenes Infections

Recombinant *Listeria Monocytogenes-Ova (*rLM-Ova*)* was kindly provided by Dr. J. Forman (UT Southwestern Medical Center, Dallas, TX). Three days prior to infection, mice were bled retro-orbitally. The mice were then infected with the appropriate dose of rLM-Ova of via tail vein injection. For innate immune response studies, the various mice were injected with 10,000 CFU rLM-Ova. For adaptive immune response studies, the mice were first primed with 2000 CFU rLM-Ova and then challenged with 200,000 CFU rLM-Ova 6 weeks later.

Three days following the primary or secondary infection, mice were euthanized by CO_2 asphyxiation. Livers and spleens were harvested. The liver was weighed, and cut in half. The liver and half of the spleen placed into sterile, purified water. The liver was dissociated using a Dounce homogenizer, and half of the spleen was dissociated through wire mesh. The volumes were then adjusted to 5 and 12 ml for the spleen and liver, respectively. Serial dilutions (neat, 1×10^{-1}, $1x\ 10^{-2}$, 1×10^{-3}) were made from the starting homogenate. Fifty µl of each lysate was added to Brain Heart Infusion (BHI) agar plates that contained 100 µg/ml streptomycin. Two plates were used per dilution. Plates were then incubated at 37°C for 36 hours. Colony forming units (CFUs) were then calculated for each tissue.

The second half of the spleen was placed into DPBS, and a single cell suspension was made as described above. RBCs were lysed, and cells were stained for cell surface proteins and analyzed by flow cytometry as previously described.

Borrelia burgdorferi Infections

Borrelia burgdorferi strain 297 (Bb-297) spirochetes were grown in Barbour-Stoenner-Kelley-II (BSK-II) medium with 6% normal rabbit serum in a 35°C incubator with 5% CO_2. All mice were 4-6 weeks old at the time of infection. Mice were intradermally inoculated with $1.2 - 1.5 \times 10^6$ Bb-297 spirochetes. To ensure mice were infected with *B. burgdorferi*, a 2 mm ear punch was taken from the mice at 14 days post-infection. The biopsies were grown in BSK media for 72 hours at 35°C incubator with 5% CO2. Dark field microscopy was used to confirm the presence of spirochetes. At 2 weeks or 6 weeks post-infection, the mice were sacrificed by halothane overdose and

subsequent cervical dislocation. The tibiotarsal and knee joints were harvested for histological examination.

Histology

Inflammatory arthritis was assessed in control and infected mice by analysis of lymphocyte infiltration into the joints. The knees and tibiotarsal joints were fixed in 10% buffered formalin (Mallinckrodt Baker, Inc., Phillipsburg, NJ) for 6 days at room temperature, inverting every other day. The tissue was then sent to the UT Southwestern Pathology Core (Dallas, TX) for paraffin embedding and sectioning. Seven μm sections were cut, and the tissue was stained with hemotoxylin and eosin. Lymphocyte infiltration into the joint was analyzed by the UT Southwestern ARC Diagnostic Unit (Dallas, TX). A score of 0 – 3 was assigned to the tissue indicating, no, mild, moderate, or severe inflammation, respectively, using a blinded scoring system.

CHAPTER III

THE CONSTITUTIVE PHOSPHORYLATION OF CD3 ζ RESULTS FROM TCR / MHC INTERACTIONS THAT ARE INDEPENDENT OF THYMIC SELECTION

Introduction

T cell development and effector functions are controlled by TCR interactions with peptide-self-MHC complexes. In developing thymocytes, TCR recognition of a select set of self-peptides embedded in MHC molecules can support both positive and negative selection processes (4). These selection processes ensure the development of mature T cells that are tolerant to self-peptide/self-MHC and responsive to foreign peptides presented by self-MHC molecules. Even before selection, the TCR appears to have an intrinsic bias toward the recognition of MHC molecules (240, 241).

Many thymocyte and peripheral T cell functions are controlled by TCR-mediated increases in the phosphorylation of intracellular proteins (4). These TCR-regulated phosphorylation cascades are initiated by at least three distinct families of protein tyrosine kinases, Src, Syk, and Tec. The functions of these kinases coordinate around the ITAMs, a signaling motif that is present in 10 copies in the invariant subunits of the TCR complex (CD3 γ, -δ, -ε, and -ζ) (3, 76). TCR interactions with peptide-MHC complexes result in a transient phosphorylation of two tyrosine residues in each ITAM ($YxxLx_{6-8}YxxL$), which allows the Zap-70/Syk protein tyrosine kinases to bind via their tandem SH2 domains. The ITAM pathway not only provides the basis for TCR signaling but is also involved in BCR and certain Fc- and activating NK cell receptors (144, 242).

The TCR complex is distinct from all other ITAM-containing receptors because it contains 10 ITAMs, whereas most other receptors contain one or two (243). Six of the ten TCR ITAMs are located in the CD3 ζζ homodimer (three ITAMs per chain). After TCR engagement, CD3 ζ appears as two distinct tyrosine-phosphorylated intermediates of 21 and 23 kDa, respectively (p21 and p23) (52). P21 is constitutively phosphorylated in thymocytes and peripheral T cells and can complex with an inactive population of Zap-70 molecules (48, 49). The constitutive phosphorylation of CD3 ζ results from TCR interactions with MHC molecules, given that p21 levels are reduced 5- to 15-fold in MHC class II/β_2-microglobulin (β_2m) double-knockout mice and are almost absent in TCRα-deficient mice (48, 244) . In addition, p21 is reduced or absent in Lck- and Zap-70-deficient mice (58, 245). The p21 that is present in developing thymocytes maintains TCR expression at a low level, in part through its interactions with the SH2 domain containing Src-like adaptor protein in association with Cbl (41, 42). Elevated levels of p21 have been correlated with autoimmune diseases and/or T cell hyperactivity (246, 247). How the constitutive phosphorylation of CD3 ζ (p21) influences T cell functions is controversial. For example, p21 was reported to enhance the sensitivity of naive T cells to foreign Ag by ~5- to 10-fold (248). In contrast, partial phosphorylation of CD3 ζ (p21-like) reduced TCR-mediated functions, eliciting T cell anergy (47, 249). Still other studies have shown that p21 is not required for positive selection, TCR signal transmission, or T cell anergy (77, 108, 142). Yet, p21 has been reported to influence the effectiveness of negative selection (140). These results leave unanswered the physiological contribution of p21 to T cell biology.

To better understand the functional role(s) of p21 in thymocytes, we have analyzed the regulation of ζ phosphorylation. We report herein that the constitutive tyrosine phosphorylation of CD3 ζ was maintained in thymocytes isolated from both class I- and class II-restricted TCR-transgenic lines when the cells developed in either a selecting or a nonselecting MHC environment. These findings suggested that the intrinsic capacity of the TCR to recognize MHC molecules before repertoire selection resulted in TCR-mediated intracellular signals that contributed to the constitutive phosphorylation of CD3 ζ. Biochemical signaling assays and intracellular staining procedures indicated that the presence of p21 had little impact on signals induced through the TCR. These findings suggest that tonic TCR interactions with MHC molecules elicit a constitutive phosphorylation of CD3 ζ that, under normal physiological conditions, is functionally inert.

Results

P21 can is detected as a constitutively phosphorylated protein in thymocytes from various MHC class I- and II-restricted TCR-transgenic lines

In most T cell lines, TCR ligation is required for the induction of two tyrosine-phosphorylated derivates of CD3 ζ, which are defined by their two distinct molecular masses of 21-kDa (p21) and 23-kDa (p23) (Figure 7 A, lane 2 vs lane 1) (46). p21 is phosphorylated on all four tyrosines in the two-membrane distal ITAMs, whereas p23 is fully phosphorylated on all three ITAMs (46). Unlike cultured T cell lines, p21 is constitutively tyrosine phosphorylated in both thymocytes and peripheral T cells due to

TCR interactions with MHC molecules (46, 49, 50). This was evident after Western blot analysis of CD3 ζ precipitates prepared from unstimulated thymocyte lysates, which revealed an abundant constitutive tyrosine phosphorylation of CD3 ζ (p21) (Fig. 7 A, lane 3). p23 was detected after TCR cross-linking, although it was present at much lower levels than p21 (Figure 7 A, lane 4). Prolonged exposures did reveal some p23 expression in unstimulated samples (data not shown).

We wanted to determine whether p21 phosphorylation resulted from TCR interactions with self-peptide/self-MHC molecules involved in positive and/or negative selection, and whether the TCR density influenced its formation (48, 244, 245). To answer these questions, thymocytes from several different TCR-transgenic lines were analyzed for the levels of p21 and the 16-kDa nonphosphorylated form of CD3 ζ (p16). Consistent with previous reports, p21 was detected in unstimulated thymocytes from C57BL/6 mice and all class I (HY, P14, OT-I, C10.4)-and class II (OTII, 5C.C7, D011.10)-restricted TCR-transgenic lines examined (Figure7 A, lanes 5, 7, and 9; data not shown and (49, 50, 169). Furthermore, stimulation of the thymocytes with agonist peptide-loaded APCs induced p23 (Figure 7 A, lanes 6, 8, and 10). Using a normalized value of 1 for the p21:p16 ratio in C57BL/6 thymocytes, the p21:p16 values ranged from 0.49 ± 0.08 (n = 3) for the HY mice to 1.1 ± 0.2 (n = 3) for the P14 line (Figure 7 B and Table II). Thymocytes from the various mice were also analyzed by flow cytometry for the cell surface expression of CD4, CD8, and CD3 (Figure 8). There was a wide range in the percentage of positively selected cells, from 15.1 ± 1.8% (P14) to 49.4 ± 3.0% (5C.C7) (Table II). The TCR density was also markedly varied, ranging from an MFI of

34 (C57BL/6) to 201 (5C.C7) (Table II). In thymocytes from wild-type C57BL/6 mice, quantitative measurements indicated that p21 represented 4.0 ± 3.1% (n = 4) of the total available pool of CD3 ζ, with most phospho-ζ associated with the TCR (Figure 9, A and B). A comparison of the p21/p16 ratios, the percentage of positively selected thymocytes, and TCR density revealed no obvious relationship between any of these parameters, leaving unresolved the mechanism by which p21 is constitutively present (Table II).

Several alternative explanations have been proposed to account for the constitutive phosphorylation of CD3 ζ including coreceptor interactions with MHC molecules and/or in vitro experimental manipulations (49, 250). To address these possibilities, we characterized the phosphorylation state of CD3 ζ in mice wherein coreceptor interactions were eliminated. The levels of p21 in thymocytes isolated from wild-type and CD4-deficient mice were determined. P21 was maintained at equivalent levels in thymocytes from CD4-null mice when compared with wild type mice (Figure 10, lane 1 vs. lane 2). In addition, the constitutive phosphorylation of p21 was analyzed in thymocytes from TCR-transgenic male and female HY mice. In male HY mice, >85–95% of thymocytes are $CD4^{-}CD8^{-}$ because of the deletion processes. P21 was constitutively present in these cells, suggesting that neither CD4 nor CD8 coreceptor molecules were necessary for its formation (Figure 10, lanes 3 and 4). This is consistent with earlier findings that p21 is present in CD4/β_2m double-knockout mice, a condition that eliminates all coreceptor interactions with MHC (48). To examine the contribution of temperature-regulated in vitro manipulations of CD3 ζ phosphorylation, one thymic lobe isolated from a mouse was immediately lysed and processed by Dounce homogenization,

Table I. *Regulation of p21 expression in C57BL/6 and TCR-transgenic mice*

Mouse Line	p21:p16 Ratio[a]	MHC Restriction	% $CD4^+CD8^-$	% $CD4^-CD8^+$	TCR MFI[b]
C57BL/6	1.0 ± 0.3	H-2^b	10.5 ± 2.0	4.7 ± 0.5	34.4 ± 4.5
HY	0.5 ± 0.1	H-2D^b	8.4 ± 1.2	15.5 ± 1.8	144.3 ± 8.5
P14	1.1 ± 0.2	H-2D^b	2.7 ± 0.2	15.1 ± 1.8	106.3 ± 4.0
OTII	1.0 ± 0.3	IA^b	28.8 ± 6.9	1.2 ± 0.5	134.0 ± 23
5CC7	0.7 ± 0.2	IE^k	49.4 ± 3.0	6.1 ± 1.4	201.0 ± 6.0

[a] The p21:p16 ratios in the various mice were determined from analyses of at least three mice per group. The ratio was normalized to a value of 1 in the C57BL/6 mice, and all other mice were compared with C57BL/6 mice. The phenotypic analyses were undertaken with 6- to 8-wk-old mice (n = 3–4 mice/line). Data are the average ± SEM.

[b] MFI, Mean fluorescence index.

eliminating any in vitro temperature and cell suspension variability, whereas the second lobe was processed under standard conditions. P21 was evident in both samples (Figure 10, lanes 5 and 6). Overall, these findings indicated that direct TCR interactions with MHC molecules were primarily responsible for the formation of p21.

The constitutive phosphorylation of p21 was present in thymocytes developing in nonselecting MHC environments

We considered two hypotheses to account for the constitutive presence of p21 in thymocytes. First, p21 could result from TCR interactions with MHC complexes independent of positive and/or negative selection. This would be consistent with the proposed structural capacity of the TCR to recognize MHC molecules independent of the peptides bound in the peptide-binding cleft (240, 241). Alternatively, p21 could result from TCR interactions with the diverse array of positively and negatively selecting peptides bound to MHC molecules (4). To address these possibilities, the phosphorylation state of CD3 ζ was compared in thymocytes isolated from several distinct TCR-transgenic lines maintained on selecting or nonselecting MHC backgrounds. All the mice were on a Rag-deficient background to ensure that TCR/MHC interactions in the thymus resulted from the transgenic TCR, with no contributions from endogenous TCR α gene products (251, 252). In initial experiments, 6-wk-old TCR-transgenic female HY/Rag mice were obtained on either selecting (H-2^b) or nonselecting (H-2^d) MHC backgrounds. At this age, only small numbers of thymocytes are positively selected (253). Mature $CD4^-CD8^+$ T cells developed only in the H-2^b female mice, while most thymocytes were blocked at the $CD4^+CD8^+$ stage in an H-2^d environment (Figure 11

A, B) (251, 252). There were no T cells detected in the lymph nodes of female HY/Rag/H2^{d} mice (data not shown). The phosphorylation state of CD3 ζ was examined in equivalent numbers of thymocytes from the two sets of mice. P21 was detected in thymocytes isolated from both the female HY/Rag/H2^{b} and HY/Rag/H2^{d} mice (Figure 11 B). By normalizing the p21:p16 ratio to 1 in the HY/Rag/H2^{b} mice (1.0 ± 0.02, n = 3), we determined that the p21:p16 ratio was higher in thymocytes not undergoing positive selection (1.5 ± 0.1, n = 3). This finding strongly supported the idea that p21 resulted from TCR interactions with MHC molecules, independent of positive and/or negative selection, and that this occurred in developing CD4^{+}CD8^{+} thymocytes.

We next characterized the phosphorylation state of CD3 ζ in a class II-restricted TCR-transgenic line (5C.C7/Rag) where the thymocytes developed in selecting (IE^{k}) or nonselecting MHC backgrounds (IA^{b}). Upwards of 55% of the thymocytes from the 5C.C7/Rag/IE^{k} line were positively selected into the CD4^{+}CD8^{-} subset (Figure 10 C). Mature CD4^{+}CD8^{-} thymocytes (0.5%) and lymph node T cells were completely absent in the 5C.C7/Rag/IE^{b} line (Figure 11 C, data not shown). Again, CD3 ζ was constitutively phosphorylated in both sets of thymocytes, although the levels of p21 were higher in the thymocytes that developed in a selecting environment (Figure 11 D). This increase may have been a consequence of the extremely high TCR density in the 5C.C7/Rag/IE^{k} mice (Figure 11 C). Using a normalized p21:p16 ratio of 1 in the 5C.C7/Rag/IE^{k} line (1.0 ± 0.2, n = 3), we determined that the ratio was 4-fold higher in thymocytes from the nonselecting environment (4.5 ± 2.6).

Based on these results, we concluded that the constitutive phosphorylation of CD3 ζ (p21) was independent of the selecting ligands available in the thymus. The data support the concept that the TCR has a structural design facilitating MHC interactions that are independent of the self-peptide/self-MHC molecules responsible for positive or negative selection.

The constitutively phosphorylated CD3 ζ subunit does not facilitate TCR signal transduction

We next addressed the functional consequences of the constitutive phosphorylation of p21 on TCR signal transmission. Biochemical studies have revealed that Zap-70 is complexed to p21, with the tandem SH2 domains of Zap-70 stabilizing p21 (46). The p21-associated Zap-70 kinase is catalytically inactive, as defined by its weak to nonexistent tyrosine phosphorylation state. Given the high levels of p21 present in thymocytes, we wanted to determine whether the presence of p21 and associated Zap-70 enhanced TCR signaling, as measured by Zap-70 activation. We compared TCR signal transduction with different CD3 ζ transgenic lines that have selected substitutions of tyrosine residues in the ITAMs (52). These lines are called CD3 ζ YF1,2, YF5,6, and YF1–6. The YF1,2 and YF5,6 lines are matched for TCR density, ITAM number, and single-positive and double-positive thymocyte percentages (108). T cells from the YF1,2 line retain p21 and the associated Zap-70 but cannot generate p23. Cells from the YF5,6 line contain weak phospho-ζ forms of 19 and 20 kDa that are detected after TCR stimulation and have no pre-existing phospho-ζ/Zap-70 complexes. All tyrosine residues

in CD3 ζ are substituted with phenylalanine in the YF1–6 line, resulting in no phospho-ζ intermediates (142).

We compared the TCR-induced changes in Zap-70 phosphorylation among the wild-type, YF1,2-transgenic, and YF5,6-transgenic lines. Before stimulation, p21 coprecipitated with Zap-70 in the wild-type and YF1,2 but not the YF5,6 line (Figure 12, lanes 1, 5, and 9). TCR ligation induced the tyrosine phosphorylation of Zap-70 at 10 and 30 min in all three lines, which diminished by 90 min (Figure 12, lanes 2–4, 6–8, and 10–12). Some phospho-CD3 δ and -CD3 ε were detected in the Zap-70 immunoprecipitates at the 10-min stimulation point (Fig. 12, lanes 2, 6, and 10). The magnitude and kinetics of Zap-70 phosphorylation were similar when comparing all three distinct lines (Figure 12, lanes 2, 3, 6, 7, 10, and 11). This was consistent with our earlier findings that agonist peptide-stimulated thymocytes isolated from HY, HY/YF1,2 and HY/YF5,6 TCR-transgenic female mice had very similar patterns and kinetics of Zap-70 and SLP-76 phosphorylation (108). The experiments suggested that the pre-existing p21/Zap-70 complex failed to enhance the magnitude of TCR signaling in thymocytes. We further explored this possibility by examining the amounts of p21 associated with activated Zap-70 before and after receptor ligation. If the constitutively phosphorylated CD3ζ/Zap-70 population contributes to TCR signaling, then the population of activated Zap-70 detected after TCR engagement should include the majority of p21. If p21/Zap-70 is excluded from newly engaged TCRs, then very little should coprecipitate with activated Zap-70. To address these possibilities, we analyzed signaling events in thymocytes from class I- or class II-restricted TCR-transgenic lines (HY and 5C.C7). Cells from the HY mice were

left untreated or stimulated for 10 minutes with agonist peptide-loaded APCs. Phospho-Zap-70 was precipitated with antisera directed against two phosphotyrosine residues that are only phosphorylated when Zap-70 is catalytically active (phosphotyrosine residues 319 and 493). These antisera do not detect nonphosphorylated Zap-70, as evidenced by the absence of Zap-70 in (Figure 13, lanes 1 and 2*)*. In unstimulated lysates, only 9 ± 5% (*n* = 4) of total p21 coprecipitated with phospho-Zap-70, and there was very little detectable phospho-Zap-70 (Figure 13, lane 1). After receptor triggering, both p21 and p23 detectably coprecipitated with active Zap-70 (Figure 13, lane 2). The amount of p21 that coprecipitated with phospho-Zap-70 increased to 13 ± 7% of the total pool of p21, suggesting that the amounts of new p21 induced after receptor ligation were small. After immunodepletion of active Zap-70, the remaining pool of total Zap-70 was immunoprecipitated (Figure 12, *lanes 3* and *4*). The amount of phospho-Zap-70 was very low in these sequential immunoprecipitates, indicating that the majority of active Zap-70 was precipitated with the anti-phospho-active mAb. A significant amount of p21 coprecipitated with nonphosphorylated Zap-70 (Figure 13, lanes 3 and 4). This indicated that the majority of the pre-existing p21/Zap-70 complex was not activated after TCR cross-linking. CD3 ζ was immunoprecipitated next (Figure 13, lanes 5 and 6). Most p21 remained in the lysates after Zap-70 depletion, and additional experiments indicated that 30–40% of p21 was not associated with Zap-70 (data not shown).

The experiments were repeated with thymocytes from the 5C.C7 TCR-transgenic line. In unstimulated cells, 14 ± 3% (*n* = 2) of total p21 coprecipitated with phospho-Zap-70 (Figure 14, lane 1). After TCR engagement, both p21 and p23 detectably

coprecipitated with phospho-Zap-70, with 24 ± 5% of total p21 present in these samples (Figure 14, lane 2). The 11% increase in p21 detected after TCR stimulation could have developed from the pre-existing population of constitutively phosphorylated CD3 ζ and/or newly formed p21 from the nonphosphorylated pool of ζ. Such experiments also suggested that more p21 and p23 are generated as more mature thymocytes are used, likely due to higher surface expression of the TCR (Figure 14, lanes 1–6). Taken together, the findings suggest that the constitutive phosphorylation of p21 and its associated pool of Zap-70 do not enhance TCR signal transmission.

To examine whether only a subset of thymocytes contained p21, we performed intracellular staining with a mAb that specifically recognizes the fifth phosphotyrosine residue in CD3 ζ (pY-142). This tyrosine is constitutively phosphorylated in p21 (46). In both unstimulated and TCR-stimulated $CD4^+CD8^+$ cells from either the C57BL/6 or YF1–6 line, pY-142 (p21) was not detected by intracellular staining (Figure 15 A). In fact, pY-142 (p21) was detected at appreciable levels only in the unstimulated $CD4^+CD8^-$ thymocytes, and these levels increased after TCR cross-linking (Figure 15 A). There were very minimal changes in the levels of phospho-pY142 in the $CD4^+CD8^-$ thymocytes from the YF1–6 line (Figure 15 A). These experiments suggested that the constitutively phosphorylated CD3 ζ was inaccessible to the anti-phospho-ζ mAb in immature thymocytes, the levels of p21 were below the detection limits, and/or the tyrosine at position 142 was not phosphorylated. To examine these possibilities, thymocytes from C57BL/6 mice were sorted into $CD4^+CD8^+$ and $CD4^+CD8^-$ subsets and lysed. The samples were then immunoblotted with the same anti-pY142 mAb (unlabeled version) as

that used for intracellular staining (Figure 15 B). The samples were also immunoblotted with anti-phosphotyrosine mAbs (Figure 15 C). P21 was detected in lysates of $CD4^+CD8^+$ cells that were isolated from wild-type but not YF1–6 mice (Figure 15 C). Taken together, the experiments suggested that p21 is inaccessible or partially blocked from detection in $CD4^+CD8^+$ thymocytes when using intracellular staining techniques. This could account for the inability of p21 to enhance TCR signaling. Second, most of the pre-existing p21/Zap-70 pool was excluded from agonist peptide or Ab induced TCR signaling, which could elicit new p21 and p23 from the previously nonphosphorylated pool of CD3 ζ.

T cell survival in thymectomized mice is regulated by ITAM numbers

The function of p21 in peripheral T cells has yet to be determined. For peripheral T cells, the constitutive phosphorylation of p21 has been correlated with peripheral T cell survival (168). In contrast, a distinct report has shown that $CD4^+$ T cells decline after their adoptive transfer into an MHC class II-deficient environment independent of p21 (169).

To address the role of p21 in regulating peripheral T cell survival, we analyzed the percentage of $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells in control and thymectomized mice 9 wk postthymectomy. Thymectomies were used to eliminate the presence of thymic emigrants to the peripheral T cell pool. Mice used for these assays included C57BL/6 mice and the CD3 ζ-transgenic lines YF1,2, YF5,6, and YF1–6. Upon thymectomy, the number of $CD4^+CD8^-$ T cells in the lymph node was reduced by 11% in C57BL/6 mice

relative to mock-treated controls ($p < 0.001$; Figure 16 A). A similar decrease occurred in mice that contained p21 but lacked p23 (YF1,2), and in mice that lacked both p21 and p23 (YF5,6). In mice that lacked all phosphorylated CD3 ζ forms (YF1–6), the number of $CD4^+CD8^-$ T cells decreased by 19%. An analysis of the $CD4^-CD8^+$ T cells revealed a decrease in the percentage of cells similar to that shown for the $CD4^+CD8^-$ T cells (Figure 16 B). There was, however, no significant decrease in the number of $CD4^-CD8^+$ T cells from the thymectomized YF1–6 line, as had been observed for the $CD4^+CD8^-$ cells ($p > 0.05$; Figure 16 B).

Next, we analyzed the survival of naive $CD4^+$ T cells, as defined by the expression of CD62L (Figure 16 C). The number of naive $CD62L^+CD4^+$ T cells in the lymph node declined by 12% in C57BL/6 mice 9 wk after thymectomy ($p < 0.05$). There was a 7% reduction of these cells in the YF1,2 line. A more substantial reduction of 17% was noticed in the YF5,6 line ($p < 0.01$). The greatest reduction of naive cells was noted in the YF1–6 mice, where a 27% reduction in T cells was detected ($p < 0.001$). We also analyzed the percentage of peripheral T cells in the spleens of control and thymectomized mice. The results were comparable with that noted in the lymph node (data not shown). We also undertook adoptive transfer experiments using CSFE-labeled T cells from the YF lines into C57BL/6 recipients. Again, we were unable to detect statistically significant differences in the survival span of the YF1,2 vs. YF5,6 T cells (data not shown). In summary, p21 did not appear to be critical for regulating T cell survival based on the comparisons of the YF1,2 and YF5,6 lines. The substantial changes seen in the

YF1–6 mice, however, suggested that the number of ITAMs available in the TCR were important for T cell survival in the peripheral lymphoid organs.

Discussion

The CD3 ζ subunit is unique among the ITAM-containing proteins in the TCR, BCR, Fc, and NK cell receptor complexes as it exists as a constitutively tyrosine-phosphorylated protein (p21) when isolated *ex vivo* (242). There has been a considerable effort to define the functional role of the different phosphorylated forms of CD3 ζ, in particular p21 (254). We provide evidence that the constitutive tyrosine phosphorylation of p21 resulted from TCR interactions with MHC molecules in the thymus, independent of the specificity of the TCR for self-peptide/self-MHC molecules. P21 appeared to be functionally inert, given that the development of mature thymocytes and the biochemical changes in Zap-70 activation assessed after TCR triggering were not significantly enhanced or inhibited by its presence. Intracellular staining with anti-phospho-ζ-specific mAbs suggested that p21 was either inaccessible or at levels too low to detect when present in a constitutively phosphorylated form in immature thymocytes.

The constitutive phosphorylation of CD3 ζ was dependent on TCR interactions with MHC molecules, with a 5- to 15-fold reduction in the magnitude of p21 phosphorylation evident in thymocytes isolated from MHC II-β_2m double-deficient mice (48). The presence of some p21 in these mice was consistent with the fact that a low level of MHC class I expression, particularly H-2D^b, is apparent in β_2m-deficient mice (255). That TCR interactions with peptide-MHC molecules elicit p21 formation was supported

by analyses of TCR α- and Zap-70-deficient the positive and/or negative selecting peptides that shape the T cell repertoire are responsible for the constitutive phosphorylation of CD3 ζ. Our current experiments suggest that the TCR interactions with selecting self-peptides/self-MHC are not required for the generation of p21. P21 was evident in thymocytes expressing either class I- or class II-restricted TCRs that were unable to interact with the selecting ligands. We also noted that $CD4^{-}CD8^{-}$ thymocytes from HY TCR-transgenic male mice and cells from CD4-deficient mice contained p21 (48, 50). Given these findings, we propose the following mechanism for the constitutive tyrosine phosphorylation of CD3 ζ. TCR interactions with MHC molecules, independent of the selection environment, activate intracellular signals involving the Src and Syk families of kinases. This is consistent with the reported ability of the TCR to recognize disparate MHC molecules before repertoire selection (240, 241). These interactions may cause an activation and/or redistribution of Lck (and to a lesser extent, Fyn), which phosphorylates the four distal tyrosine residues in the ITAMs of CD3 ζ. Upon their tyrosine phosphorylation, the two C-terminal ITAMs in CD3 ζ are complexed by the tandem SH2 domains of Zap-70, which stabilize p21. CD4 interactions with MHC class II interactions are hypothesized to activate Lck, which in turn phosphorylates CD3 ζ in immature thymocytes (49, 256). In addition, CD8 can bind to MHC class I molecules in immature thymocytes, implicating a possible role for CD8-associated Lck to phosphorylated CD3 ζ (257). Our findings indicate that p21 is independent of CD4 and/or CD8 coreceptor interactions with MHC molecules. However, both CD4 and CD8 could contribute to p21 by stabilizing appropriate TCR/MHC encounters. It remains very

interesting that the Zap-70 that is precomplexed to p21 does not become activated. Current experiments are addressing this issue.

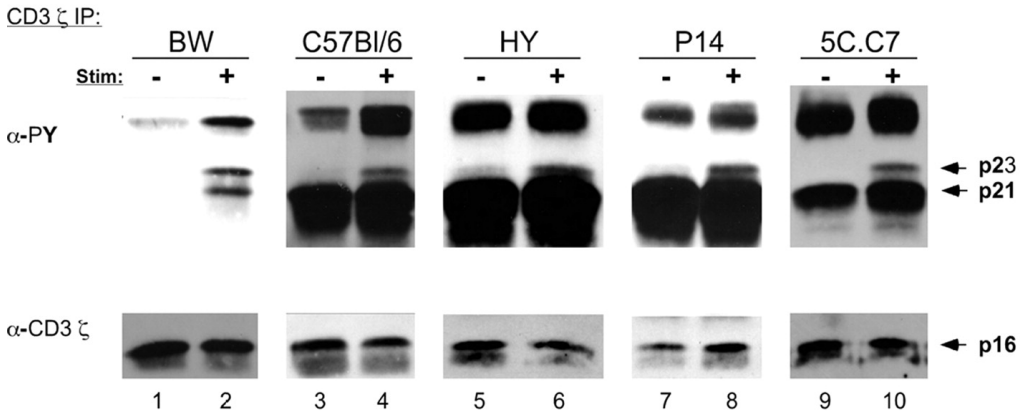
Three cellular fates are classically defined for developing thymocytes: death by neglect if the TCR cannot recognize self-peptide/self-MHC; positive selection; or negative selection (4). Our results suggest an additional step during thymopoiesis, wherein T cell recognition of MHC, independent of positive or negative selection and/or coreceptor expression, induces a biochemical signal resulting in CD3 ζ phosphorylation. This likely reflects the structural capacity of the TCR in the prerepertoire population of thymocytes to recognize peptide/MHC complexes. The consequence of this interaction leading to phospho-CD3 ζ is currently being elucidated but is also likely to contribute to some as yet unidentified peripheral T cell function.

The constitutively phosphorylated p21 could not be detected in $CD4^+CD8^+$ thymocytes by intracellular staining with anti-phospho-ζ-specific mAbs. Attempts to detect this constitutive pool with TAT fusion proteins containing the tandem SH2 domains of Zap-70 linked to GFP or pure 2SH2-GFP were also unsuccessful ((258) and data not shown). Only after TCR triggering or by Western immunoblotting was phospho-ζ detected. These results suggested that p21 was modified, preventing its detection. This could be a result of its distinct intracellular distribution within the cytoskeleton (259, 260). Alternatively, the p21 could be interacting with distinct SH2 domain containing proteins such as SHP-1, Shc, Sts-1, Sts-2, and/or Src-like adaptor protein in addition to Zap-70 (260, 261). The idea that p21 is excluded from de novo signaling events is

consistent with the inability of p21 to enhance TCR signal transduction elicited by Abs or peptides. A recently proposed model suggests that endogenous and agonist peptide/MHC complexes function cooperatively in amplifying TCR signal transduction and subsequent effector functions (262, 263). Our findings also imply that the constitutively phosphorylated CD3 ζ subunit does not contribute to the agonist response. These interpretations are extremely divergent from that published for peripheral T cells where it was proposed that the presence of p21 enhanced peripheral T cell responses 10-fold over T cells wherein p21 was reduced (248, 264). Additional experiments are necessary to account for these discrepancies but may relate to the particular TCR-transgenic line used and/or the *in vivo* Ab manipulations. Finally, p21 has been linked to peripheral T cell survival in some but not all studies. Using thymectomy procedures, we were unable to identify a role for p21 in T cell survival (168, 169). However, such experiments are limited because recent reports have indicated the presence of more than one functional thymus in mice (265). Transfer of the various YF series of mice into appropriate MHC-deficient hosts that express or lack the selecting allele could be another way in which to address this question.

In summary, the constitutive phosphorylation of p21 occurs in thymocytes and peripheral T cells in a mechanism that does not require selecting ligands and/or coreceptor molecules. Under certain disease conditions or autoimmune diseases, changes in the levels of p21 could contribute signals that enhance or inhibit autoimmune progression (173, 246).

A.



B.

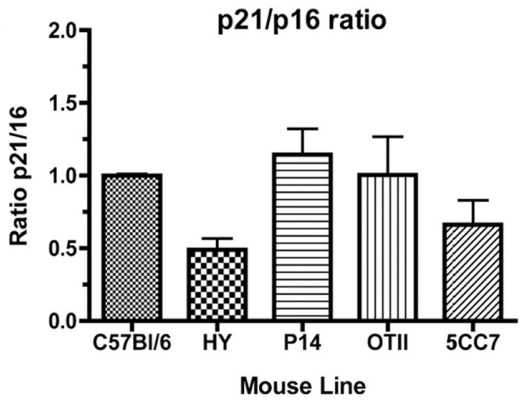


Figure 7: P21 and p23 phosphorylation in cell lines and mice. The constitutive phosphorylation of p21 occurs in thymocytes from all class I- and class II-restricted TCR-transgenic lines examined. *A*, T cells from the BW T cell lines or thymocytes from normal C57BL/6 mice or the TCR-transgenic lines HY, P14, OT-II, and 5C.C7 were left untreated (*lanes 1*, *3*, *5*, *7*, and *9*) or stimulated for 10 min with anti-CD3 ε mAbs (*lanes 2* and *4*) or agonist peptide-loaded APCs for 10 min (*lanes 6*, *8*, and *10*). The cells were lysed in Triton X-100 lysis buffers, and CD3 ζ was immunoprecipitated. The precipitates were resolved by SDS-PAGE and subsequently immunoblotted with anti-phosphotyrosine (-PY) and anti-CD3 ζ mAbs. *B*, The ratios of p21 to p16 were determined from CD3 immunoprecipitates processed as in *A*. For statistical analyses, the lysates from all the mice were processed and analyzed in the same experiment (n = 3 mice/line). Thymocytes from the OTII line were analyzed in a separate experiment and compared with C57BL/6 (n = 4).

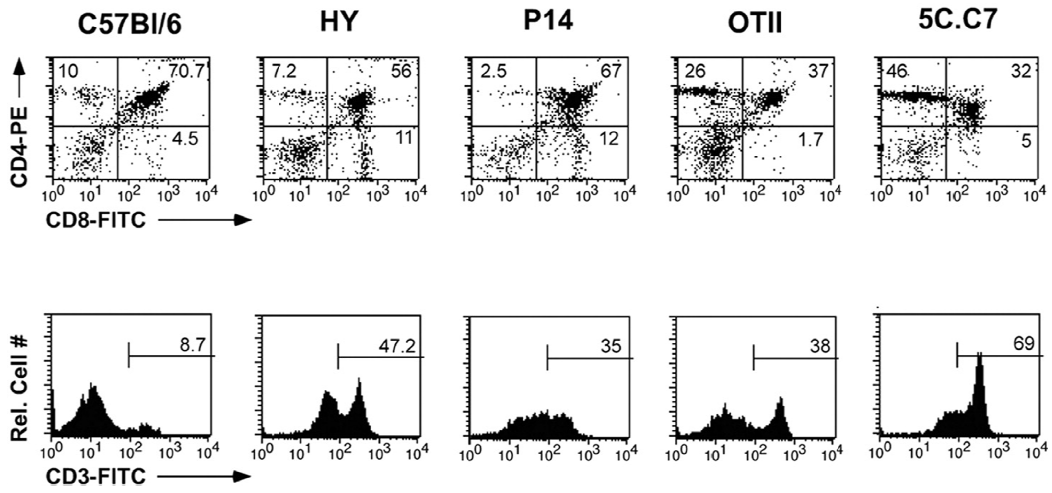


Figure 8: T cell development in distinct TCR trasngenic mice. Thymocytes from the indicated mice were harvested, stained with fluorochrome-labeled mAbs, and analyzed by flow cytometry. Percentages of cells in individual quadrants are indicated. Data are representative of two to three independent experiments. Rel. Cell #, Relative cell number.

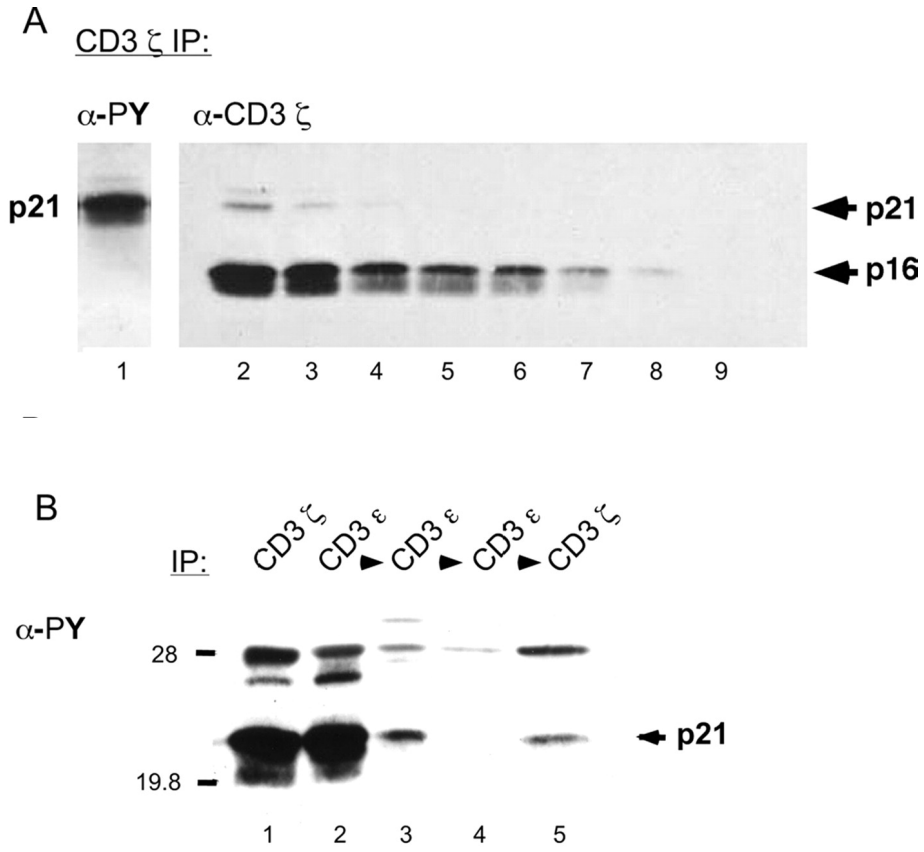


Figure 9: The constitutive phosphorylation of p21 represents a small proportion of total CD3. *A*, The CD3 subunit was immunoprecipitated (IP) from lysates prepared from varying numbers of C57BL/6 thymocytes: 1 x 10^8 (*lane 1*); 3 x 10^8 (*lane 2*); 5 x 10^7 (*lane 3*); 2.5 x 10^7 (*lane 4*); 1 x 10^7 (*lane 5*); 0.5 x 10^7 (*lane 6*); 0.25 x 10^7 (*lane 7*); or 0.125 x 10^7 (*lane 8*) cell equivalents. *Lane 9* is an Ab control lane with no thymocyte lysates. The samples were immunoblotted with anti-phosphotyrosine ((-PY; *lane 1*) or anti-CD3 ζ mAbs (*lanes 2–7*). The weak band at 22 kDa is TCRη (CD3η), an alternative splice variant of ζ. Data are representative of three independent experiments. *B*, The CD3 subunit was either directly immunoprecipitated from lysates prepared from C57BL/6 thymocytes (1 x 10^8, *lane 1*) or was sequentially immunoprecipitated with anti-CD3 ε mAbs three times (1 x 10^8 cells; *lanes 2*, *3*, and then *4*) followed by an anti-CD3 ζ mAb to detect residual p21 not associated with the TCR complex (*lane 5*). The samples were immunoblotted with anti-phosphotyrosine mAbs. Data are representative of two independent experiments.

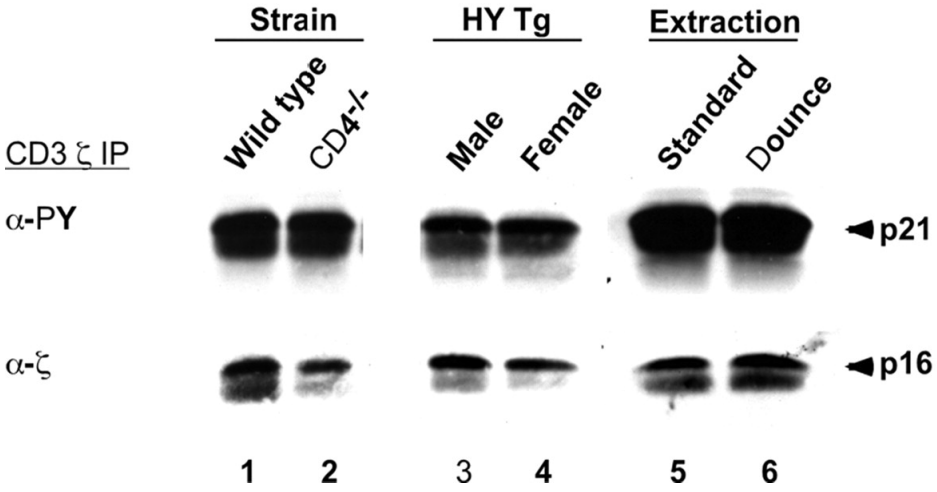


Figure 10: Phosphorylation of CD3 ζ is not dependent or enhanced by co-receptor signaling or temperature. Thymocytes from wild-type (*lane 1*), CD4-deficient (*lane 2*), HY TCR-transgenic (Tg) male (*lane 3*) and female HY TCR-transgenic mice (*lane 4*) were analyzed for the presence of p21 as described in Figure 8. Individual thymic lobes from an individual mouse were processed and analyzed for p21 by normal cell separation and processing (*lane 5*) or by direct Dounce homogenization of the lobe immediately after its removal from the thymus (*lane 6*).

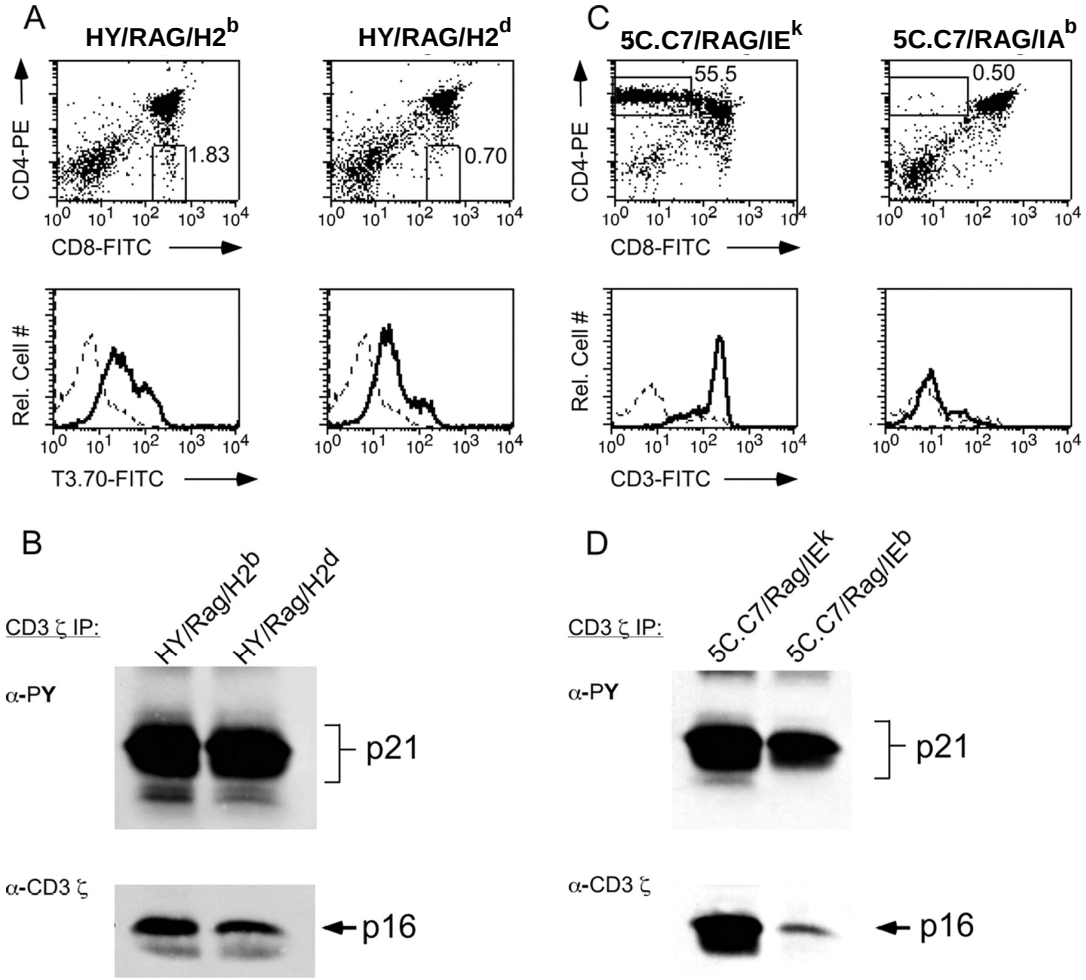


Figure 11: p21 expression is induced by TCR interactions with MHC molecules that are independent of the selecting environment. *A*, Thymocytes from female HY/RAG/H-2^b and HY/RAG/H-2^d mice were stained with a combination of fluorochrome-labeled mAbs as indicated. The stained aliquots were analyzed by flow cytometry. The dashed line in the histograms represents fluorescence levels with control Abs. *B*, The CD3 ζ subunit was immunoprecipitated (IP) from lysates of thymocytes obtained from female HY/RAG/H-2^b and HY/RAG/H-2^d mice (5 x 10^7 cell equivalents/sample). The precipitates were resolved by SDS-PAGE and subsequently immunoblotted with anti-phosphotyrosine (-PY) followed by anti-CD3 ζ mAbs. For statistical calculations, at least three littermates were used per transgenic line. *C*, Thymocytes from 5C.C7/RAG/H-2^k and 5C.C7/RAG/H-2^b mice were analyzed by flow cytometry as indicated. *D*, The CD3 ζ subunit was immunoprecipitated from equivalent numbers of cells (6 x 10^7 cell equivalents/sample), and it was analyzed as described in *B*, Data are representative of three sets of mice. Rel. Cell #, Relative cell number

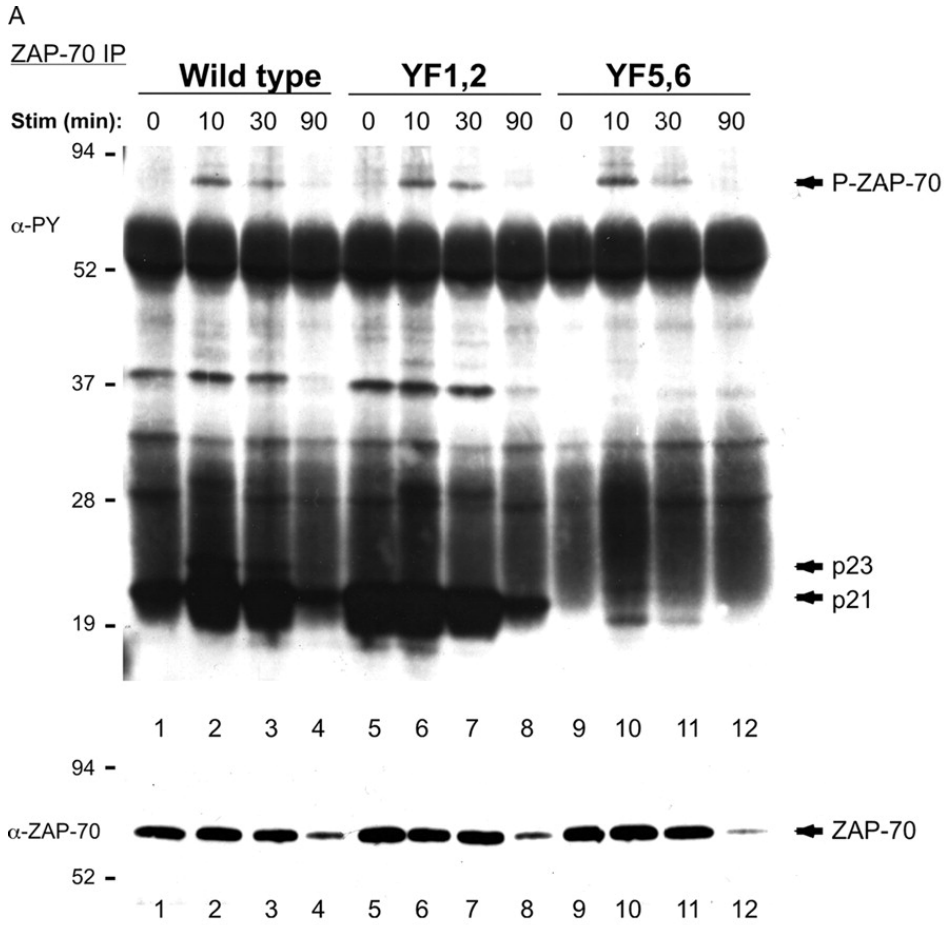


Figure 12: The constitutive phosphorylation of p21 does not augment or inhibit ZAP-70 phosphorylation following TCR crosslinking. Thymocytes from wild-type (*lanes 1–4*), YF1,2 (*lanes 5–8*), or YF5,6 (*lanes 9–12*) mice were either untreated (*lane 1*, *5*, and *9*) or stimulated (Stim.) with anti-CD3 ε mAbs for 10 min (*lanes 2*, *6*, and *10*), 30 min (*lanes 3*, *7*, and *11*) or 90 min (*lanes 4*, *8*, and *12*). At these time points, the cells were lysed, and Zap-70 was immunoprecipitated (IP) with an anti-Zap-70 mAb. The samples were processed for Western blotting with anti-phosphotyrosine (-PY) followed by anti-Zap70 mAbs. Data are representative of three independent experiments.

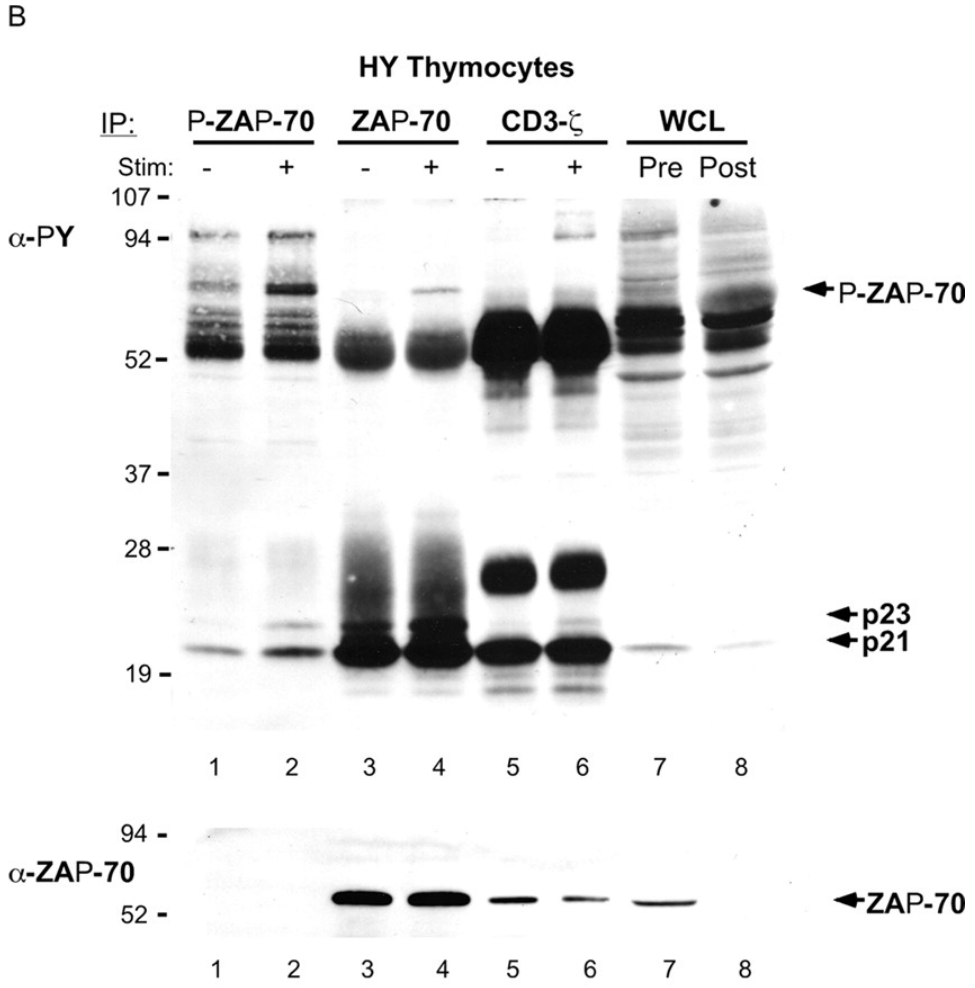


Figure 13: The constitutive pool of p21 is not involved in *de novo* TCR signal transduction in thymocytes from HY TCR trasngenic mice. Thymocytes from female HY TCR-transgenic mice were stimulated with control or agonist peptides for 10 min. The cells were lysed, and the lysates were sequentially precipitated with anti-phospho-Zap-70 polyclonal antisera (*lanes 1* and *2*), followed by anti-Zap-70 antisera (*lanes 3* and *4*) and then anti-CD3 ζ mAbs (*lanes 5* and *6*). The immunoprecipitates were processed for Western immunoblotting with anti-phosphotyrosine and anti-Zap-70 mAbs. An aliquot of the lysates, prepared before and after the anti-Zap-70 immunoprecipitations was analyzed for the levels of Zap-70 (*lanes 7* and *8*). Data are representative of four independent experiments.

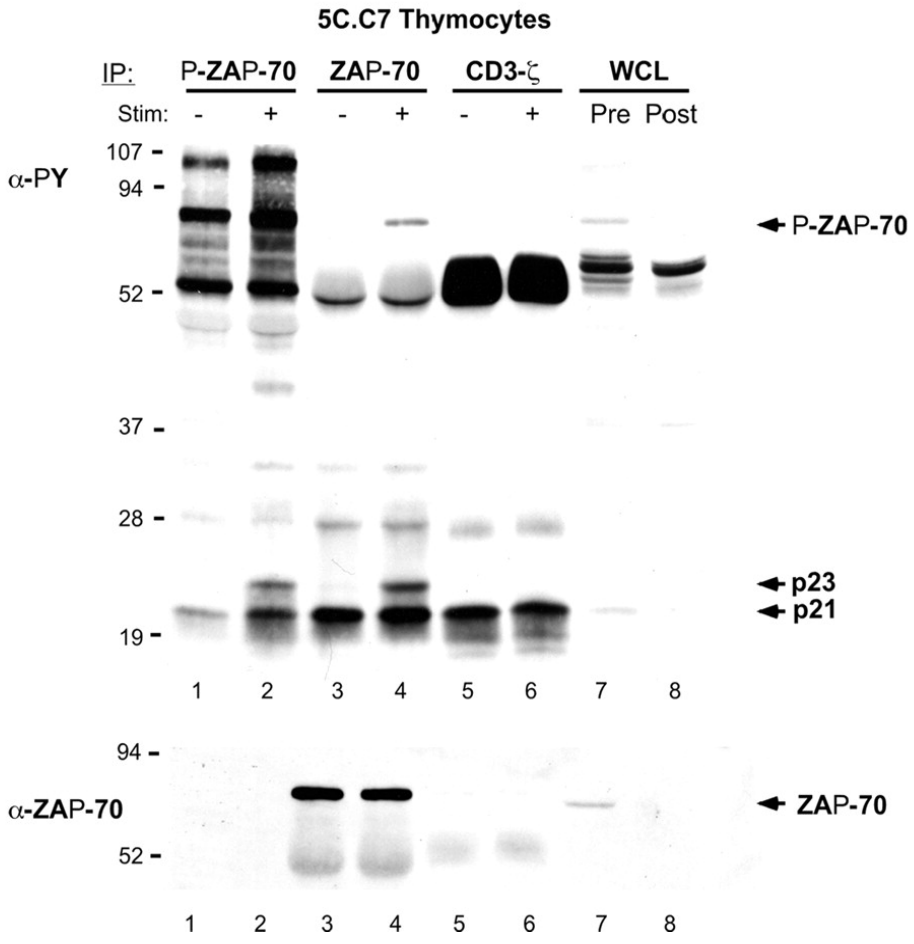


Figure 14: The constitutive pool of p21 is not involved in *de novo* TCR signal transduction in thymocytes from 5C.C7 TCR transgenic mice. Thymocytes from 5C.C7 mice were stimulated with control or agonist peptides for 10 min. Phospho-Zap-70 (*lanes 1* and *2*), Zap-70 (*lanes 3* and *4*), and then CD3 (*lanes 5* and *6*) were sequentially immunoprecipitated and processed as in Figure 13. The levels of Zap-70 in WCL before and after immunodepletion are shown in *lanes 7* and *8*. Data are representative of two independent experiments. WCL, Whole cell lysate.

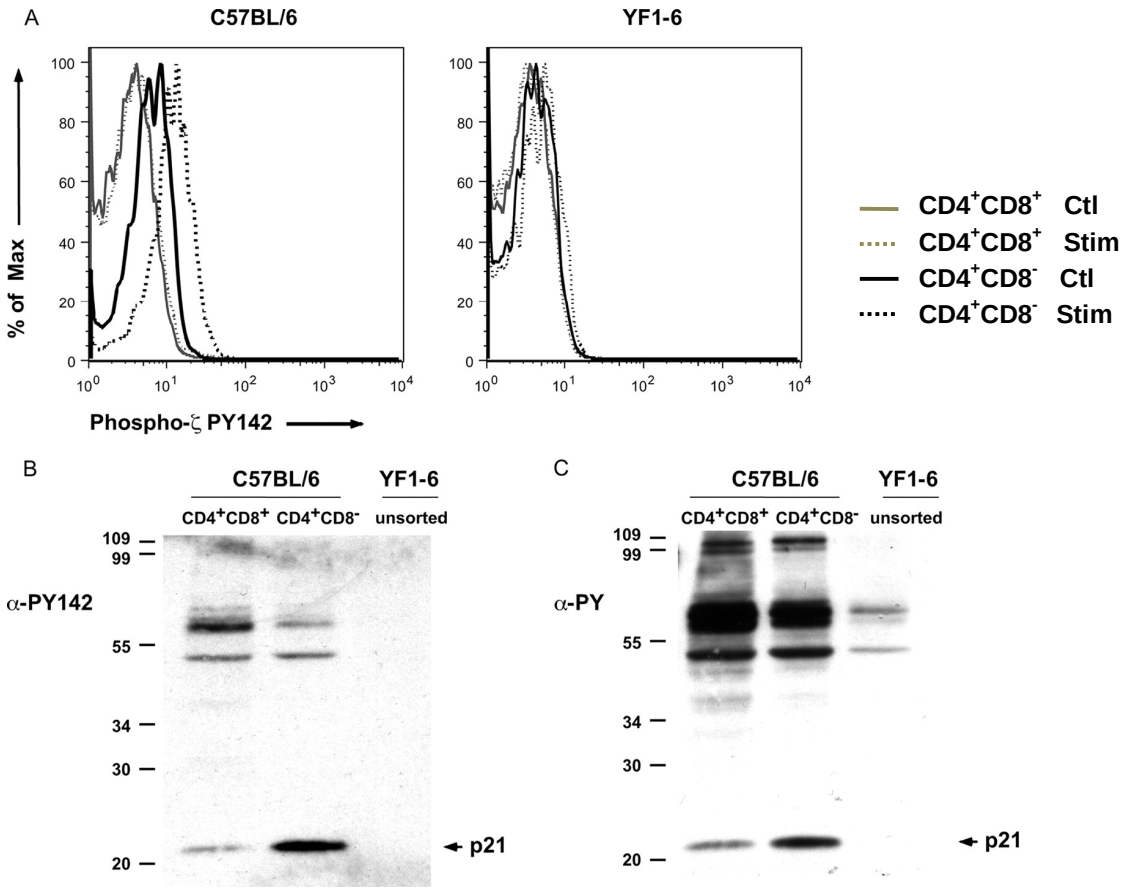


Figure 15: p21 is not detected in immature thymocytes by intracellular staining. *A*, Thymocytes from C57BL/6 or YF1–6 mice were left untreated or stimulated for 10 min. The cells were stained with Abs against CD4 and CD8 and subsequently processed for intracellular staining with anti-phospho-(Y143)-specific mAbs. The $CD4^+CD8^+$ and $CD4^+CD8^-$ were electronically gated and analyzed for phospho-ζ by flow cytometry. Data are representative of three independent experiments. $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes from wild-type mice were isolated by cell sorting, lysed, and processed by Western immunoblotting with B, anti-phospho-Y142, or C, anti-phosphotyrosine. The samples were compared with those isolated from YF1–6 mice (*B* and *C*). Max., Maximal; Ctl, control.

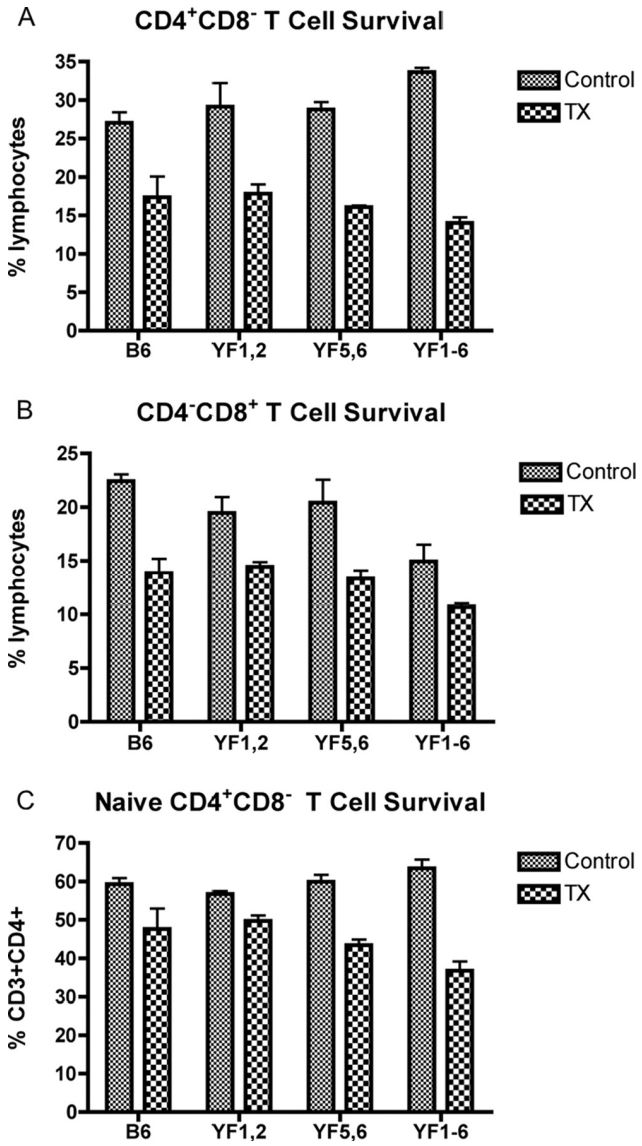


Figure 16: ITAM numbers in the TCR complex regulate T cell survival. Four-week-old mice were thymectomized (TX). Nine weeks postthymectomy, the mice were euthanized and analyzed for the absence of a thymus. The lymph nodes were removed and processed. Aliquots of the cells were stained with the indicated fluorochrome-labeled mAbs and analyzed by FACS analyses. The percentage of *A,* $CD4^+CD8^-$, *B,* $CD4^-CD8^+$, and *C,* naive $CD62L^+CD4^+$ T cells in the lymph nodes were plotted for control and thymectomized mice. B6, C57BL/6. Data are average ± SEM

CHAPTER IV

iNKT CELL DEVELOPMENT REQUIRES A FULL COMPLEMENT OF FUNCTIONAL CD3 ITAMS

Introduction

Invariant natural killer T (iNKT) cells participate in immune responses to various bacterial and viral infections and reduce the severity of some autoimmune diseases (5, 175). The ability of iNKT cells to regulate B cell, dendritic cell, and T cell responses is partly due to their rapid and substantial production of IFN-γ and IL-4 within minutes of TCR engagement (175, 266). Thus, these cells are thought to provide a critical link between the innate and adaptive immune system. In fact, iNKT cells are proposed to control the extent of inflammatory arthritis following *Borrelia burgdorferi* infections (267, 268).

Contrasting conventional T cells, which express a diverse repertoire of Vα and Vβ subunits, iNKT cells express a semi-invariant αβ TCR containing a Vα14Jα18 rearrangement of the TCR α chain paired with a β subunit that is limited to Vβ8.2, Vβ7 or Vβ2, in mice (Vα24Jα18/Vβ11 in humans). This TCR reacts with glycolipid complexes that are bound to the MHC class Ib molecule, CD1d. iNKT cells develop in the thymus from the same $CD4^+CD8^+$ (DP) precursor cell as conventional T cells (196, 198, 229). However, unlike conventional T cells, the positive selection of iNKT cells requires invariant αβ TCR interactions with glycolipid/CD1d complexes expressed on $CD4^+CD8^+$ thymocytes (5). Thus, $J\alpha18^{-/-}$ and $CD1d^{-/-}$ animals lack iNKT cells (213, 269,

270). Furthermore, iNKT cell development requires homotypic interactions between SLAM family receptors such as SLAM and LY108, using signaling pathways involving SAP and the protein tyrosine kinase Fyn (198, 220, 271).

The signaling pathways required for iNKT cell development are poorly understood. For conventional T cells, TCR-driven signals mediated by a signaling motif found in one or more copies of the cytoplasmic tail of the CD3 subunits, termed an immunoreceptor tyrosine-based activation motif (ITAM), are required for both positive and negative selection processes. Following receptor engagement by selecting ligands expressed in the thymus, the ITAMs are phosphorylated on tyrosine residues by the Scr-family of protein tyrosine kinases (PTKs). As phospho-proteins, the ITAMs are complexed by the PTK ZAP-70. Most conventional T cells can be positively selected with as few as 4 functional ITAMs (108, 140, 142). While TCR $\zeta^{-/-}$, ZAP-$70^{-/-}$ and $Fyn^{-/-}$ mice lack iNKT cells, the number of functional ITAMs required for iNKT cell development has not been addressed (116, 129, 220, 271, 272). Some experimental evidence suggests that strong TCR-driven signals are needed for iNKT cell development. For example, mice lacking the PTK Itk have limited iNKT cell development (82, 221). This is consistent with the observation that the elimination of the transcription factor early growth response 2 (Egr2), a target of Ca^{++}/calcineurin/NFAT signaling, results in a significant reduction in iNKT cells in the thymus and periphery (224).

To explore the functional contribution of the CD3 ITAMs in iNKT cell development, we analyzed a series of CD3 ζ transgenic mice in which selected tyrosine to phenylalanine substitutions were introduced in the first (YF1,2), third (YF5,6), or all

three ITAMs of CD3 ζ (YF1-6). In this report, we show that iNKT cell development is almost completely abrogated in mice lacking as few as two of ten functional CD3 ITAMs. The developmental block occurred at an early iNKT cell developmental stage, as evidenced by the increased cell death in developing iNKT cells. The few iNKT cells that formed in the CD3 ζ ITAM substituted lines contained more $CD4^{-}CD8^{-}$ (DN) subsets relative to the $CD4^{+}CD8^{-}$ ($CD4^{+}$) populations, opposite to that noted in wild type mice. In spite of a near absence of iNKT cells, there was no difference in the onset of inflammatory arthritis in YF mice compared to controls following infection with the Lyme disease causing spirochete, *B. burgdorferi*. Taken together, these data suggest that a full complement of functional CD3 ITAMs is necessary for proper iNKT cell development, but that iNKT cells are not critical for controlling immune mediated arthritis following *B. burgdorferi* infection.

Results

CD3 ζ ITAMs are required for iNKT cell development

The development of αβ T cell occurs in mice containing as few as 4 functional CD3-encoded ITAMs (144). For example, T cell development is relatively normal in mice containing tyrosine to phenylalanine substitutions in the first (YF1,2), third (YF5,6), or all three CD3 ζ ITAMs (YF1-6) (Figure 5 and Figure 17A) (52). Much less is known about the ITAM requirements for iNKT cell development. To determine if the development of these cells was also permissive with reduced numbers of functional

ITAMs, we analyzed the YF series of mice for the presence of iNKT cells. These mice retain 8/10 (YF1,2 and YF5,6) or 4/10 (YF1-6) functional ITAMs, with the YF1,2 and YF5,6 differing only in the presence or absence of the constitutively phosphorylated 21 kDa form of CD3 ζ (Figure 5) (46, 52). The iNKT cells were detected with glycolipid-loaded CD1d tetramers (PBS57) combined with anti-CD3ε mAbs. The percentage of iNKT cells, defined as $CD3^{intermediate}$ and CD1d $tetramer^{+}$, was significantly reduced in the thymus, spleen, liver, and bone marrow of the YF1,2 (0.02%, 0.02%, 1.2 %, 0.02%), YF5,6 (0.02%, 0.05%, 0.30%, 0.02%) and YF1-6 (0.05%, 0.07%, 0.6%, 0.02%) mice compared to wild type controls (0.4%, 0.7%, 15.6%, 0.2%) (Figure 18). The lack of iNKT cells was also reflected in a statistically significant decrease in absolute number of these cells in the thymus and spleen of YF1,2, YF5,6, and YF1-6 lines compared to the wild type controls (Figure 19). Interestingly, the mice that lacked only 2 of the 10 CD3-encoded ITAMs (YF1,2 and YF5,6) had a more severe reduction in iNKT cell numbers compared to those missing 6/10 ITAMs (YF1-6). This is likely a consequence of the increased TCR density in the YF1-6 lines, which occurs because of decreased CD3 ζ ubiquitination and degradation (Figure 17B) (41, 42). Regardless of these effects, our results demonstrate that normal iNKT cell development requires a minimum of 9-10 CD3-encoded ITAMs.

iNKT cells from mice lacking two or more CD3 ζ ITAMs are blocked early in development

Like conventional αβ T cells, iNKT cell development progresses through a number of stages defined by the expression of particular cell surface markers. To

determine the stage at which iNKT cell development was blocked in YF series of mice, thymocytes were isolated and stained with mAbs against the heat stable antigen (HSA,CD24) and the CD1d tetramer. The percentage of iNKT cells in the HSA^{lo} compartment was significantly reduced in thymocytes from the YF1,2 (0.01%), YF5,6 (0.01%) and YF1-6 (0.04%) lines compared to wild type mice (0.43%) (Figure 20 A). This reduction was consistent with the statistically significant increase in the percentage iNKT cells (electronically gated $CD3^{intermediate}$ $tetramer^{+}$) that expressed high levels of HSA (HSA^{hi}) in the YF1,2 (36%, *p<0.0001*), YF5,6 (33%, *p<0.0001*) and YF1-6 (19%, *p<0.001*) mice compared to wild type mice (3%) (Figure 20 B). Following the down-regulation of HSA, iNKT cells mature in three progressive stages defined by CD44 and NK1.1 (Stage I: $CD44^{-}NK1.1^{-}$; Stage II: $CD44^{+}NK1.1^{-}$; Stage III $CD44^{+}NK1.1^{+}$). To examine which maturation stage was affected by the modifications to the CD3 ζ ITAMs, iNKT cells (gated as $CD3^{intermediate}$ $tetramer^{+}$ HSA^{lo}), isolated from the thymus and spleens of the different mice, were analyzed for the expression of CD44 and NK1.1. More iNKT thymocytes were arrested at Stage I in the YF1,2 (12%), YF5,6 (13%) and YF1-6 (11%) lines compared to wild type mice (3%) (Figure 21). This block was also noted in the splenocyte populations (Figure 21). Contrasting the differences in Stage I, the percentages of cells at Stage II and III in both the thymus and spleen were comparable between the YF lines and the wild type controls. Taken together, these findings indicate that a deficiency of one or more CD3 ζ ITAMs severely impairs iNKT cell development, but the cells that do develop can proceed through the later stages without undergoing significant cellular expansion.

In wild type mice, most iNKT cells express the CD4 coreceptor molecule ($CD4^+CD8^-$ or $CD4^+$), with a small subset developing into $CD4^-CD8^-$ cells (DN) (Figure 22) (198, 209). Contrasting this, all the mice lacking one or more functional CD3 ζ ITAMs exhibited an inverted ratio of $CD4^+$ to DN iNKT cells in both the thymus (1.4 versus 0.3-0.6) and the spleen (5.8 versus 0.2-0.7) (Figure 22). Overall, these data show that iNKT cells from CD3 ζ transgenic mice lacking one or more functional CD3 ζ ITAMs are developmentally impaired in the thymus, resulting in enhanced percentage of DN iNKT cells in the periphery.

iNKT cells from mice lacking two or more functional CD3 ζ ITAMs have increased cell death

Mice deficient in the protein tyrosine kinase Itk and the transcription factor Egr2 have reduced iNKT cell numbers due, in part, to increased cell death in the thymus (221, 224). To examine whether a similar mechanism was occurring in the YF sets of mice, the tetramer positive cells were stained with Annexin V and 7AAD to detect dead cells. The iNKT cells from of the thymus of the YF1,2 (43 %) and the YF5,6 (13 %) mice had a statistically significant increase in the percentage of Annexin V^+ $7AAD^+$ cells compared to the wild type controls (3%) (Figure 23 A, B). Interestingly, in spite of an equivalent TCR density and functional ITAM number, the YF1,2 line exhibited much greater cell death than the YF5,6 mice. The difference between these two lines is the presence of the constitutively phosphorylated CD3 ζ subunit of 21 kDa, or p21, in the YF1,2 mice (52). Notably, the constitutive presence of p21, in the absence of other phospho-ζ intermediates, can attenuate T cell development (144). While there was a higher rate of

cell death in the YF1-6 mice relative to C57BL/6 controls, this did not reach significance, consistent with the presence of more iNKT cells in the YF1-6 mice relative to the YF1,2 and YF5,6 lines.

The most likely interpretation of our findings was that TCR signaling in the iNKT cells required a full complement of CD3 ITAMs to promote the efficient development. A second explanation to account for the reduced NK T cell numbers is a change in the cell surface expression of CD1d, LY108, and SLAM on DP thymocytes. This would result in decreased selection of iNKT cells. The thymocytes from all the mice were analyzed for the expression of CD1d, LY108 and SLAM (Figure 24 A, B). Notably, there was no difference in the expression of any of these three proteins on DP thymocytes among the various mice, indicating that the block in iNKT cell development was most likely a consequence of inefficient signaling through the TCR.

iNKT cells are not necessary to prevent inflammatory arthritis following Borrelia burgdorferi infection

The YF series of mice had severe reductions in iNKT cell numbers. Since iNKT cells are proposed necessary for controlling inflammatory arthritis in mice infected with the spirochete *B. burgdorferi*, we were interested in assessing whether the severity of arthritis in the YF sets of mice was increased (267, 268). Wild type, YF1,2 and YF1-6 lines were infected intradermally with 1.2 – 1.5 x 10^6 *B. burgdorferi* spirochetes. At 2 and 6 weeks post-infection, the knees and tibiotarsal joints were sectioned and stained with hematoxylin and eosin. Tissues were then analyzed for arthritis by assessing lymphocyte infiltration into the joints. The tissue was scored for arthritis severity using a

scale from 0 - 3, where the intervals indicate no, slight, moderate and severe inflammation, respectively, as evidenced by the degree of lymphocyte infiltration into the joints. Interestingly, there was no difference in the degree of inflammation in the tibiotarsal or knee sections at 2 weeks or 6 weeks post-infection in YF1,2 or YF1-6 mice compared to wild type mice (Figure 25 A-D, Figure 26 A-D). These experiments suggested that iNKT cells are not the principal effector populations that control inflammatory responses to Lyme arthritis.

Discussion

Although conventional αβ T cells can develop with as few as 4 functional CD3-encoded ITAMs, our study shows that iNKT cell development requires a full complement of CD3 ITAMs. Thus, mice lacking one functional CD3 ζ ITAM, effectively eliminating 2 of the 10 TCR/CD3 ITAMs, had a severe block in iNKT cell maturation and/or expansion. These findings suggest that the TCR signaling strength required for iNKT cell development is relatively weak when the TCR engages glycolipid/CD1d complexes. This interpretation is consistent with previous studies showing that conventional αβ T cells with low avidity TCRs require a full complement of CD3 ζ ITAMs for effective positive selection (107, 108, 144). The fact that some iNKT cells develop normally in the YF sets of mice described herein could be explained in one of two ways. First, a subset of iNKT cells might express a TCR with sufficient affinity for the selecting glyolipids expressed in the thymus (192-195). For example, Vβ7-expressing iNKT cells are

preferentially selected by iGb3, in an *in vitro* culture system, since the Vβ7 is a higher affinity receptor (204, 273). Second, a full complement of TCR ITAMs might actually be required for the proper expansion of the iNKT cells once they are positively selected. Comparing the wild type and YF sets of mice, the developmental patterns are not dramatically different when analyzing StageI-III. Rather, all the iNKT cells in the YF lines exhibited a statistically significant increase in the number of cells undergoing cell death. In particular, the YF1,2 lines had the most appreciable extent of cell death, with the residual iNKT cells detected in the thymus of these mice expressing relatively lower TCR densities. The fact that the YF1,2 line differs from the YF5,6, despite having an equivalent TCR density and ITAM number, is consistent with our early studies comparing conventional T cell development in these mice. Thus, the YF1,2 line retains a constitutively phosphorylated CD3 ζ subunit that is complexed to an inactive population of ZAP-70 molecules. When analyzed in the setting of HY TCR transgenic male mice, the p21/ZAP-70 pool attenuates negative selection (140). To support this, we have shown that the constitutive pool of p21/ZAP-70 is not involved in *de novo* TCR signaling (274). Thus, the existence of p21/ZAP-70 complexes in the YF1,2 line could diminish the magnitude of TCR signaling even more than the YF5,6 mice.

Mechanistically, the TCR-ITAM signaling pathway involves the activation of downstream PTKs such as ZAP-70 and Itk (76, 82). These kinases are required for TCR-mediated elevations in intracellular calcium levels. Notably, both ZAP-70 and Itk-deficient mice have a block in iNKT cell development (211, 221). Recent experiments have revealed a direct requirement for TCR-mediated calcium responses in the activation

of calcineurin leading to iNKT cell selection and/or expansion (224). Itk functions downstream of the TCR by amplifying calcium signaling pathways through PLC-γ induction (82). The calcium/calcineurin pathway contributes to the expression of the Egr2, a transcription factor required for iNKT cell development. Impressively, the developmental arrest of the iNKT cells in the Itk- and Egr2-deficient animals phenocopies that noted in our YF series of mice. In unpublished data from our lab, we have carried out proliferation with α-galactosylceramide, a potent agonist of iNKT cells, and measured cell expansion by CFSE dilution in YF1,2, YF1-6 and wild type mice. We found that while the percentage of YF1-6 iNKT cell increase consistent with a decrease in CFSE and cellular proliferation, the percentage of iNKT cells from YF1,2 mice decreased and very few CFSE negative cells were detected compared to wild type mice. The reason for this is still under investigation; however, one explanation could be due inefficient induction of Egr2, resulting in a higher rate of cell death. Our additional observation that the elimination of 6/10 functional CD3 ITAMs (YF1-6 line) had an iNKT cell selection/maturation defect less severe than the YF1,2 or YF5,6 lines is explained by the increased TCR density previously described in these mice. In summary, strong TCR driven intracellular signals, regulated by both ITAM numbers and TCR densities, are required for efficient iNKT cell development.

iNKT cells are reported necessary for preventing the arthritis that develops in mice following infections with the spirochete, *B. burgdorferi* (267, 268). These findings would suggest that a more severe arthritis should develop in mice deficient in iNKT cells. However, the YF1,2 or YF1-6 mice exhibited similar pathology in the tibiotarsal and

knee joints at 2 and 6 weeks post-infection compared to wild type. We considered three explanations to account for these results. First, iNKT cells are not the principal cell population regulating arthritis following exposure to *B. burgdorfe*ri. For example, several studies suggest that *B. burgdorferi* specific antibody production by B cells is critical for mediating protection as serum transfer from infected to uninfected animals can ameliorate arthritis (275). A second explanation is the differences in the haplotype of the mice studied. BALB/c mice are tolerant to low but not high doses of *B. burgdorferi*, whereas C57BL/6 mice have been shown to be tolerant even at high doses of infection (276). Furthermore, $RAG^{-/-}$ C57BL/6 mice maintained resistance to arthritis, unlike $RAG^{-/-}$ BALB/c mice, despite the lack of T and B cells and a similar bacterial burden (277). All of our mice were maintained on a C57BL/6 background. Thus, it is possible that, in more susceptible strains of mice, iNKT cells could provide efficient protection from or immunoregulation of the disease. This protection could be mediated by early cytokine production by iNKT cells, as early IL-4 production correlates with decreased susceptibility to arthritis whereas IFN-γ production has been associated with increased severity of arthritis (277-282). The small number of iNKT cells that remain in the YF1,2 and YF1-6 mice could have thwarted the development of inflammatory arthritis. However, due to the fact that $RAG^{-/-}$ C57BL/6 mice remain resistant to arthritis suggests that these cells are not necessary for arthritis prevention. Furthermore, iNKT cells in the YF1,2 and YF1-6 mice are mostly DN, a subset which has been associated with a Th1, rather than the protective Th2 response. Thus, these data suggest that the prevention of arthritis in C57BL/6 mice is not dependent on iNKT cells. In order to confirm this, *B. burgdorferi* infection studies using $Ja18^{-/-}$ mice on a C57BL/6 background are necessary

in order to formally answer this question. Taken together, these data show that early iNKT cell development is dependent on signaling through a full complement of CD3 ITAMs, but iNKT cells are not necessary for the prevention of Lyme arthritis.

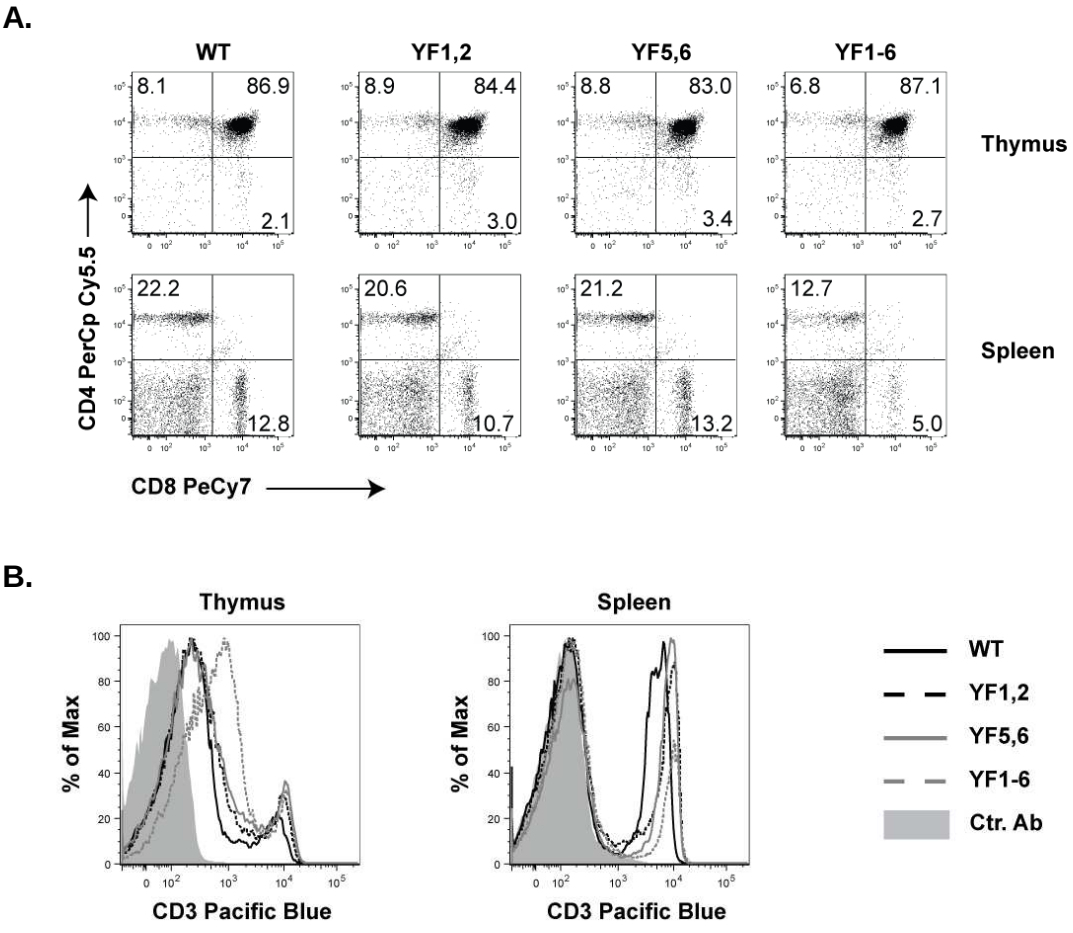


Figure 17: Conventional CD4$^+$ and CD8$^+$ T cells develop normally in mice lacking two or more CD3 ζ ITAMs. T cells in the thymus and spleen were stained with *A*, anti-CD4 PerCp-Cy5.5 and -CD8 PE-Cy7 and *B*, CD3 Pacific Blue. Histograms gated through total lymphocyte gate.

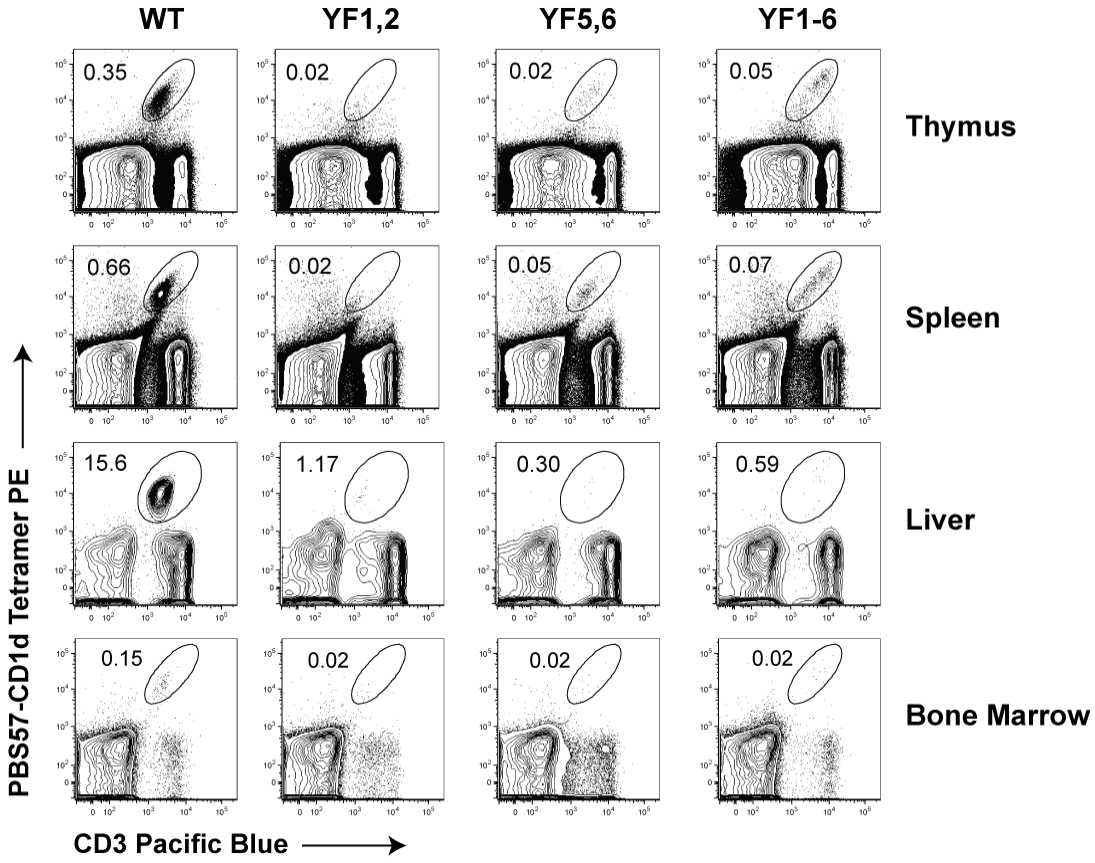


Figure 18: iNKT cells are reduced in mice lacking one or more CD3 ζ ITAMs. The percentage of iNKT cells in the thymus, spleens and livers of the indicated mice were identified by staining cells with anti-CD3 ε-Pacific Blue in combination with PBS57-loaded CD1d tetramer-PE and analyzed the cells by flow cytometry. iNKT cells are represented in oval gates.

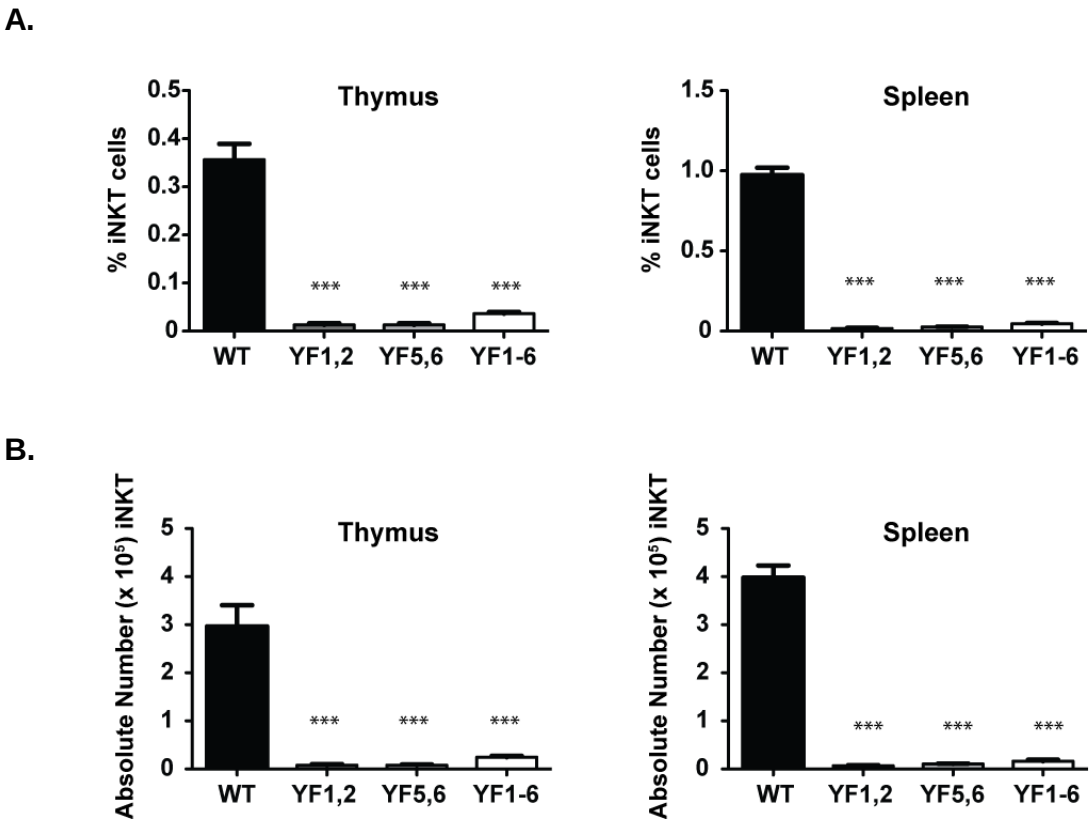


Figure 19: The percentage and absolute number of iNKT cells is significantly reduced in the mice lacking one or more CD3 ζ ITAM. iNKT cells were stained as in Figure 18. *A*, The percentage and *B*, absolute number of iNKT cells from WT, YF1,2, YF5,6 and YF1-6 mice were compared with at least 3 mice per group. Data is consistent with 2 - 3 independent experiments. ****p = 0.0001*; values were generated by one-way ANOVA.

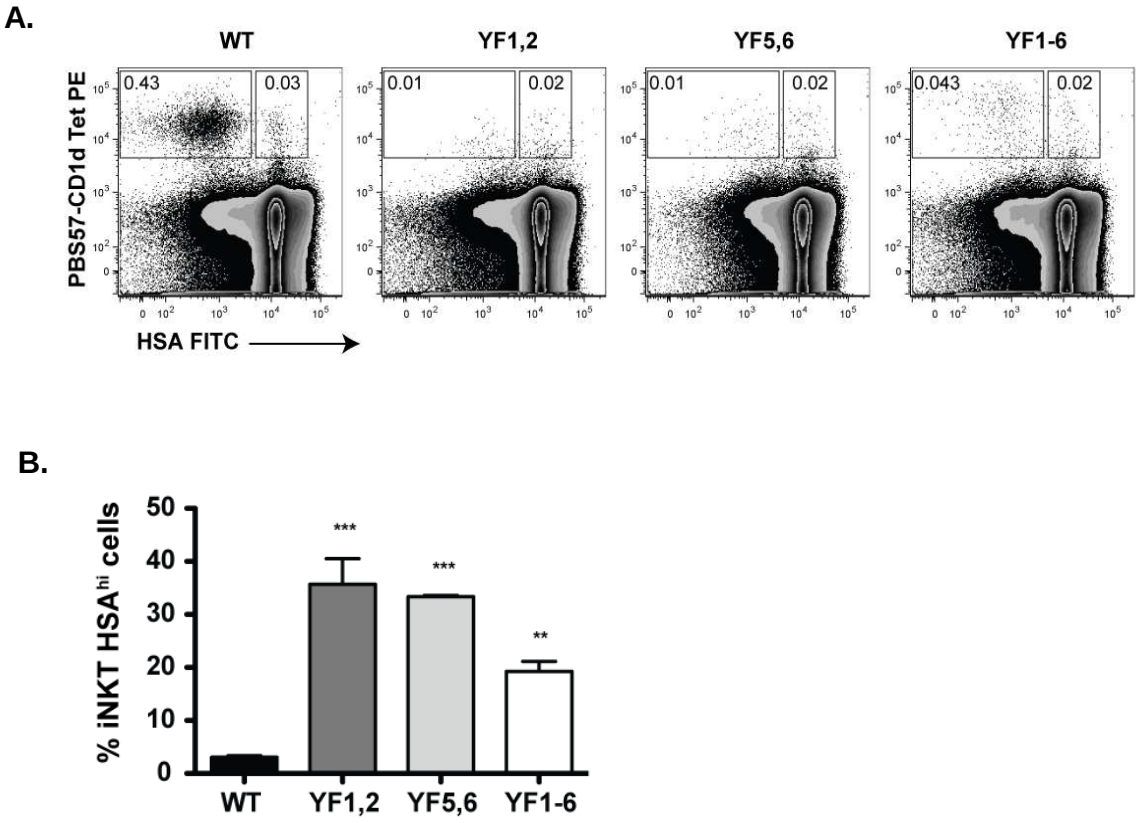


Figure 20: iNKT cell development is blocked at an early stage of thymopoeisis in mice lacking one or more functional CD3 ζ ITAMs. Thymocytes or splenocytes from wild type, YF1,2, YF5,6, and YF1-6 mice were stained with anti-CD3 ε-Pacific Blue, PBS57-CD1d tetramer-PE and anti-HSA FITC. *A*, The percentage of HSA^{lo} iNKT cells was analyzed by gating on HSA^{lo} PBS57-CD1d $tetramer^{+}$ lymphocytes (right gate). *B*, The bar graph represents the average percentage of HSA^{hi} iNKT cells. Cells were first gated electronically on $CD3^{intermediate}$ CD1d $tetramer^{+}$. Statistics were generated using a Student t test. *** $p < 0.001$, ** $p < 0.01$

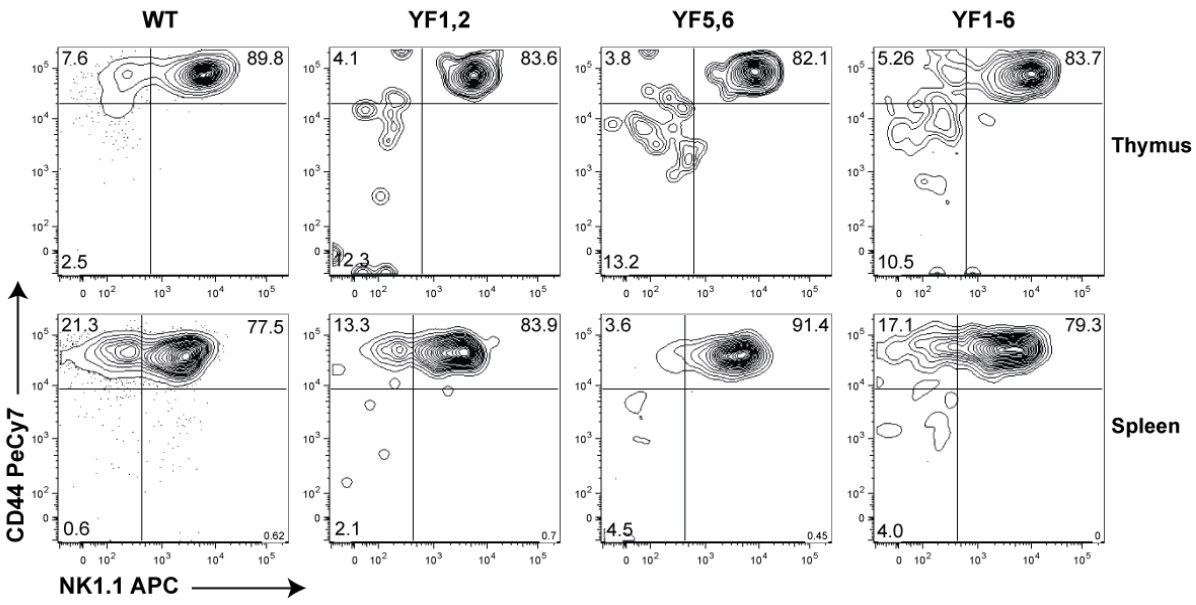


Figure 21: Increased percentage of iNKT cells are CD44^{-}NK1.1^{-} in mice lacking two or more CD3 ITAMs. Thymocytes or splenocytes from wild type, YF1,2, YF5,6, and YF1-6 mice were stained with anti-CD3 ε-Pacific Blue, PBS57-CD1d tetramer-PE, anti-HSA FITC, -CD44 PeCy7, and –NK1.1 APC. Stage I, II, and III iNKT cells were analyzed in the thymus and spleen of indicated mice as indicated in corresponding quadrants labeled I, II, and III in the upper left FACs plot. Quadrants represent CD3^{intermediate} CD1d tetramer^{+} HSA^{lo} cells.

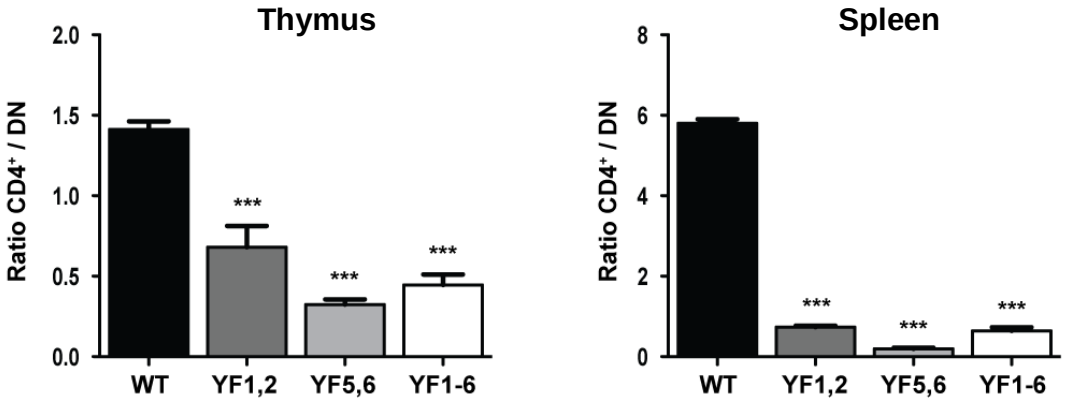


Figure 22: iNKT cells are skewed to the DN lineage in mice lacking two or more functional CD3 ζ ITAMs. The percentage of CD4 SP and DN iNKT cells was analyzed on $CD3^{intermediate}$ CD1d $tetramer^{+}$ HSA^{lo} cells. The bar graph represents the ratio of CD4 SP / DN iNKT cells. Data are representative of at least 3 independent experiments. Statistics were generated by 1-way ANOVA. ***p<.0001, ** p<0.001.

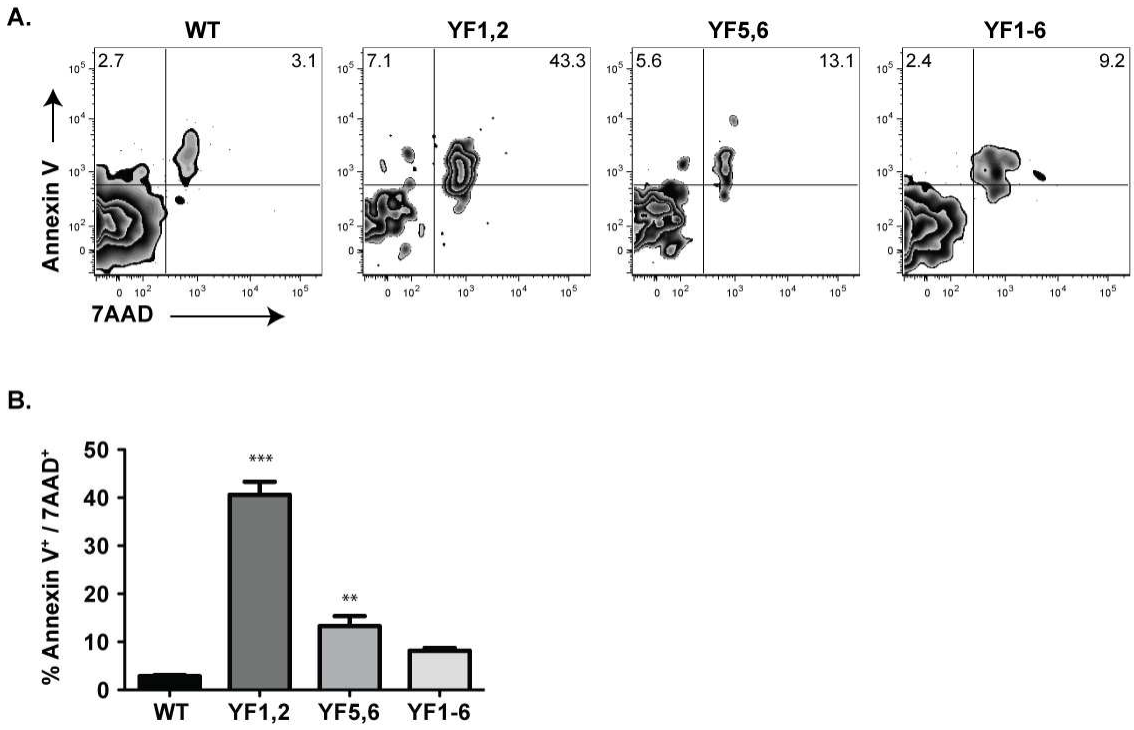


Figure 23: iNKT cells from mice lacking two or more CD3 ζ ITAMs have an increase in cell death compared to controls. *A,* Thymic iNKT cells from the indicated mice were stained with anti-CD3 ε, PBS57-CD1d tetramer, Annexin V APC and 7AAD, and analyzed by flow cytometry. The percentage of cells is represented in each quadrant. *B,* Bar graph indicates the percentage of iNKT cells that are Annexin V^+ $7AAD^+$ using at least 2-3 mice/group. Statistics were generated by 1-way ANOVA. *** $p < 0.0001$, ** $p < 0.001$.

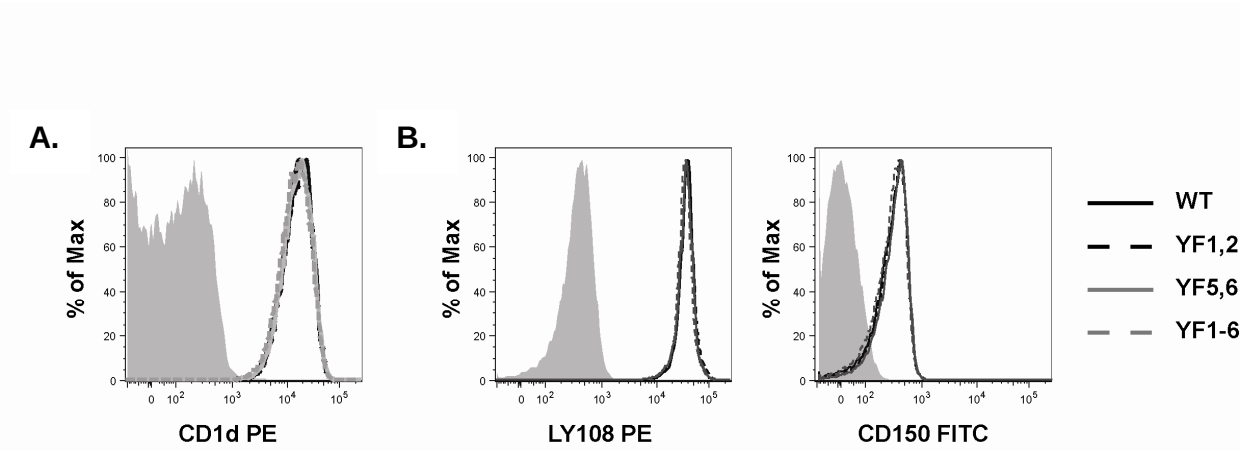


Figure 24: DP thymocytes from CD3 ζ transgenic mice express equivalent levels of CD1d, LY108 and CD150. Thymocytes were stained with anti-CD4 PerCp-Cy5.5 and -CD8 PE-Cy7 plus *A*, anti-CD1d PE *B*, -LY108 PE, or -SLAM FITC. Histograms were generated after gating on $CD4^+CD8^+$ thymocytes. Grey shaded histograms represent fluorescence minus one (FMO) controls.

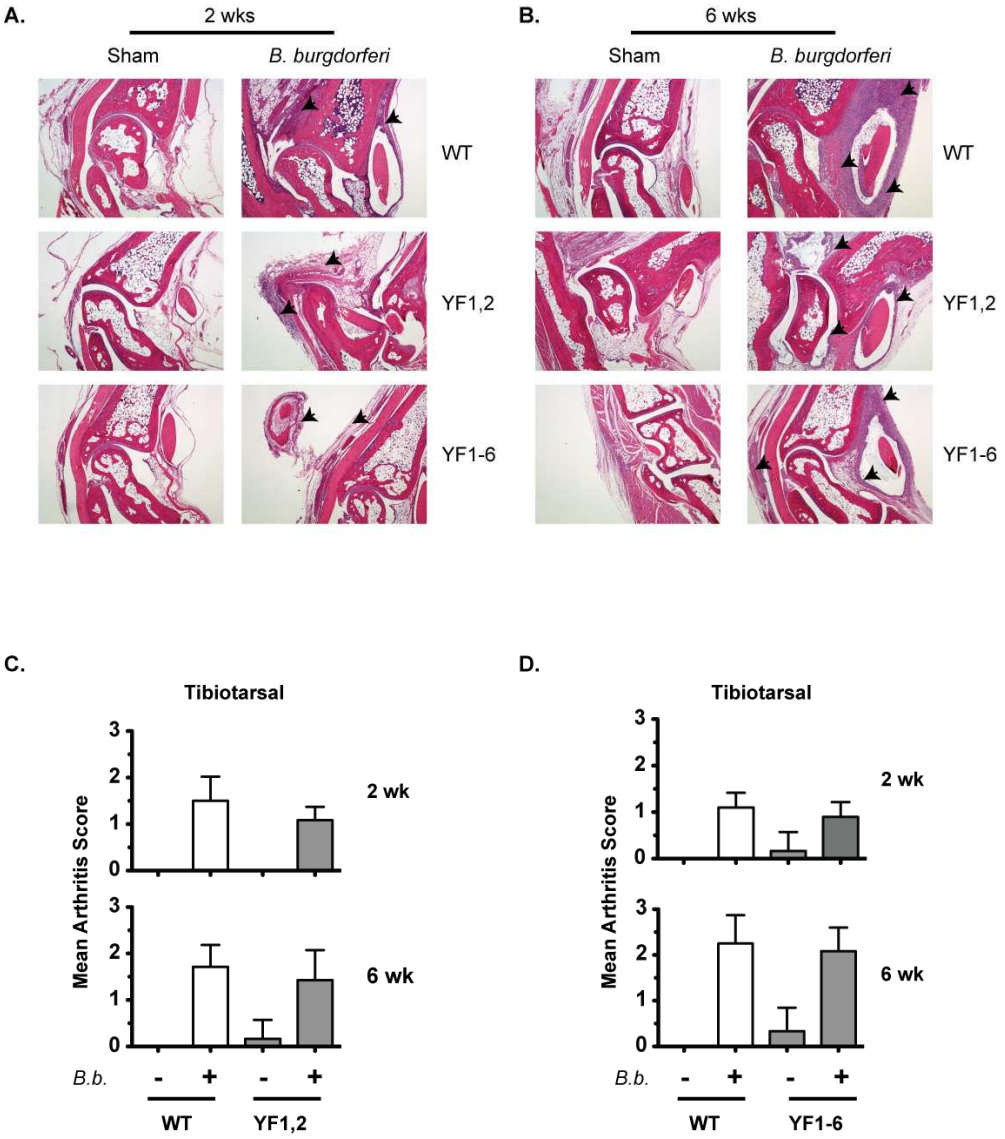


Figure 25: iNKT cells are not necessary to prevent inflammatory arthritis following *B. burgdorferi* infection. 6 week old mice were inoculated intradermally with 1.2 – 1.5 x 10^6 *B. burgdorferi* spirochetes. Right and left tibiotarsal joints were isolated, paraffin embedded, and 7 μm sections were cut and stained with hematoxylin and eosin. *A ,B* Digital pictures taken of tibiotarsal joints from indicated mice. *C, D* Joints were scored for arthritis on a scale from 0 – 3 indicating no, slight, moderate or severe inflammation. Bar graphs indicated the mean arthritis score at 2 or 6 weeks post infection. 3 shams and 6 - 9 infected mice were analyzed per group, per time point. Arrows indicate sites of inflammation. Graphs represent scoring from both right and left joint sections.

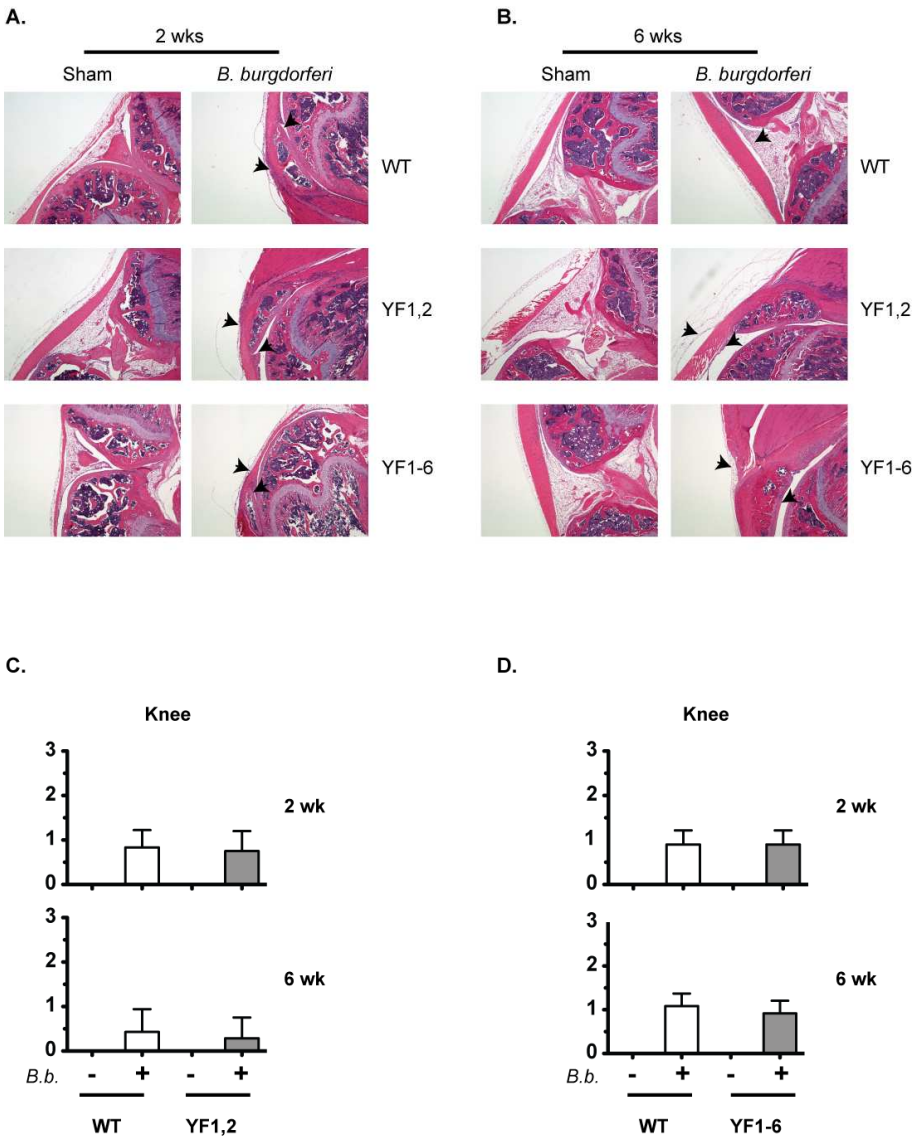


Figure 26: iNKT cells are not necessary to prevent inflammatory arthritis following *B. burgdorferi* infection. 6 week old mice were inoculated intradermally with 1.2 – 1.5 x 10^6 *B. burgdorferi* spirochetes. Right and left knee joints were isolated, paraffin embedded, and 7 μm sections were cut and stained with hematoxylin and eosin. *A, B* Digital pictures taken of knee joints from indicated mice. *C, D* Joints were scored for arthritis on a scale from 0 – 3 indicating no, slight, moderate or severe inflammation. Bar graphs indicated the mean arthritis score at 2 or 6 weeks post infection. 3 shams and 6 - 9 infected mice were analyzed per group, per time point. Arrows indicate sites of inflammation. Graphs represent scoring from both right and left joint sections.

CHAPTER V

DISCUSSION

The TCR recognizes both self- and foreign-peptides complexed to MHC molecules. Ligand-mediated signaling by this receptor is critical for both T cell development and the initiation of T cell mediated immune responses. The TCR signals through ten ITAMs, distributed throughout the CD3 γ, δ, ε, ζ subunits.

A proportion of CD3 ζ exists in a constitutively phosphorylated state *in vivo*. TCR interactions with MHC molecules are necessary for constitutive phosphorylation of p21. Mapping studies have shown that p21 requires phosphorylation of the 4 tyrosine residues the two membrane distal ITAMs (46). P21 is absent or reduced in TCR α, Lck, and ZAP-70 deficient mice, but maintained in $CD4^{-/-}$ and $Fyn^{-/-}$ lines. In order to determine whether p21 arises from self-ligand interactions *in vivo*, we monitored the phosphorylation state of CD3 ζ in thymocytes developing in distinct MHC class I and class II restricted TCR transgenic mice. In this system, thymocytes developed in a selecting or a non-selecting environment. Under selecting conditions, T cells could undergo positive selection and develop into mature CD4 or CD8 SP T cells. Under non-selecting conditions, cells were blocked at the DP stage of development. The experimental finding showed that p21 phosphorylation resulted from TCR recognition of MHC molecules, as it could be detected in both the selecting and non-selecting environments.

Previous reports provided conflicting data about the role of p21 in TCR signaling and function. Some reports suggest that p21 enhances the sensitivity of naive T cells to

foreign antigen, others support a role for p21 in T cell anergy (47, 248, 249). Furthermore, some studies have shown that p21 is not required for positive selection, TCR signal transmission, or T cell anergy; however, we have recently found a role for p21 in negative selection (108, 140, 142). We further analyzed the role of p21 in TCR signal transduction in thymocytes prior to and following stimulation. Our results indicated that the constitutive pool of p21/inactive ZAP-70 complexes were not involved in *de novo* TCR signaling, but that the formation of new p21/activated ZAP-70 complexes could be formed following TCR crosslinking. Interestingly, a large pool of p21 was immunoprecipitated with anti-CD3 ζ in ZAP-70 depleted lysates, suggesting that p21 could be complexed to proteins other than ZAP-70. This data is supported by previous findings in which we analyzed the level of ZAP-70 phosphorylation in HY/CD3 ζ transgenic mice prior to and following stimulation with peptide/MHC complexes. We have previously shown that wild type HY, HY/YF1,2, and HY/YF1-6 contained comparable levels of phosphorylated ZAP-70 following agonist stimulation (108). There was a slight increase in the level of activated ZAP-70 in the HY/YF5,6; however, this difference did not reach significance. Because the HY/YF1,2 and HY/YF5,6 mice contained comparable TCR surface expression and total ITAMs, this data suggests that the presence of p21 in the HY/YF1,2 mice could have slightly attenuated signaling, possibly by sequestering inactive ZAP-70 away from active signaling complexes. The YF series of mice was initially generated in order to determine the role of the various phospho-ζ derivatives in T cell development, signaling, and function. While most T cell development remains intact in all of the YF mice, we did notice a difference in the development of iNKT cells.

Early iNKT cell development is dependent on the same signaling pathways as conventional T cell development. As such, pTα, CD3 ζ, ZAP-70 and Lck deficient mice have poor iNKT cell development (116, 122, 129, 210-212). These cells develop from the same DP precursor population as conventional T cells, and as a result, all T cell signaling leading up to positive selection is likely similar. Consequently, iNKT cells diverge from conventional T cells during positive selection due to the expression of a canonical Vα14Jα18 TCR that is specific for glycolipid/CD1d complexes (283). Interestingly, there are some differences in the signaling and co-stimulatory requirements for iNKT cells and conventional T cells, endowing iNKT cells with a unique phenotype and functional properties (5). Conventional T cells develop with only 4/10 functional CD3 ITAMs (107-109, 142, 145). Contrasting this, iNKT cell development was almost completely abrogated in YF1,2, YF5,6 and YF1-6 lines. These findings suggest that iNKT cells rely on stronger TCR signals, implying that the Vα14Jα18 TCR has a very low affinity for selecting glycolipid/CD1d complexes. iNKT cells have an activated, memory phenotype even when developing in germ-free mice (284). Development of such cells likely requires more strict control during selection, such that mature iNKT cells do not pose a threat to the host when released into the periphery. Consistent with this, iNKT cells express lower levels of the TCR on their surface. Furthermore, in the final stages of maturation, iNKT cells upregulate a number of inhibitory NK cell markers, such as NK1.1, which correlate with a decrease in proliferation, suggesting that these receptors could keep iNKT cells quiescent in the periphery (208, 209).

T cell development requires signals through the TCR following recognition of peptide/MHC or glycolipid/CD1d complexes. Some recent evidence suggest that the

TCR evolved specifically recognize self and to support the emergence of self-tolerant T cells. Draper, an ancient ITAM immunoreceptor expressed on phagocytic glial cells in *D. melanogaster,* recognizes "modified self" proteins presented by dead or dying neurons, leading to the phagocytosis and clearance of these cells. The Draper signaling pathway is dependent on a Src family kinase and the Syk homolog, Shark, for proper signal transduction. This was likely the predecessor from which other ITAM-based immunorecptors evolved, including the TCR, suggesting that the TCR evolved in order to recognize and induce tolerance to self-proteins. Data presented in this thesis support this role for the TCR. We have shown that pre-selection T cells generate signals through the TCR following recognition of self-MHC molecules. Furthermore, p21 is sequestered from TCR signaling and likely regulates signaling during development by sequestering key signaling proteins, or by recruiting proteins that actively attenuate signaling. Furthermore, we have shown that the development of highly reactive innate effector iNKT cells requires a full complement of CD3 ITAMs in order to develop properly, and that additional signal attenuation by p21 additionally impairs development.

While Draper contains a single ITAM, the TCR is endowed with 10 ITAMs. The CD3 γ, δ, and ε proteins are highly homologous and are thought to have arisen by gene duplication, while CD3 ζ ITAMs are duplications of Fcγ (243). This suggests that over time, as the species became more complex, so did the TCR. More importantly, the multiple ITAMs within the TCR likely evolved for the specific purpose of generating and integrating signals that are necessary for the development of self-tolerant T cells.

What is Role of the Constitutively Phosphorylated P21 in TCR Signaling and Selection?

The pre-repertoire of T cells express TCRs that readily recognize MHC molecules (240, 241, 285). Following the mutation of three conserved residues on a Vβ8.2 restricted TCR that are necessary for binding conserved regions of MHC molecules located outside of the peptide binding groove, positive selection is impaired (285). Three conclusions can be drawn from such reports. First, MHC reactivity is not imparted by positive selection, but is present prior to selection. Second, MHC reactivity is the basis of positive selection as mutations of the amino acids that are critical for MHC binding reduce the efficiency of selection. Third, negative selection limits MHC reactivity by deleting T cells that are highly MHC reactive, as cells developing in an environment with limited negative selection show degenerate MHC cross-reactivity. Indeed, we identified p21 in the pre-selection thymocytes that were unable to undergo positive or negative selection (Figure 11 A-D). Thus, there are two sequential steps in T cells selection, the appropriate recognition of self-MHC followed by the detection of selecting self-peptides or self-glycolipids.

Thymocyte selection occurs in an environment in which TCR surface expression is actively maintained at a low level in developing DP thymocytes. This process is fostered by the recruitment of the E3 ligase c-Cbl to the CD3 ζ ITAMs, by the adapter molecule SLAP (41, 42, 150). This causes the ubiquitination and subsequent degradation of CD3 ζ, eventually leading to a reduction in TCR expression. This is likely a purposeful event in order to keep TCR signaling potentials low during selection. This is consistent with the fact that the DP thymocytes are more sensitive to stimulation

with low affinity antagonists than mature SP T cells and that low avidity ligands can induce deletion of thymocytes (286, 287). Furthermore, microRNA 181a, which increases TCR sensitivity to stimulation by negatively regulating the expression or function of phosphatases, is highly expressed in DP thymocytes (288). Due to the fact that TCR surface expression is kept very low prior to selection, low signaling potentials likely mediate proper selection. However, lowering signaling below a certain threshold adversely impacts both positive and negative selection. This is evident by data showing that efficient negative selection of conventional αβ T cells expressing low affinity TCRs requires a full repertoire of functional CD3 ITAMs (107-109, 140, 145). In one case, we found that potentially autoreactive $CD8^{int}T3.70^{+}$ T cells were released into the periphery of male HY/YF1,2 transgenic mice which express p21 in the absence of p23 (140). These cells also exhibit high levels of CD5 compared to other HY/YF mice, suggesting that these cells are in a more activated state. HY/CD3 ζ male mice expressing wild type, YF5,6 or YF1-6 CD3 ζ have a peripheral population of $T3.70^{+}$ cells that are $CD8^{lo}$, rendering them unresponsive to peptide/MHC complexes (140). The emergence of potentially autoreactive HY-specific T cells in HY/YF1,2 mice but not HY/YF5,6 mice, might be due to additional signal attenuation provided by p21 in HY/YF1,2 mice. Yet, if this is true, HY/YF1-6 mice should have also released these cells into the periphery, as mutation of 6 rather than 2 ITAMs would more profoundly attenuate signaling. However, the HY/YF1-6 mice have been shown to have much more severe impairment in positive selection (108). Thus, while negative selection was likely impaired YF1-6 mice, the impairment in positive selection could have prevented development of autoreactive T cells. Despite the emergence of potentially autoreactive cells in the periphery, HY/YF1,2

male mice did not show overt signs of autoimmunity. This could also be due to reduced signaling through the TCR as a result of mutations in two ITAMs. Furthermore, since HY/YF1,2 mice contain the constitutively phosphorylated p21, this might further attenuate TCR signaling, thereby keeping autoreactive cells quiescent in the periphery.

There are instances; however, where autoimmunity does result from lower TCR signaling thresholds. SKG is a spontaneous mutation in BALB/c mice, in which a mutation in the C-terminal SH2 domain ZAP-70 results in inflammatory arthritis (138). This mutations lead to a decrease in positive and negative selection. As such, HY^{skg} male mice have a large percentage of $TCR^{hi}CD8^{+}$ HY specific T cells, in almost comparable percentages to female HY mice. This suggests that although signaling through the TCR was reduced, sufficient signals were generated, allowing for activation of these cells following peptide/MHC recognition in the periphery. This might be especially critical since mature SP cells have higher TCR surface expression. The constitutive phosphorylation of p21 was detected in these cells but at much lower levels compared to wild type mice, likely due to the inability of $ZAP\text{-}70^{skg}$ to form stable interactions with phospho-ζ. Thus, autoimmunity could have resulted due to a lower level of p21 expressed in the periphery. Alternatively, p21 alone might not attenuate signaling enough to prevent the activation of autoreactive cells, when a full functional repertoire of CD3 ζ ITAMs is available for signaling.

The mechanism by which p21 could attenuate TCR signaling is unclear. P21 could sequester a population of inactive ZAP-70 molecules away from active TCR signaling components and/or recruit other proteins that negatively regulate signaling

pathways. This could limit signaling and promote thymocyte survival. The Epstein Barr virus expresses the ITAM containing protein, LMP2A. LMP2A sequesters Syk and ZAP-70 in infected B and T cells, respectively (30, 35). As a result, TCR signaling is reduced through the TCR, impairing host immune responses. Alternatively, when the LMP2A ITAMS were mutated, Syk association with LMP2A was prevented, and normal signaling within the B cell was restored. Furthermore, we have shown that p21/ZAP-70 complexes were not recruited to active signaling complexes (Figure 13 and 14). This suggests that p21 could attenuate signaling by sequestering ZAP-70.

Alternatively, another model, called receptor desensitization, was observed for the FcαRI receptor (44). This activity required low affinity binding interactions between monomeric IgA to the FcαRI receptor which limited receptor clustering, as high affinity IgA complex binging to FcαRI led to receptor aggregation and activation. Signal attenuation by FcαRI required association with the ITAM containing protein, Fcγ, which recruited the phosphatase SHP-1. This resulted in signal attenuation of the heterologous receptor FcγRI, due to dephosphorylation of the signaling proteins, Syk, LAT and Erk. P21 is also induced via low affinity interactions between the TCR and MHC molecules. Furthermore, TCR signaling is regulated by a number of phosphatases, such as SHP-1 (52). Thus, p21 could recruit phosphatases that to dampen signaling through adjacent TCRs.

P21 detection in DP T cells

In order to truly analyze the fate of T cells expressing p21, it is necessary to determine whether some or all cells express p21 and to determine the level of expression per cell. The only method to detect p21 in DP thymocytes is through Western blot analysis. Thus, p21 is detected in pooled T cells. We analyzed p21 by flow cytometry, using an anti-phospho-ζ (Y142) antibody that is specific for the fifth phospho-tyrosine residues in the CD3 ζ ITAMs. P21 was not detected in DP thymocytes from wild type C57BL/6 mice, but could be detected in SP T cells (Figure 15 A). Since SP T cells have much higher surface TCR expression, one explanation could be that p21 was not detected due to lower TCR surface expression in the DP cells. However, we also analyzed p21 expression using this antibody in wild type HY mice, which have much higher TCR surface expression in DP thymocytes. P21 was also undetectable in these cells (data not shown). This suggests that p21 was occluded, possibly by forming complexes with ZAP-70 or other signaling proteins, thereby blocking binding of the antibody to the phospho-ITAMs.

The fact that we detected p21 in cells prior to selection in both selecting and non-selecting backgrounds suggests that p21 results from recognition of conserved regions of MHC molecules. Therefore, one way to determine whether p21 is expressed in all cells would be to take advantage of the Vβ8.2 TCR system, in which known TCR residues mediate TCR recognition of MHC molecules (285). It would be useful to determine whether p21 is maintained in Vβ8.2 mice that contain that the mutations in the TCR that abrogate MHC recognition. If p21 was eliminated, it would also suggest that

all cells express some level of p21, since all T cells likely bind the conserved MHC regions. However, even if all T cells express p21, a possibility still remains that the level of p21 expression per cell would be the more important factor. In order to truly answer these questions, techniques would need to become available that would allow direct detection and measurement of p21 on a per cell basis.

CD3 ζ ITAMs in the regulation of iNKT cell selection and/or development

A full complement of functional CD3 ζ ITAMs is required for iNKT cell development. As such, mice with mutations in only one CD3 ζ ITAM, leaving 8 of the 10 CD3 ITAMs intact, have a significant reduction in the percentage and absolute number of iNKT cells in the thymus, spleen, liver, and bone marrow (Figure 18, 19 A, B). This could be due to inefficient induction of Ca^{++} signaling pathways in response to selecting ligands. In support of this, the Tec tyrosine kinase, Itk, and the transcription factor, Egr2, are required for iNKT cell development as mice lacking these molecules have reduced iNKT cell numbers due to an increase in cell death (221, 224). Itk functions downstream of the TCR and augments TCR signaling by increasing the activation of PLC-γ (82). This in turn, increases intracellular Ca^{++} levels. Egr2 is induced by NFAT, a transcription factor reliant on Ca^{++}/calcineurin signaling pathways for its activation (224, 225). One possible explanation is that YF mice could have reduced calcium responses contributing to the inefficient induction of Egr2. For the YF1,2 and YF1-6 mice, this could be due to inefficient recruitment to the immunological synapse, thereby dampening signaling through the TCR (289). Furthermore, there might be a more profound attenuation in the YF1,2 mice due to the expression of p21. This is

supported by the fact that iNKT cells from these mice had extremely low TCR surface expression and the highest rate of cell death, even when compared to the YF5,6 mice, which lack p21. P21 could additionally attenuate signaling in these mice by sequestering ZAP-70, further attenuating signaling in these mice, resulting in even lower expression of Egr2. However, the constitutive phosphorylation of p21 in iNKT cells has not been assessed. P21 could be assessed by Western blot analysis of sorted iNKT cells; however, this would require breeding the YF mice to the Vα14Jα18 transgenic line in order to increase iNKT numbers. It will be important to examine Itk activation in all of the YF iNKT cells in order to determine whether this pathway is disrupted by the ITAM mutations. In order to do this, phospho-Itk could be analyzed in iNKT cells by western blot or flow cytometry before and after stimulation with iGb3. Moreover, it is necessary to determine whether aberrant Egr2 expression accounts for the iNKT cell developmental block. Egr2 expression could be analyzed in iNKT cells from the various YF mice prior to and following stimulation with iGb3 (the endogenous ligand). Alternatively, iNKT cells could be analyzed following ectopic expression of Egr2 in order to determine whether this restores normal development. This could be done by retroviral transduction of Egr2 in DP precursor cells followed by intrathymic injection into a wild type host.

In preliminary experiments, the proliferation of iNKT cells was compared in YF1,2, YF1-6, and wild type mice. iNKT cells that were enriched from the spleen were stimulated *in vitro* with 100 ng/ml of α-GalCer, a potent agonist for iNKT cells. Cells were harvested after 24 or 96 hours of stimulation, and proliferation was analyzed by flow cytometry using a CFSE dilution assay. YF1-6 iNKT cells proliferate as well or

slightly better than wild type mice at both time points, as evidenced by similar (24 hour) or higher (96 hour) rates of CFSE dilution (Figure 27 B). YF1,2 iNKT cells has the lowest rate of cell division at both time points. There were almost no iNKT cells detected in the YF1,2 line by 96 hours of stimulation, whereas iNKT cells were detected in wild type and YF1-6 lines (Figure 27 A). As noted in developing thymocytes, the YF1,2 iNKT cells analyzed in the CFSE assay could be undergoing a higher rate of cell death. Staining with Annexin V and 7AAD staining following stimulation would confirm this. The YF5,6 line should also be included as a control for the YF1,2 mice, in order to distinguish between the effects of CD3 ITAM number versus the presence of the constitutively phosphorylated p21. To correlate these finding with the developmental profiles of thymocytes, using the endogenous, selecting ligand iGb3 would be useful, as α-GalCer is a potent agonist. Lastly, it would be interesting to analyze phospho-proteins, such as ZAP-70, SLP-76, Itk, and PLC-γ in order to determine whether TCR signaling is impaired in iNKT cells when the number of functional ITAMs is reduced. This could be determined by flow cytometric analysis of phospho-proteins. Alternatively, if adequate cell numbers could be acquired, this could also be determined by Western blot analysis in sorted iNKT cells.

Lastly, it remains unclear whether a full complement of CD3 ITAMs is required for normal iNKT cell development. In order to determine this, iNKT cells should be analyzed in mice carrying YF mutations in either the CD3 γ or CD3 δ ITAM. Since this would only eliminate one ITAM in the TCR complex, nine ITAMs would remain

functional. If there were reductions in these mice, this would suggest that all ten CD3 ITAMs must be functional to support the proper development of iNKT cells.

Are CD3 ζ ITAMs important for the development of other innate effector T cell populations?

Preliminary data suggest that the development of other innate effector T cells is also dysregulated in the YF series of mice. $CD8^+$ T cell development was analyzed in wild type and YF1-6 mice (Figure 17 A). A two-fold reduction was consistently noted in peripheral $CD8^+$ T cells is observed in the YF1-6 mice compared to wild type mice (Figure 17 A). We further analyzed the function of $CD8^+$ T cells by analyzing the types of cytokines produced following *in vitro* TCR stimulation. T cells from the C57BL/6 and YF1-6 were stimulated with an anti-CD3 ε antibody, and analyzed IFN-γ production 24 hours later by intracellular cytokine staining and flow cytometry. There was a 2-fold reduction in the percentage of IFN-γ producing $CD8^+$ T cells in YF1-6 mice compared to controls (Figure 28). Because IFN-γ production is required for efficient innate immune responses to *Listeria monocytogenes* infection, we also compared the ability of wild type and YF1-6 mice to clear primary *L. monocytogenes* infection (290-293). We used a recombinant version of *L. monocytogenes* that expresses ovalbumin (rLM-Ova). Mice were injected with 10, 000 CFU of rLM-Ova. On day three post-infection, the total colony forming units (CFU) were calculated in the spleen and liver. There was no difference in the ability of wild type and YF1-6 mice to clear rLM-Ova from the spleen as similar CFUs were calculated (Figure 29). The YF1-6 line did, however, demonstrate

a slight impairment in the clearance of bacteria from the liver, although this difference never reached statistical significance. We also analyzed the ability of wild type and YF1-6 mice to mount an efficient memory response to rLM-Ova. Mice were infected with 2000 CFU of rLM-Ova, rested for 6 weeks, and rechallenged with 200,000 CFU. On day 3 p.i., the CFUs in the liver and spleen were calculated. Both the YF1-6 and the C57BL/6 mice cleared infection. These results demonstrate that the YF1-6 mice were able to mount efficient primary and secondary immune responses to rLM-Ova infection, or that these responses are not dependent on IFN-γ production by $CD8^+$ T cells.

To further characterize $CD8^+$ T cells from YF1-6 and WT mice, we stained splenocytes and lymph node cells for cell surface markers that are used to identify naïve and memory T cell populations. The expression of CD44 and CD62L was analyzed on $CD8^+$ T cells in order to identify naïve ($CD62L^+CD44^-$) and memory ($CD62L^+CD44^+$, effector memory, EM; $CD62L^-CD44^+$, central memory, CM) subsets. In the lymph node, the percentage of naïve T cells was reduced from 55% to 16% in WT and YF1-6 mice respectively (Figure 30; $p < 0.05$). In addition, the percentage of EM and CM cells were significantly higher in the YF1-6 (13%, 27%, respectively) compared to WT mice (5%, 13%; $p < 0.05$). In the spleen, naïve $CD8^+$ T cells were reduced in YF1-6 (16% versus 29%), although this difference did not reach significance (Figure 30). The percentage of EM and CM cells were significantly increased in the YF1-6 (34%, 29%) compared to WT (15%, 12%, $p<0.05$) mice. Next, innate IFN-γ production was analyzed in $CD8^+$ T cells following stimulation with recombinant IL-12 (rIL-12) and rIL-18. A similar percentage of total $CD8^+$ T cells from the YF1-6 mice (38%) produced IFN-γ compared

to wild type mice (28%) (Figure 31, top). However, there was a significant increase in the percentage of $CD44^{hi}CD8^{+}$ T cells that produced IFN-γ in YF1-6 mice (31%) compared to wild type controls (16%) (Figure 31, middle). When IFN-γ production was assessed in the $CD44^{lo}CD8^{+}$ T cells, there was no difference in the YF1-6 and wild type mice (Figure 31, bottom). Further experiments will be needed in order to confirm these results.

It is interesting to note that naïve $CD8^{+}$ T cells are almost completely absent in $ITK^{-/-}$ mice. Yet, the total percentage of $CD8^{+}$ cells in these mice is normal (221). The $CD8^{+}$ T cell compartment was almost exclusively made up of innate $CD8^{+}$ T cells, which were characterized by high expression of CD44, NK1.1, and CD122. The pattern of $CD8^{+}$ and iNKT cell development in Itk deficient animals is similar to our YF1-6 mice. Our phenotype is likely not as profound as that seen in Itk deficient mice since some Itk signaling is likely intact due to the ability of YF1-6 T cells to signal through 4 CD3 ITAMs.

Innate $CD8^{+}CD44^{+}$ T cells isolated from $Itk^{-/-}$ mice rescue INF-γ null mice from an otherwise lethal *L. monocytogenes* infection, and $Itk^{-/-}$ mice clear *L. monocytogenes* more quickly than wild type mice due to an increase in IFN-γ secretion (294). Since the YF1-6 mice have an increase in $CD8^{+}CD44^{+}$ T cells with an increased capacity to secrete IFN-γ following innate cytokine stimulation, this could have masked any deficit that might have occurred following *L. monocytogenes* in these mice. Several groups have

shown a role for various innate $CD8^+$ T populations in the clearance of *L. monocytogenes* infection (294-297).

Future experiments should be directed at determining whether the "memory" $CD8^+$ T cells from our YF1-6 mice are actually innate effector T cells, by further characterizing the expression of CD44, NK1.1, and CD122. Furthermore, it would be interesting to determine whether innate $CD8^+$ T cells populations are also increased in YF1,2 and YF5,6 mice, as they exhibit a similar defect in iNKT cell development. It would be interesting to determine whether YF1-6 mice clear rLM-Ova as efficiently as WT mice if the $CD44^+CD8^+$ T cells are depleted prior to infection. Furthermore, these cells could be transferred into IFN-γ null mice in order to determine whether the $CD44^+CD8^+$ T cells from YF1-6 mice can rescue these mice from lethal rLM-Ova infection.

Are iNKT cells necessary for the prevention of Lyme arthritis?

The role of iNKT cells in the control of Lyme arthritis following *B. burgdorferi* infection is controversial. Some reports that suggest that iNKT cells are not required for the prevention of inflammatory arthritis following *B. burgdorferi* infection, as $Rag^{-/-}$ C57BL/6 mice, but not $Rag^{-/-}$ BALB/c mice, remain resistant to arthritis (276, 277). Other reports, however, suggest that iNKT cells are critical for preventing the onset of inflammatory arthritis as both $Ja18^{-/-}$ and $CD1d^{-/-}$ mice are susceptible to arthritis (267, 268). Our data supports the former findings, as YF mice, despite a profound lack of iNKT cells, remained resistant to arthritis following high dose *B. burgdorferi* infection.

Because different strains of mice have been shown to be more susceptible to inflammatory arthritis following *B. burgdorferi* infection, the possibility remains that iNKT cells could provide some level of protection or immunoregulation of the disease in susceptible strains of mice (276, 277, 298). The protection offered by iNKT cells is likely due to cytokine secretion by these cells during infection. In both mice and humans, resistance to Lyme arthritis correlates with increased IL-4 (Th2) while IFN-γ (Th1) is associated with more progressive disease (279, 280, 282). IL-10 secretion is also correlates with improved disease outcome in mice and humans (299, 300). Borrelia-specific antibody production and marginal zone B cells are also critical for the control of *B. burgdorferi* infection and prevention of Lyme arthritis (275, 301-303). In the $Ja18^{-/-}$ model of Lyme arthritis; however, *B. burgdorferi* specific antibodies were higher than those produced by WT mice. Thus, it is unlikely that iNKT cells are necessary to control the humoral immune response during infection. Instead, it appears that these cells act as true regulatory cells and attenuate the inflammatory response to *B. burgdorferi* in susceptible hosts, possibly by secreting high levels of IL-4 and/or IL-10.

Model for innate effector T cell development

The development of T cells bearing low affinity TCRs requires signaling through a full complement of CD3 ITAMs for efficient positive and negative selection. iNKT cells appear to be especially sensitive to signals generated through the TCR, as disruption of only two ITAMs significantly reduces iNKT cell development. Thus, we propose the following model to account for the development of low affinity T cells (Figure 32). In developing T cells, recognition of selecting peptide/MHC or glycolipid/CD1d complexes

results in the phosphorylation of all of the CD3 ITAMs by Lck. This results in the recruitment of ZAP-70 to the phospho-ITAMs and subsequent activation. ZAP-70 then phosphorylates LAT, resulting in the recruitment and activation of PLC-γ. This would increase intracellular calcium within the T cells, allowing for the activation and translocation of NFAT into the nucleus, where it could be retained in order to promote efficient positive selection.

In the case of innate effector cells, Itk would be recruited to the active signaling complex via its association with LAT, either directly or indirectly via interaction with SLP-76. Activated Itk would then phosphorylate and activate PLC-γ, resulting in increased and/or sustained calcium signaling in the developing iNKT cells. This would lead to increased activation of NFAT, and allow for sufficient upregulation of Egr2. Egr2 would then go on to regulate the transcription of genes important for iNKT cell selection, development and survival.

In mice in which one or more CD3 ζ ITAMs are mutated, signaling potential through the TCR decreases resulting reduced calcium signaling within the cell. This could lead to NFAT export from the nucleus, and the transcription of genes important for selection and development would be arrested. In the case of iNKT cells, this could be a result of inefficient expression of Egr2.

Additional attenuation of TCR signaling found in the YF1,2 mice, due to the constitutive p21 phosphorylation, could further reduce signaling through this pathway, resulting in a more severe iNKT and conventional T cell phenotype in these mice.

Reduced signaling, on the other hand, would favor the emergence autoreactive T cells or innate $CD8^+$ T cells.

Overall Conclusions

The TCR signals through 6 signaling chains that contain 10 ITAMs. The reason for such a complex immunreceptor is unclear, but is likely necessary to promote the development of numerous T cell subsets that are self-tolerant. This is supported by the fact that the ancient ITAM-based immunoreceptor, Draper, recognizes "modified self" on of dying neurons, inducing the phagocytosis and clearance of these cells in order to promote self-tolerance. While the function of this receptor is slightly different than the TCR, it sets a precedent for an ITAM-based immunoreceptor that promotes self tolerance following intracellular signals mediated by ITAM-based signaling pathways. Thus, these data suggest that the TCR evolved in order to recognize self-antigens from thymocytes undergoing necrosis and apoptosis and translate these events into intracellular signals that lead to the development of self-tolerant T cells. However, the predecessor of the TCR contained a single signaling chain that included a single ITAM. The reason for 10 ITAMs in the TCR is unknown; however, substantial evidence suggests that these ITAMS are specifically required to overcome signaling thresholds and/or direct signaling pathways that are necessary for efficient positive and negative selection.

T cell development occurs in an environment in which TCR expression is maintained at very low levels. Thus, 10 ITAMs might be necessary to additively increase ITAM signaling in order to provide efficient signals for selection. This is supported by

data from mice in which some or all CD3 ITAMs have been mutated or eliminated in T cells bearing low affinity TCRs, resulting in inefficient positive and negative selection of several subsets of cells. Furthermore, the fact that the TCR is organized into two distinct signaling modules, the CD3 γε/δε heterodimer and the CD3 ζζ homodimer, suggests that signal discrimination might also be necessary to ensure efficient selection. This type of signaling could be required for the induction the distinct signaling pathways involved in positive versus negative selection. The constitutive phosphorylation of CD3 ζ in developing thymocytes could also provide some level of signal attenuation and/or receptor desensitization, which likely further contributes to the appropriate selection of T cells. Taken together, these data indicate that TCR signaling during thymocyte development is a highly regulated process, as even subtle reductions can disrupt development. Thus, the evolution of the TCR complex was a precise and deliberate event in order to provide multiple and meaningful layers of signal transduction during thymocyte development, thereby directing the development self-tolerant T cells.

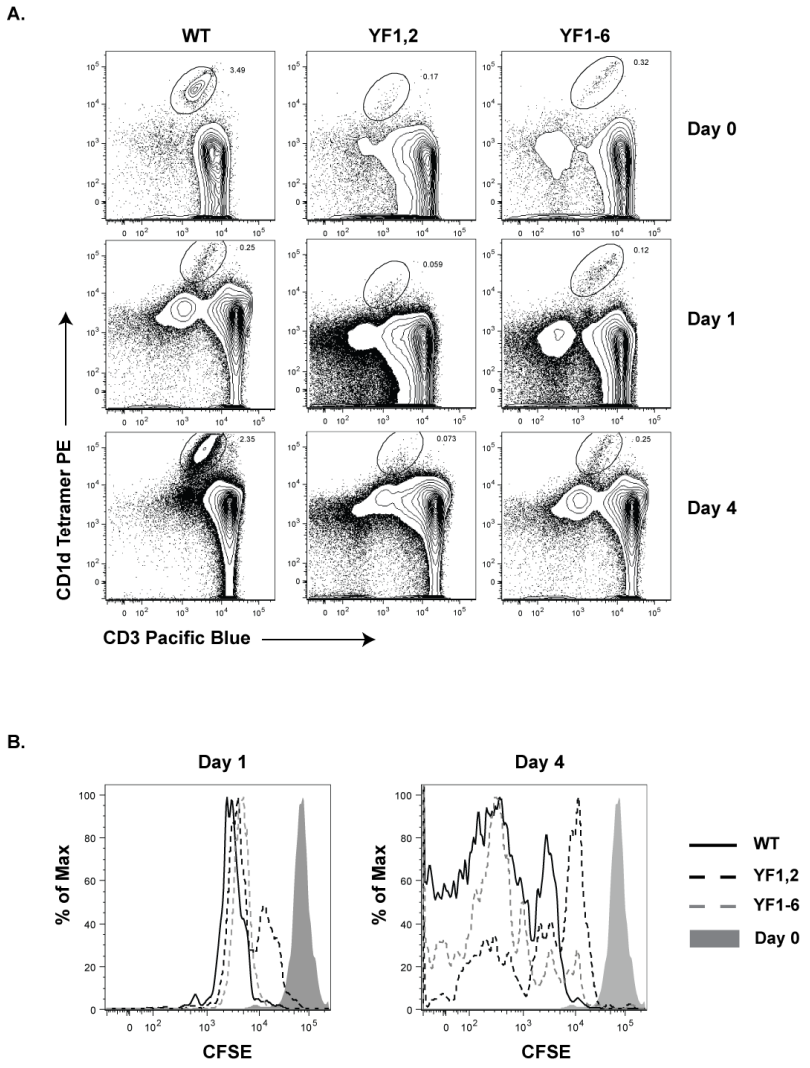


Figure 27: iNKT cells from YF1,2 mice proliferate less efficiently than WT and YF1-6 iNKT cells. Splenocytes were isolated from the indicated mice. B220 cells were depleted using anti-B220 magnetic beads. The remaining cells were CFSE labeled. 500,000 cells were plated per well of a 96 well round bottom plate. Six wells were plated per mouse. Cells were stimulated with 100 ng/ml α-GalCer. *A.* Cells were harvested after 96 hours of stimulation, and stained with anti-CD3 ε Pacific Blue, -B220 and CD11b APC-Cy7, unloaded CD1d tetramer APC, and PBS57-loaded CD1d tetramer PE. B220, CD11b, and APC tetramer positive cells were gated out. The iNKT cells were then identified as $CD3^{intermediate}$ CD1d $tetramer^{+}$ cells. *B.* Histograms represent iNKT cells.

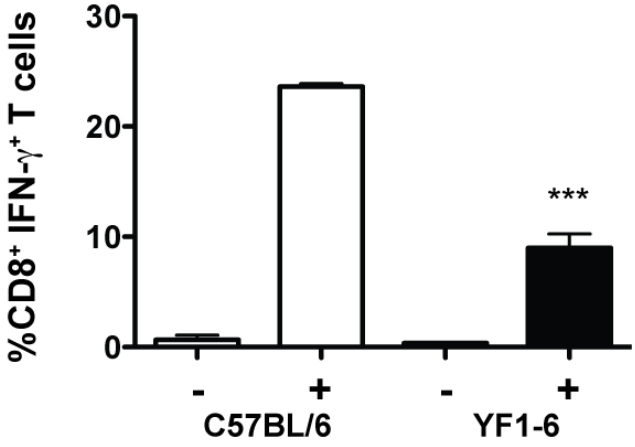


Figure 28: Fewer $CD8^+$ T cells from YF1-6 mice produce IFN-γ following anti-CD3 stimulation. Splenocytes were harvested from the indicated mice. Red blood cells were lysed. 1 x 10^6 cells were added to each well of a 24 well plate. Cells were stimulated with soluble anti-CD3ε mAb for 24 hours. Cells were harvested and stained with anti-CD4 APC, anti-CD8 FITC. Cells were then washed and permeablized using BD Cytofix/Cytoperm kit. Cells were then stained intracellularly with anti-IFN-γ PE. Bar graphs represent the percentage of $CD8^+$ T cells producing IFN-γ. Data are representative of at least 3 independent experiments with 3-5 mice per group. Statistics generated by Student's t test. $p < 0.002$

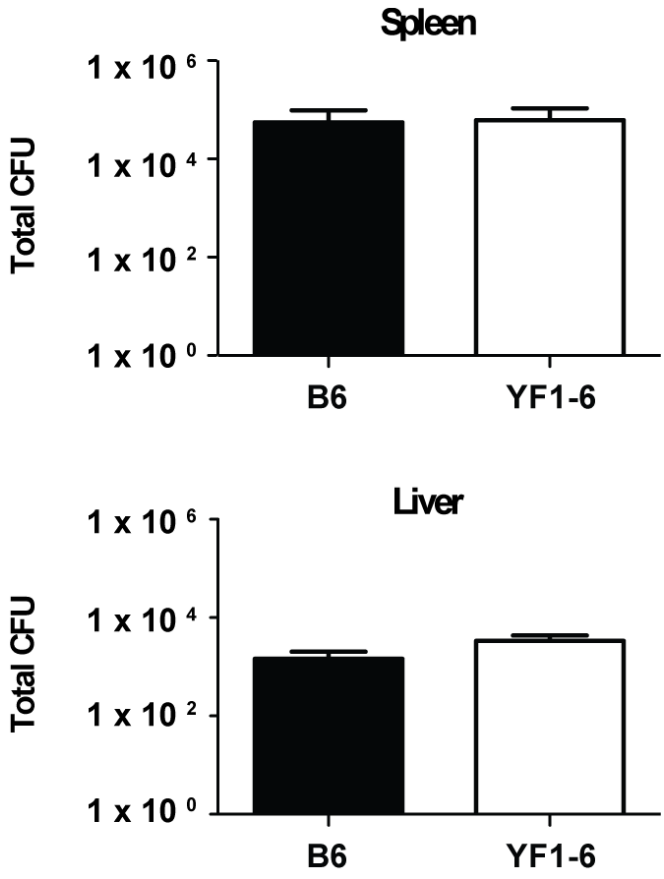


Figure 29: Clearance of primary rLM-Ova infection is not dependent on CD3 ζ ITAMs. C57BL/6 (B6) or YF1-6 mice were injected with 10,000 CFU of rLM-Ova. Three days post-infection, mice were sacrificed, and spleen and livers were harvested and cells were lysed in sterile water. Serial dilutions of lysates were made and total colony forming units (CFU) were calculated in each organ.

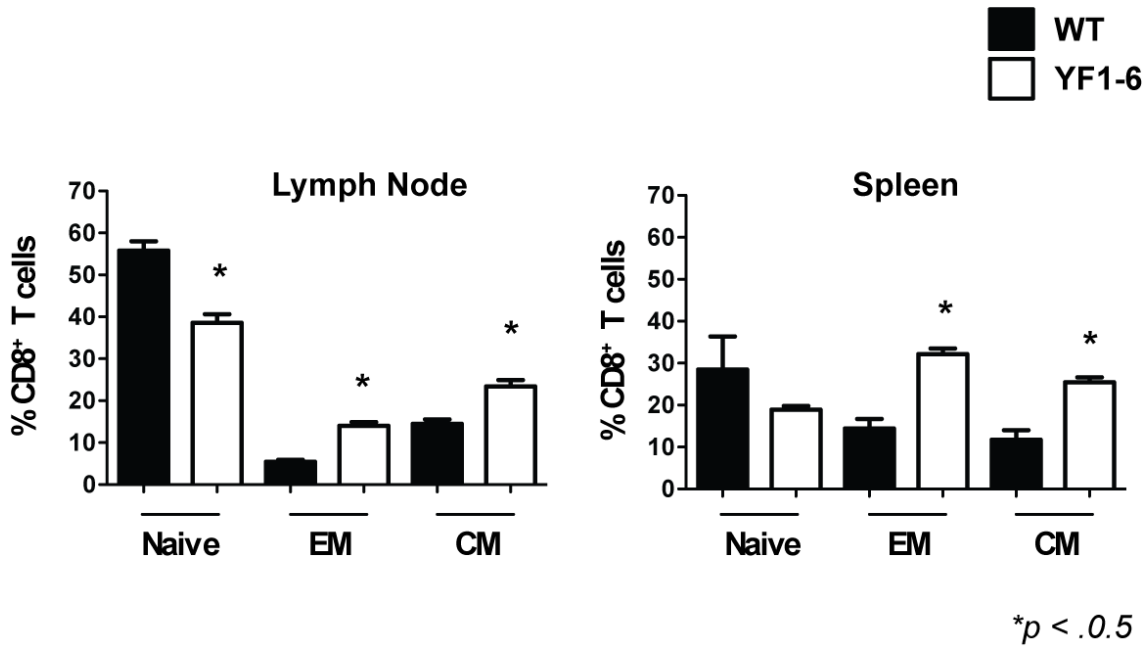


Figure 30: Effector and central memory CD8$^+$ T cells are increased in YF1-6 mice compared to WT mice. Splenocytes and lymph node cells were harvested. Red blood cells were lysed in the spleen. Cells were stained with anti-CD4, anti-CD8, anti-CD44, and anti-CD62L. Cells were gated on CD8$^+$ T cells and analyzed for the expression of CD44$^+$CD62L$^-$ effector memory (EM), CD44$^+$CD62L$^+$ central memory (CM), and CD44$^-$CD62L$^+$ naïve CD8$^+$ T cells. * $p < 0.05$. Statistics were generated by Student t's test.

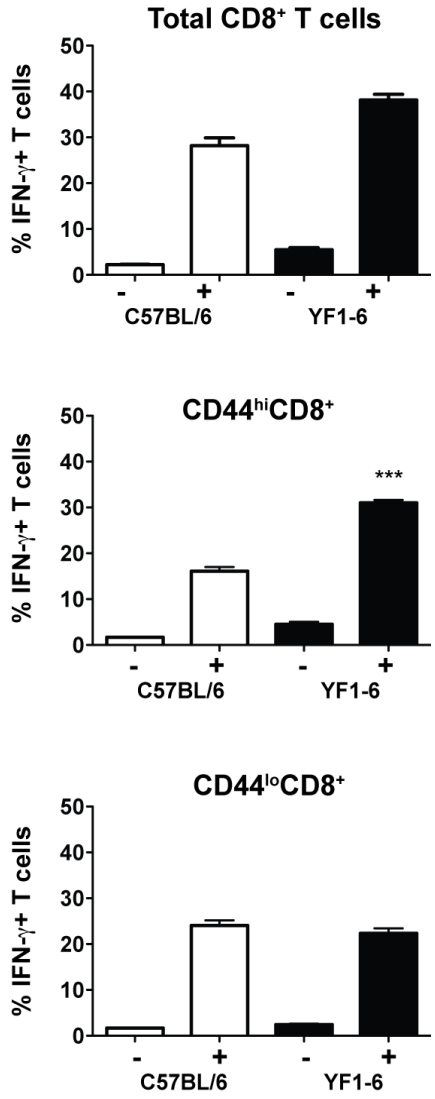


Figure 31: CD44$^+$CD8$^+$ T cells produced higher levels of IFN-γ following IL-12 and IL-18 stimulation *in vitro*. Splenocytes were harvested and red blood cells were lysed. 1 x 10^6 cells were plated per well of a 6 well plate. Recombinant IL-12 and IL-18 were added. Cells were stimulated for 24 hours at 37 degrees Celsius. Cells were harvested, and stained with anti-CD4 and –CD8. Cells were washed and then fixed and permeablized using BD Cytofix/Cytoperm kit. Cells were then stained intracellularly using anti-IFN-γ PE. Cells were analyzed by flow cytometry using the BD FACSCalibur. Plots represent the percentage of CD8$^+$IFN-γ$^+$ T cells. Each dot represents a mouse. Statistics were generated using the Student t's test. ** $p < 0.005$.

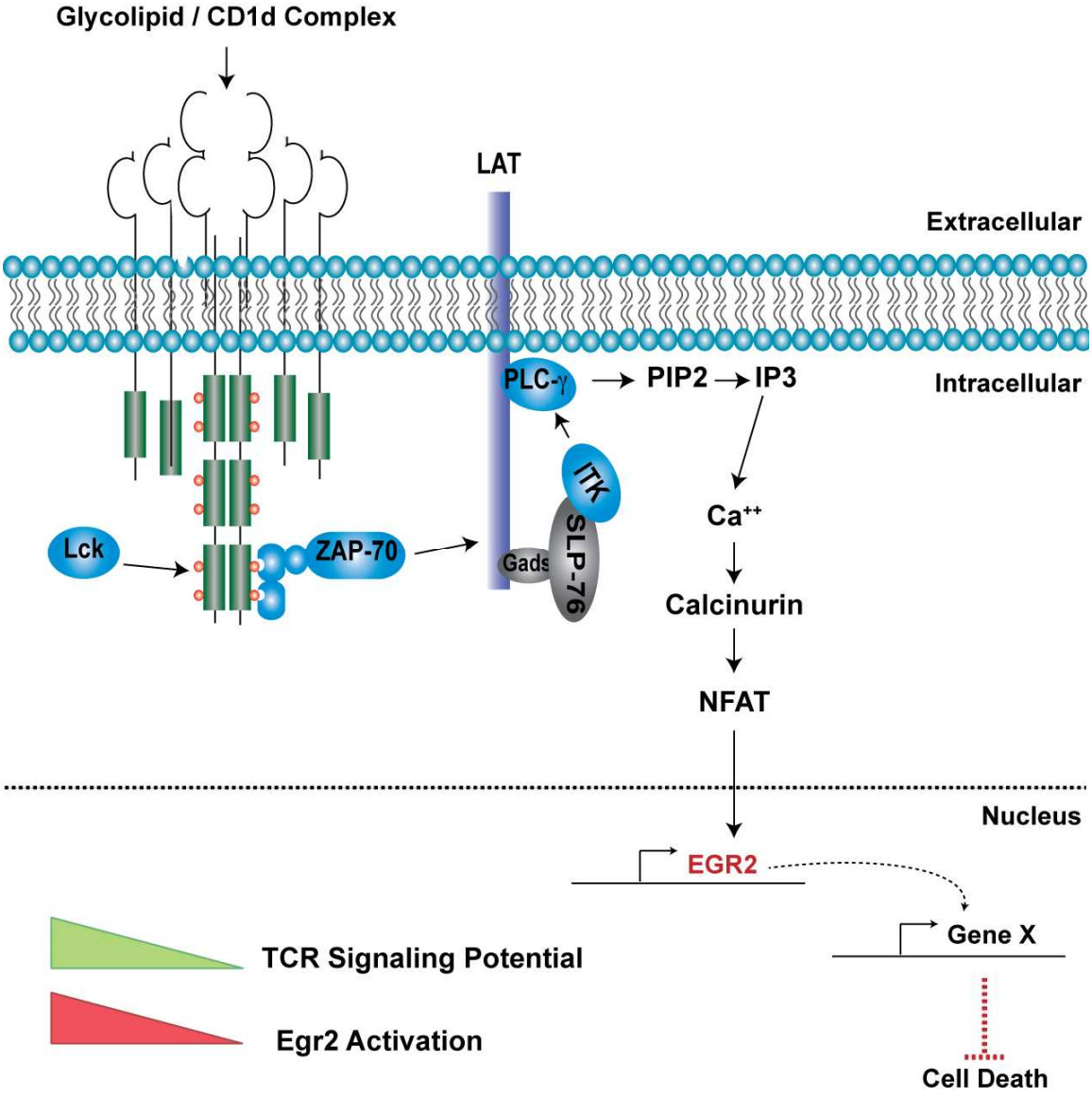


Figure 32: iNKT cell signaling in CD3 ζ wild type mice. Once the TCR is engaged by glycolipid/CD1d complexes, CD3 ζ ITAMs are biphosphorylated by Lck, leading to the recruitment of ZAP-70 via its tandem SH2 domains. This leads to an intracellular signaling cascade that results in the recruitment of Itk to the signaling complex, enhancing the activation of PLC-γ. This amplifies TCR signaling by increasing intracellular calcium signaling. This results in the activation of calcineurin which goes dephosphorylates and activates NFAT, which translocates into the nucleus. NFAT then upregulates Egr2 expression, a transcription factor that regulates genes that are necessary for iNKT cell selection, development and/or survival.

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