THE FUNCTIONS OF THE TCR ζζ MODULE IN T CELLS

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Acknowledgments

There are a number of people that I would like to acknowledge for all of their support during my years in graduate school at UT Southwestern. I am very grateful to Dr. Nicolai van Oers, who welcomed me as his first graduate student in the lab. Nicolai provided an outstanding learning environment for me to learn how to think critically and become an independent scientist.

I would also like to give special thanks to all of my family, especially my parents, Tom and Judy Pitcher, who have been a constant source of love and encouragement. My siblings, Sue, Brent and Dani and their families have also encouraged and motivated me as I have continued my education, even though I moved across the country to do so.

Last, but not least, I would also like to thank the many friends that I have made while living in Texas. You have helped maintain my sanity, especially during the frustrating times and you always made me see the humor in everything. I will never forget all of the great times that we had!

THE FUNCTIONS OF THE TCR ζζ MODULE IN T CELLS

By

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2005

THE FUNCTIONS OF THE TCR ζζ MODULE IN T CELLS

Publication No. _____

Lisa Anne Pitcher, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2005

Supervising Professor: Nicolai S. C. van Oers, Ph.D.

The αβ T cell receptor complex (TCR) has the unique ability to discriminate and differentially respond to peptide/MHC ligands encountered on the surface of antigen presenting cells. The ligation of the TCR with peptide/MHC complexes is translated into intracellular signals through a conserved sequence motif, termed ITAM, or immunoreceptor tyrosine-based activation motif, which are present in one or more copies in the cytoplasmic portions of the TCR ζ and CD3 γ, δ and ε chains. A distinctive feature of the TCR complex is that it contains ten ITAMs, in contrast to other antigen receptor complexes which contain two or four ITAMs. The ten TCR ITAMs are distributed as

one in each CD3 chain and three in the TCR ζ subunit (TCRαβ εδεγζζ). It has been proposed that the TCR is comprised of two autonomous signaling modules, TCR ζζ and CD3 γε/δε. Following receptor ligation, TCR ζ is the most heavily tyrosine-phosphorylated subunit of the TCR, developing into two stable intermediates of 21- and 23-kDa (p21 and p23). Based on the number of ITAMs it contributes, TCR ζζ was initially presumed to be the predominant signaling module in the TCR complex, with p21 and p23 being linked to virtually all aspects of T cell biology. To clearly define the functions of the TCR ζζ module, we generated a series of TCR ζ transgenic mice, with modified ζ molecules, that selectively express p21 alone, no p21 or p23, or no phospho-ζ intermediates. In a wild type or the P14 TCR transgenic system, T cell development was completely normal in the TCR ζ transgenic lines. Surprisingly, when peripheral T cells were analyzed for their functionality in response to various stimuli, including peptide/MHC-, T cell mitogen- and superantigenic- stimulation, equivalent dose response curves were observed, regardless of phospho-ζ. Notably, these data also eliminated a possible inhibitory role for the partially phosphorylated p21 intermediate of TCR ζ. To more carefully examine the roles of p21 and p23, the TCR ζ transgenics were mated to a second TCR transgenic line (HY), wherein a direct dependence on the TCR ITAMs for thymocyte selection has been noted. In this system, important roles for phospho-ζ during T cell selection were revealed. Specifically, TCR ζ ITAMs functioned additively during positive selection events. These selection events appear to be independent of traditional signaling pathways, as the signaling capacity of unselected T cells in the absence of all phospho-ζ was equivalent to T cells with wild-type TCR ζ subunits. These results imply that the CD3 γε/δε module forms the predominant signaling module in the TCR complex.

Our studies also identified a unique role for p21 during negative selection events. The select expression of p21 in T cells attenuated negative selection of thymocytes, resulting in the generation of a population of potentially autoreactive cells. Based on these data, a revised model of TCR signal transmission is proposed. Within this model, the TCR ζζ and CD3 γε/δε modules contribute both redundant and unique functions to T cells. The CD3 γε/δε module is primarily responsible for classical TCR-mediated signaling pathways leading to T cell activation. The TCR ζζ and CD3 γε/δε modules contribute redundant functions to thymocyte positive selection. These redundant functions are mediated by the ten TCR ITAMs. Phosphorylated intermediates of the TCR ζζ module also contribute to thymocyte positive selection, likely through alternative signaling pathways. In addition, the TCR ζζ module functions in a unique manner during thymocyte negative selection, with p21 attenuating negative selection of thymocytes. Furthermore, preliminary evidence suggests novel roles for the TCR ζζ module in the maintenance of peripheral T cells and in the adaptive immune response to bacterial pathogens.

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Abbreviations

Ab: antibody

APC: antigen presenting cells

APL: altered peptide ligand

BCR: B cell antigen receptor

CAST: CD3ε associated signal transducer

CFSE: Carboxyfluorescein diacetate succinimidyl ester

Con A: Concanavalin A

CPM: counts per minute

CTLA-4: Cytotoxic T lymphocyte antigen 4

DAG: diacylglycerol

DC: dendritic cell

DIG: detergent insoluble glycolipid-enriched domains

DN: double negative

DP: double positive

EBV: Epstein Barr Virus

ERK: extracellular regulated MAP kinase

FACS: fluorescence activated cell sorter

FcR: Fc receptor

FITC: fluorescein isothiocyanate

Grb2: growth factor binding protein 2

GEM: glycolipid enriched membrane domain

HSC: hematopoietic stem cell

IFN γ: interferon gamma

Ig: immunoglobulin

IL-2: interleukin 2

IP_3: inositol 4,5 trisphosphate

ITAM: immunoreceptor tyrosine-based activation motif

JNK: jun N-terminal kinase

kDa: kilodalton

LAT: linker of activated T cells

LM: *Listeria monocytogenes*

mAb: monoclonal antibody

MAPK: mitogen activated protein kinase

MFI: mean fluorescence intensity

MHC: major histocompatability complex

NK: natural killer

PCR: polymerase chain reaction

PE: Phycoerythrin

PI3K: Phosphoinositide-3 kinase

PIP_2: phosphatidyl 4,5 bisphosphate

PLCγ1: phospholipase C γ1

pMHC: peptide/MHC

PTB: protein tyrosine binding

PTK: protein tyrosine kinase

PTPase: protein tyrosine phosphatase

RAG: recombinase activating gene

SDS-PAGE: SDS polyacrylamide gel electrophoresis

SEB: Staphylcoccal Enterotoxin B

SH2: Src homology 2

SH3: Src homology 3

SLP76: SH2 domain containing leukocyte protein of 76-kDa

SLAP2: Src-like adaptor protein 2

SOS: son of sevenless

TCR: T cell receptor

Tg: transgene

TN: triple negative

TRIM: TCR-interacting molecule

ZAP-70: zeta associated protein of 70-kD

Chapter I. Introduction

T cells are an important constituent of the adaptive immune response against bacterial and viral pathogens. T cells develop in the thymus into two cell types ($CD4^+$ and $CD8^+$T cells) based on their MHC restriction and specificity. T cells specifically recognize an assortment of small peptide ligands embedded within the major histocompatability complexes (MHC) expressed on the surface of antigen presenting cells (APCs). T cell recognition of peptide/MHC ligands is mediated through the T cell receptor complex (TCR). An essential feature of the TCR is the ability to detect and differentiate small variations in the peptide ligands presented by MHC molecules and translate them into a series of intracellular signaling events. These signaling events can induce a wide range of T cell responses, from T cell proliferation and differentiation to cytolytic function and the secretion of cytokines.

The Structure of the T Cell Receptor Complex

The T cell receptor is a multimeric complex comprised of the antigen specific subunits, TCR α and β (or the related TCR γ and δ) and six noncovalently associated signaling subunits, CD3 γ, δ and ε and TCR ζ (αβγεδεζζ; Figure 1)(1, 2). All of the TCR subunits, TCR α and β, CD3 γ, δ and ε and TCR ζ, are the products of distinct genes that are assembled in a highly orchestrated fashion. The TCR α and β genes comprise multiple gene segments, which are rearranged by a series of recombination and joining

events that occur during T cell ontogeny. The recombination of the TCR α and β genes serves to create a diverse TCR repertoire capable of responding to an extensive array of microbial pathogens (1, 3). The recombined TCR α and β subunits, which range from 80-90-kDa in size, contain extracellular immunoglobulin-like domains, membrane-spanning regions containing a highly conserved lysine residue, and very short cytoplasmic tails of 4-12 amino acids (1, 2). In addition to the conserved lysine residue, TCR α contains an arginine residue within the transmembrane domain that is critical for TCR assembly. Noncovalently associated with the TCR α and β subunits are four distinct invariant chain proteins, CD3 γ, δ and ε and TCR ζ. The CD3 γ, δ and ε share high sequence and structural homology to each other. These genes are closely linked in a region on chromosome 11q23 in humans and chromosome 9 in mice (2). The CD3 γ, δ and ε subunits share a similar structure, comprised of an extracellular immunoglobulin-like domain, a short transmembrane region of 27 amino acids and a cytoplasmic domain of 45-55 amino acids. Within the cytoplasmic domains of CD3 γ, δ and ε is a semi-conserved sequence motif, termed an immunoreceptor tyrosine-based activation motif (ITAM). Based on similar sequence and structure, it is likely that the CD3 γ, δ and ε invariant chain genes arose from a common ancestor by a series of gene duplications and transpositions (4, 5). CD3 γ and δ exist as glycosylated proteins that migrate at 21- and 25-kDa, respectively in mouse or 25-28- and 21-kDa in human (2). CD3 ε exists in the TCR complex as an unglycosylated protein of 22- or 23-kDa in mouse and human. The TCR ζ/η gene is located on human and mouse chromosome 1 and differs structurally from the TCR α and β or CD3 γ, δ and ε subunits (4, 6). TCR ζ/η belong to a family of proteins which includes TCR ζ, TCR η and the FcRγ subunits. The TCR ζ/η subunits are

products of alternative splicing of the TCR ζ/η gene which contain a short extracellular domain (6-9 amino acids), a membrane-spanning region and long cytoplasmic domains. Within the cytoplasmic tails of TCR ζ/η are multiple copies of the ITAM motif. TCR ζ is a 16-kDa protein that contains three ITAMs within a 113 amino acid cytoplasmic domain. In contrast, TCR η is a splice variant of TCR ζ that contains two ITAMs in a 155 amino acid cytoplasmic domain and migrates at 22-kDa (7, 8). The FcRγ is a related family member that contains only one ITAM in its cytoplasmic domain(4). In most T cell, 90% of TCR complexes contain disulfide-linked homodimers of the TCR ζ subunit. In some circumstances, the TCR complex can contain heterodimers of the TCR family members TCR ζ/η or FcRγ (9, 10).

The cell surface expression of a fully assembled TCR complex is required for the functions of both immature and mature T cells. The uncertainties regarding the stoichiometry of the TCR complex and whether it exists as a monovalent or divalent structure have led to multiple models of T cell activation (11, 12). Recent data indicates that the TCR is a monovalent structure that is assembled in a highly orchestrated fashion that usually takes place in the endoplasmic reticulum and sometimes in the Gogli apparatus (13-15). The generation of a heterotrimeric complex containing CD3 δ and ε and TCR α involves interactions between critical glutamic acid residues in the transmembrane regions of CD3 δ and ε with the conserved lysine residue in the transmembrane domain of TCR α (15, 16). The generation of the TCR β/CD3 γε heterotrimer follows TCR α/CD3δε formation and involves the aspartic acid and glutamic acid residues in the CD3 ε and γ, respectively, that interact with the conserved lysine in

the TCR β transmembrane domain (15). The ectodomains of the CD3 γ and ε also contribute to the specific interactions involved in TCR assembly. Dimerization of the TCR ζ subunit and association with the partial TCR complex (αβγεδε) is the last step in TCR assembly. The TCR ζζ homodimer requires the aspartic acids at position six in the transmembrane regions of TCR ζ for interaction with the arginine residue in TCR α. The addition of the TCR ζζ homodimer to the partial TCR complex can occur in either the endoplasmic reticulum or the Golgi apparatus. The formation of the fully assembled monovalent TCR complex facilitates the trafficking of the TCR complex to the T cell surface (TCR αβεδεγζζ)(13, 14, 16).

ITAMs in the T Cell Receptor Complex

A feature common to all of the TCR invariant signaling subunits (TCR ζ and CD3 γ, δ and ε) is the presence of one or more copies of a semi-conserved amino acid sequence motif within their cytoplasmic tails. This conserved motif, termed an ITAM, contains two critically-spaced tyrosine residues amidst a series of semi-conserved amino acid residues (D/ExxYxxL/I x_{6-8} YxxL/I). (17, 18). The ITAMs are present as single copies in each of the CD3 γ, δ and ε subunits while they are triplicated in the TCR ζ subunit, yielding up to ten ITAMs in the TCR complex (αβεδεγζζ) (Figure 1).

The ITAM motif is critical for signal transmission through the TCR complex. ITAMs also form the basis of signaling through other antigen receptors, including the BCR, certain activating NK receptors (through DAP12/KARAP) and Fc receptors

(FcεRIγ and FcεRIβ), all of which encompass at least two ITAMs (4). The importance of the ITAMs is demonstrated by the conservation of this sequence motif throughout evolution. ITAMs have been identified in mammals, birds, amphibians and fish (4). In addition, the ITAM sequence has been identified in proteins from at least five viruses. These include Epstein-Barr Virus (LMP2A), Kaposi's sarcoma-associated herpesvirus (K1), bovine leukemia virus (gp30), a mutant form of Simian Immunodeficiency virus (nef) and hantavirus pulmonary syndrome-causing Hantavirus (19-23). The ITAM-containing viral proteins contribute to viral interference with host cell signaling processes, enhancing viral propagation and immune evasion.

Proximal Events in T Cell Receptor Signal Transmission

The ITAMs translate extracellular binding events into intracellular signaling events. This is primarily achieved through the reversible phosphorylation of the two tyrosine residues in the ITAMs. The phosphorylation of the tyrosine residues in the ITAMs promote interactions with SH2 domain-containing proteins involved in intracellular signal transmission. Decades of research have resulted in a well-defined model of signal transmission through the TCR. The recognition of as few as one to three peptide/MHC complexes on antigen presenting cells by the TCR initiates a series of intracellular signaling events (Figure 2) (24, 25). Ligation of the TCR αβ heterodimer by its cognate peptide/MHC ligand expressed on the APC causes the redistribution and activation of the Src family protein tyrosine kinases (PTKs). The Src PTKs expressed in

T cells, Lck and Fyn, are comprised of unique NH_2-terminal regions followed by a Src homology 3 domain (SH3), a Src-homology 2 domain (SH2) and a kinase domain (26). Lck and Fyn, are targeted to the plasma membrane as a result of myristoylation and palmitoylation on the glycine and cysteine residues, respectively, in the NH_2-terminal domains. A portion of Lck is directly associated with the cytoplasmic tails of the CD4 and CD8 T cell coreceptors. Fyn can also associate, at low stoichiometry, with the CD4 and CD8 coreceptors (27-29). Lck and Fyn are negatively regulated by the phosphorylation of a regulatory tyrosine in the kinase domain. Following receptor ligation, the negative regulatory tyrosine is dephosphorylated by CD45 protein tyrosine phosphatase (PTPase)(30). Upon dephosphorylation, the Src kinases undergo a conformational change, enabling the autophosphorylation of an activating tyrosine residue within the kinase domain, generating catalytically active Src PTK. This catalytically active Src PTK subsequently phosphorylates the two critically-spaced tyrosine residues within the ITAMs of the TCR ζ and CD3 γ, δ and ε subunits (29, 31-36). While both Lck and Fyn are expressed in T cells and localized to the plasma membrane, it has been shown that Lck is the Src PTK primarily responsible for phosphorylating the TCR ITAMs (33, 37). The biphosphorylated ITAMs complex with ZAP-70 and Syk PTKs through high affinity (2.1×10^{-8} M) interactions with tandem SH2 domains (38-43).

ZAP-70 and Syk are members of a second family of PTKs involved in TCR-mediated signal transmission. They are cytoplasmic PTKs containing two NH_2-terminal SH2 domains and a COOH-terminal kinase domain. Despite belonging to the same

family of PTKs, ZAP-70 and Syk exhibit several differences. First, the expression of ZAP-70 is restricted to T cells and NK cells, while Syk is expressed in B cells, mast cells, neutrophils, macrophages and platelets (44). Syk is also expressed in some T cell subsets, including immature thymocytes and γδ T cells. Second, the intrinsic catalytic activity of Syk is nearly 100-fold greater than that of ZAP-70, due to differences in the amino acid sequence of the catalytic domain (45). In addition, the catalytic activation of ZAP-70 requires Src family kinases (Lck or Fyn), while Syk can autophopshorylate in the absence of Src (46). Third, Syk and ZAP-70 display distinct binding to the phosphorylated ITAMs through the tandem SH2 domains (41, 42, 47). This is likely due to a 23 amino acid interdomain linker that is present in Syk, which enhances binding of the SH2 domains of Syk to phospho-ITAMs. In fact, Syk can weakly bind to monophosphorylated ITAMs. Despite these differences, both ZAP-70 and Syk have similar high affinities for the biphosphorylated TCR ITAMs (38). ZAP-70/Syk are activated following binding to the phospho-ITAMs. Catalytic activation of ZAP-70 involves transphosphorylation by Lck and subsequent autophosphorylation events (46, 48). The catalytic activity of ZAP-70 is essential for TCR-mediated signal transmission. This was documented by the analysis of several human patients with ZAP-70 mutations and the characterization of ZAP-70 deficient mice (36). A ZAP-70 deficiency in mice obstructs the development of αβ T cells at the $CD4^+CD8^+$ stage, indicating a requirement for ZAP-70 activity for TCR-mediated signaling during thymocyte selection events. In addition, point mutations in ZAP-70 that decreases the ability of ZAP-70 to complex with the TCR subunits can severely reduce the efficiency of thymocyte selection events, leading to autoimmunity (49). Despite different activation requirements, catalytically

active ZAP-70/Syk subsequently tyrosine phosphorylate a number of downstream targets involved in a complex intracellular signaling cascade. Substrates of ZAP-70/Syk that are involved in TCR signal transmission include LAT, SLP76, Vav and PLCγ1 (37, 50-52).

A critical target of ZAP-70 PTK and a central mediator of TCR-mediated signal transmission is the linker for activation of T cells (LAT). LAT is a membrane-bound, palmitoylated adaptor protein of 36-38 kDa that is constitutively localized into glycolipid enriched membrane domains (GEMs) by two cysteine residues in its cytoplasmic tail (53, 54). ZAP-70 directly phosphorylates LAT on six cytoplasmic tyrosine residues. As a tyrosine-phosphorylated protein, LAT functions as a molecular scaffold, providing multiple docking sites for SH2 domain-containing proteins. Phospho-LAT is essential for the recruitment of several signaling adaptor/effector proteins to the plasma membrane, including SLP76, Grb2, Gads and PLCγ1 (51, 55). Analysis of LAT-deficient Jurkat T cell lines and LAT-deficient mice have indicated that LAT is essential for TCR mediated signaling. In the absence of LAT, T cells are defective in TCR-mediated calcium mobilization, IL-2 production and CD69 upregulation. Since T cell development is blocked at the TN3 stage ($CD3^-CD4^-CD8^-CD44^-CD25^+$) in LAT-deficient mice, it was impossible to determine the function of LAT in mature T cell activation events *in vivo*. The use of LAT transgenic mice, bearing various mutations in the tyrosine residues in LAT, revealed crucial roles for LAT in the recruitment of PLCγ1, SLP76/Gads and Grb2/SOS to the membrane signaling complex and activation of downstream signals (54).

PLC γ1 is recruited to the LAT membrane complex through binding of the SH2 domain of PLCγ1 to the phosphorylated tyrosine 136 of LAT (26, 51, 53, 56). PLCγ1 is catalytically activated following tyrosine phosphorylation by ZAP-70. Active PLCγ1 catalyzes the hydrolysis of phosphatidyl 4-5 bisphosphate into the second messengers, diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3). The production of DAG activates Ras, in a Ras-GRP dependent manner, and leads to the activation of PKC and the MAPK signaling pathway. The second messenger IP_3 induces the release of intracellular calcium stores. The production of DAG and IP3 from PIP2 are important in the activation of signaling molecules and transcription factors further downstream in the signaling cascade.

The phosphorylation of LAT by ZAP-70 PTK at tyrosine 191 provides a second docking site for SLP76 (SH2 domain containing leukocyte protein of 76-kDa). SLP76 is a 76-kDa adaptor protein that is expressed in hematopoietic cells. Though enzymatically inert, SLP76 contains three potential tyrosine phosphorylation sites, a central proline rich region and a COOH-terminal SH2 domain (51, 53, 55). SLP76 is constitutively complexed with Gads, a member of the Grb2 family of adaptor proteins, through proline-SH3 interactions (53). Following TCR stimulation, the SLP76/Gads complex binds phospho-LAT through the SH2 domain of SLP76. SLP76 is subsequently tyrosine-phosphorylated by ZAP-70 PTK. Phosphorylated SLP76 functions as a second scaffolding protein, recruiting other proteins to the LAT-based membrane signaling complex, including Vav, Nck, Itk and ADAP (53). These proteins are involved in TCR-mediated cytoskeletal rearrangements and T cell adhesion.

The membrane proximal signals that involve the TCR ITAMs, Src and ZAP-70/Syk PTKs and the key adaptor/effector proteins LAT, SLP76 and PLCγ1 activate a complex signaling cascade in T cells. This signaling cascade involves multiple transcription factors, including NF-AT, NFκB and AP-1 that induce a series of T cell activation events. Early events in T cell activation are the upregulation of CD69 and CD25 on the T cell surface, while later T cell activation events include proliferation and cytokine secretion.

TCR ζ and CD3 ITAMs in TCR Signal Transmission

Chimeric receptors were initially used to characterize the functions of the TCR ζ and CD3 ε ITAMs in T cell signal transmission. These chimeric receptors contained the extracellular and transmembrane domains of heterologous receptors (IL-2Rα (Tac) or CD8) linked to the cytoplasmic domains of the CD3 ε subunit and either full-length or partial TCR ζ molecules. MAb-mediated cross-linking of the extracellular domains of the chimeric receptors expressed on a variety of lymphocyte cells types, induced the tyrosine phosphorylation of the TCR ζ ITAMs and the initiation of downstream intracellular signals, including the activation of ZAP-70 and PLCγ1 and mobilization of intracellular calcium stores. The signals generated by the CD8/ζ chimeric receptors in T cell lines were sufficient to promote IL-2 production, CD69 upregulation, and cytolytic effector function (57-63). While a single TCR ζ ITAM could induce intracellular

signaling events, the triplication of the TCR ζ ITAM in the chimeric receptors enhanced select intracellular signals, indicating an additive function for the three TCR ζ ITAMs (59). The CD3 ε ITAM, when present in chimeric receptors, was sufficient to initiate PTK activation and IL-2 production (60). Importantly, the functions of the CD3 ε and TCR ζ chimeric receptors were completely abrogated by mutation of either of the two tyrosine residues in the TCR ζ or CD3 ε ITAMs (60, 63).

When expressed as transgenes in RAG-2-deficient mice, the *in vivo* stimulation of the chimeric receptors containing either TCR ζ or CD3 ε could partially restore T cell development to the $CD4^+CD8^+$ stage (64). However, no mature $CD4^+$ or $CD8^+$ T cells developed in these lines. When present as transgenes in wild-type mice, the crosslinking of chimeric receptors containing either TCR ζ or CD3 ε ITAMs induced the proliferation of mature T cells (64). These data suggested that the TCR ζ and CD3 ε ITAMs could confer similar functions, leading to the activation of PTKs and IL-2 production.

Notably, a few studies also revealed important distinctions between the TCR ζ and CD3 ε ITAMs in the activation of T cell signaling pathways. In one report, a Tac/ζ chimeric receptor containing all three TCR ζ ITAMs, when expressed as a transgene in mice, was unable to activate naïve cells. However, this TCR ζ chimera was sufficient to generate signals required for the activation of previously primed T cells (65). These data implied distinct requirements for the activation of naïve and effector/memory cells. Moreover, the protein tyrosine phosphorylation of different substrates was induced following stimulation of either the TCR ζ or CD3 ε chimeric receptors (60). Taken

together, the available information suggest that the signals mediated by TCR ζ and CD3 ε can induce both similar and distinct functions. It is important to note that the use of chimeric receptors may not have fully defined the roles for the TCR ζ and CD3 ITAMs when they are present within an intact receptor complex containing all of the TCR/CD3 subunits.

To initially examine the functions of TCR ζ and CD3 subunits within the intact TCR complex, cell lines containing either full-length TCR ζ and CD3 subunits or truncated TCR ζ subunits were used. The BW5147 thymoma line, lacking TCR surface expression as a result of deficiencies in TCR ζ and CD3 δ was reconstituted with wild-type CD3 δ and either full-length or truncated TCR ζ constructs lacking one or all functional ITAMs. In all BW cell lines containing wild-type or mutant TCR ζ constructs, normal TCR surface expression was restored. In addition, IL-2 production following mAb or superantigen stimulation of the TCR was roughly equivalent in all of the lines, regardless of functional TCR ζ ITAMs (61). However, the BW cells reconstituted with the truncated TCR ζ molecules showed markedly impaired signal transmission when stimulated through the Thy-1 and Ly6 receptors (61). Based on these data obtained from the analyses of TCR ζ and CD3 ε chimeras it was proposed that the TCR complex is comprised of two autonomous signaling modules, CD3 γε/δε and TCR ζζ (61). In the absence of a functional TCR ζζ module, the CD3 γε/δε module was competent to transmit signals leading to T cell activation. In some studies, an intact TCR ζζ module appeared to be required to couple stimulation of Thy-1, Ly6 and CD2 receptors to downstream signaling pathways (61, 66, 67). The notion that the TCR complex is comprised of two

independent signaling modules would imply that the two modules can function independently during TCR-mediated signaling. Based on these data, the two signaling modules function through similar, ITAM-mediated pathways, leading to T cell activation.

Molecular Interactions with the TCR ζ and CD3 γ, δ and ε subunits

The presence of the semi-conserved ITAM motif within the TCR ζ and CD3 γ, δ and ε subunits of the TCR would suggest that the TCR signaling subunits interact with similar adaptor or effector proteins. It is clear that all of the phosphorylated TCR ζ and CD3 ITAMs can associate with the tandem SH2 domains of ZAP-70/Syk PTK, most with similar nanomolar affinities (2.1×10^{-8}M) (38, 68). The exception is the third TCR ζ ITAM, which binds ZAP-70/Syk with three-fold less efficiency. This suggests that a functional redundancy exists within the ten ITAMs of the TCR complex, all of which can bind to the ZAP-70 PTK. Given that the ten ITAMs could are capable of complexing ZAP-70, the ten ITAMs could also function for signal amplification, potentially binding to ten molecules of ZAP-70/Syk. The binding of up to ten ZAP-70 molecules could function as the ITAMs that were triplicated in chimeric receptors and amplify signals that generated through a single ITAM (59). In fact, using bacterially expressed and *in vitro* phosphorylated TCR ζ, phospho-ζ was identified to form a 1:3 molar complex with the tandem SH2 domains of ZAP-70 (69). This supported the notion of functional redundancy within the ITAM-containing subunits.

Nevertheless, the nonconserved amino acid sequences within the ITAMs, particularly at positions Y+1 and Y+2, might facilitate the association of distinct signaling molecules through SH2 or PTB domain-containing partners. Using phospho-peptides containing the TCR ζ and CD3 γ, δ and ε ITAMs as affinity ligands, a hierarchy of interactions with distinct proteins was identified. In contrast to ZAP-70, which interacts with all phosphorylated TCR ITAMs, the adaptor protein Shc selectively complexed with the phospho-ITAMs of CD3 γ and δ and the first and third TCR ζ ITAMs. No association was observed with Shc and the phospho-peptides of the second TCR ζ ITAM or CD3 ε ITAM (68). A second study confirmed the interaction between phospho-ζ and Shc (70). The binding of Shc to phospho-ζ occurs through phospho-ITAM/SH2 interactions. This association is a necessary event for pre-TCR mediated activation of Ras during T cell development in the thymus (71). The adaptor protein Grb2 and the p85 subunit of PI3K bound variably to the phosphorylated ITAMs of TCR ζ, CD3 γ and δ, but were unable to complex the phospho-CD3 ε ITAM. This data indicated that the TCR ITAMs can complex with both similar and distinct proteins. In addition, this suggests that while the TCR ζ and CD3 ITAMs may be functionally equivalent, they may also function differentially through separate molecular interactions. However, since high concentrations of TCR phospho-peptides were used as affinity ligands, the physiological relevance of some of these particular interactions still awaits validation.

Aside from the ITAM regions, the amino acid sequences of the TCR ζ and CD3 γ, δ and ε subunits are very divergent. These sequence differences provide distinctions

between the invariant chains. Analysis of the CD3 ε subunit revealed an NH_2-terminal region rich lysine residues followed by a proline-rich region, a potential site for interaction with Src homology 3 (SH3) domains (4). In addition, the cytoplasmic tail of CD3 ε contains an NPxY motif that lies partially within the ITAM. The NPxY sequence represents a second potential PTB binding site in CD3 ε. In contrast, the CD3 γ and δ subunits lack the proline-rich and NPxY regions found in CD3 ε. However, CD3 γ and δ contain dileucine internalization motifs in their cytoplasmic tails. Unique to the cytoplasmic tail of CD3 γ are two serine residues. Serine residues represent potential phosphorylation sites that can subsequently interact with proteins containing phospho-serine binding domains (14-3-3, WW and MH2) (72). These regions that provide unique sequences to the TCR ζ and CD3 subunits may also facilitate diverse molecular interactions with the TCR subunits. In fact, additional studies revealed distinct protein interactions with the various TCR/CD3 subunits. These interactions were mediated by the ITAMs as well as regions that lie outside of the ITAMs. In resting human cells, a portion (of TCR ζ associates with the actin cytoskeleton (73). This interaction, mediated by a sequence in the COOH-terminus of TCR ζ, may be involved in the localization of the TCR into higher ordered lipid raft structures. Alternatively, this interaction of TCR ζ with the actin cytoskeleton may function to maintain TCR stability, actin polymerization or TCR internalization. The interaction between TCR ζ and TRIM, or TCR receptor interacting molecule, is thought to be involved in modulating TCR expression levels, thus regulating T cell activation (74). Following TCR ligation, TCR ζ can interact with SLAP2 or CTLA-4, two negative regulators of TCR signaling (75, 76). The mechanisms used by SLAP2 and CTLA-4 to downmodulate TCR signaling are not yet known.

Unc119 is a critical activator of the Src-family kinases, Lck and Fyn that has been identified to interact with TCR ζ (77). Although the interaction between TCR ζ and Unc119 is unknown, the activation of Src PTKs by Unc119 appears to be essential for full T cell activation and IL-2 production. Collectively, these studies revealed multiple proteins that associate specifically with the TCR ζ subunit, while few had identified proteins that interact exclusively with the CD3 subunits. In a search for proteins that specifically associated with CD3 ε, a novel protein was isolated from a cDNA library from human T cells. CAST, CD3 ε associated signal transducer, constitutively associates with a transmembrane proximal region in the cytoplasmic tail of CD3 ε. Although the function of this interaction is not clearly defined, it appears to be involved in TCR-mediated NF-AT activation and IL-2 production (78). An interaction between the proline rich region of CD3 ε and the SH3 domain of the adaptor protein Nck has been described recently (79). This interaction, which occurs immediately following TCR ligation, precedes the tyrosine phosphorylation of the TCR ITAMs. It is proposed that the association of CD3 ε with Nck may be involved in actin cytoskeletal rearrangements during T cell activation.

In summary, these data indicate that the TCR ζ and CD3 subunits are capable of complexing both similar and distinct proteins involved in various T cell functions. Through these interactions, the invariant TCR subunits may confer both redundant and unique signaling capabilities to T cells. A compilation of the proteins that interact with the TCR invariant subunits presented in Table I.

TABLE I. Molecular Interactions with the TCR ζζ and CD3 γε/δε Signaling Modules

TCR Module	Associated Molecule	Function	Means of Association[a]
TCR ζ	ZAP-70 (38, 80)	Activation	$((PO_4)_2$ITAM$)$-$(SH2)_2$
	Syk(38, 41)	Activation	$((PO_4)_2$ITAM$)$-$(SH2)_2$ or $(PO_4$-ITAM$)$-$(SH2)$
	p85 (PI3K) (68)	Activation	$(PO_4$-ITAM$)$-$(SH2)$
	Fyn (35)	Activation	$(PO_4$-ITAM$)$-$(SH2)$
	TRIM (74)	Receptor Expression	Unknown-N-terminus
	Unc-119 (77, 81)	Src PTK Activation	Unknown-TM
	Shc (70)	Activation	Unknown
	SLAP2 (75)	Negative Regulator of TCR expression	$(PO_4$-ITAM$)$-$(SH2)$ Unknown
	CTLA-4 (76)	Signal inhibition	
	Actin (73)	Actin polymerization	Unknown COOH-terminal tail
CD3 ε	ZAP-70 (Syk) (38)	Activation	$((PO_4)_2$ITAM$)$-$(SH2)_2$
	Fyn (35)	Activation	Unknown-N-terminus
	Nck (79)	Cytoskeleton, ?	Proline/SH3
	CAST (78)	Activation	Unknown

CD3 γ	ZAP-70 (Syk) (38) p85 (PI3K) (68) Shc(68) Grb2(68) Fyn (35)	Activation Activation Activation Activation Activation	$((PO_4)_2$ITAM)-$(SH2)_2$ $(PO_4$-ITAM)-(SH2) $(PO_4$-ITAM)-(SH2) $(PO_4$-ITAM)-(SH2) Unknown-N-terminus
CD3 δ	ZAP-70 (Syk)(38) p85 (PI3K) (68) Fyn(68) Shc(68) Grb2(68)	Activation Activation Activation Activation Activation	$((PO_4)_2$ITAM)-$(SH2)_2$ $(PO_4$-ITAM)-(SH2) Unknown-N-terminus $(PO_4$-ITAM)-(SH2) $(PO_4$-ITAM)-(SH2)

[a.] The invariant chain sequence and the effector protein regions responsible for their interaction are indicated consecutively

The TCR ζζ Signaling Module

The TCR ζζ module was originally presumed to be the predominant signaling component of the TCR complex. Contributing six of the ten ITAMs to the complex, TCR ζ is one of the more heavily tyrosine-phosphorylated proteins following TCR

ligation (31, 32). Furthermore, TCR ζ appears as two distinct tyrosine-phosphorylated intermediates that migrate at 21- and 23-kDa following TCR ligation (31). The 21-kDa form of TCR ζ, referred hereafter as p21, is constitutively expressed in both thymocytes and peripheral T cells and associates with an inactive pool of ZAP-70 PTK (43, 82). It is widely believed that the constitutive expression of a portion of total cellular TCR ζ (5-10%) as the p21 intermediate in the periphery is reflective of constant weak interactions with self-peptide/MHC complexes (82-84). This differs from the CD3 subunits, which do not exist in a constitutively tyrosine-phosphorylated state and are difficult to detect as phosphorylated proteins following TCR ligation (34). A portion of TCR ζ can also appear as a second tyrosine-phosphorylated intermediate of 23-kDa (p23) following receptor ligations, which complexes with active ZAP-70 molecules (82). The p21 and p23 phospho-ζ intermediates have been linked to virtually all biological functions of T cells, from T cell development to T cell activation and the induction of anergy (37, 85, 86).

Formation of the 21- and 23-kDa Tyrosine-Phosphorylated Forms of TCR ζ

While it was clear from that p21 and p23 represent tyrosine-phosphorylated intermediates of TCR ζ, the distinct differences between p21 and p23 were not clearly defined in initial reports (31). TCR ζ contains six potential tyrosine phosphorylation sites within the three ITAMs. These tyrosine phosphorylation sites are designated as tyrosines one through six, beginning at the membrane proximal ITAM (Figure 3). A number of groups had attempted to identify the sites of tyrosine phosphorylation involved

in the generation of p21 and p23 in T cells (87-90). It was suggested by these studies both that p21 and p23 could be the result of partial tyrosine phosphorylation of the TCR ζ ITAMs. In one report, the 21-kDa phospho-ζ intermediate was proposed to contain monophosphorylated ITAMs (90). This was based on studies using phospho-specific antibodies to detect the phosphorylated ITAMs in TCR ζ, which yielded inconsistent results. Thus, it remained unclear which tyrosine residues in the TCR ζ ITAMs were phosphorylated in the 21- and 23-kDa phospho-ζ intermediates. Using a series of biochemical techniques, including mutational analysis and cell transfections, mass spectrometry and the generation of TCR ζ transgenic mice, van Oers *et al* definitively determined the sites of TCR ζ phosphorylation that were involved in the formation of p21 and p23 (91). These experiments established that p21 was a result of phosphorylation of the four tyrosine residues in the membrane distal ITAMs phosphorylated (tyrosine residues 3 through 6). Thus, p21 was a partially phosphorylated TCR ζ intermediate. The 23-kDa phospho-ζ intermediate was determined to be fully phosphorylated on all six tyrosine residues in the TCR ζ ITAMs. These studies also indicated that the stable expression of both p21 and p23 TCR ζ intermediates was dependent on the binding of the tandem SH2 domains of ZAP-70 PTK to the bi-phosphorylated ITAMs (91). Based on the analyses of mutated TCR ζ molecules in cell lines and mice, it was proposed that the phosphorylation of TCR ζ occurs in an ordered, stepwise fashion. The initiating event in the formation of p21 and p23 is the phosphorylation of the membrane distal tyrosine (Y6) in the third TCR ζ ITAM by the Src PTK, Lck. The tyrosine phosphorylation of TCR ζ then proceeds in ascending order to the third tyrosine, resulting in the formation of p21. The subsequent phosphorylation of tyrosines two and one (Y2 and Y1) generates the

fully tyrosine-phosphorylated 23-kDa TCR ζ intermediate (Figure 3). Based on this model, the membrane distal tyrosine residue in TCR ζ (Y6) is critical for the formation of both p21 and p23, while Y1 and Y2 are dispensable for the formation of p21. This is supported by studies with TCR η, a splice variant of TCR ζ. TCR η, which is difficult to detect as a phospho-protein, lacks the COOH-terminal tyrosine residue (Y6) (2, 92). As a consequence of the alternative splicing and the lack of the membrane distal tyrosine, TCR η is unable to form either p21 or p23. Since the 21- and 23-kDa tyrosine phosphorylated intermediates of TCR ζ have been linked to virtually all aspects of T cell biology, the mapping of p21 and p23 have important implications for studying the roles of the TCR ζζ module in T cells. The specific contributions of p21 and p23 to T cell development, T cell activation events and antagonism of T cell responses will be discussed.

T Cell Development

The majority of αβ T cells that develop in the thymus come from bone marrow derived hematopoietic stem cells (HSC). During T cell ontogeny, thymocytes proceed through a series of developmental stages. These developmental stages are characterized by phenotypic changes in cell surface markers, including CD44, CD25, CD3, CD4 and CD8 (3, 10, 26). The HSCs that enter the thymus are classified as triple negative (TN) cells, lacking surface expression of CD3, CD4 and CD8 (TN; $CD3^-CD4^-CD8^-$). Transition through four distinct TN stages is characterized phenotypically by the surface expression of CD44 and CD25. The initial TN1 stage contains cells that have not yet committed to the T cell lineage and retain the capacity to develop into T cells, NK cells

or thymic dendritic cells. These TN1 cells express CD44 on the surface but lack expression of CD25 ($CD44^{+}CD25^{-}$). Signaling through the IL-7 receptor drives the expression of CD25 on the cell surface and transition to the TN2 stage. The germline rearrangement of the TCR β chain is initiated at TN2 stage ($CD44^{+}CD25^{+}$) and continues through the TN3 ($CD44^{-}CD25^{+}$) stage. The rearrangement of the TCR β genes is mediated by the recombinase activating genes (RAG) (1, 3, 10, 93). The productive rearrangement of TCR β marks a developmental checkpoint, where TCR β associates with a TCR α-like chain called pre-TCR α or pTα and promotes transition to the TN4 stage ($CD44^{-}CD25^{-}$). At this developmental checkpoint, marked by the surface expression of the pre-TCR, a sharp reduction in recombination events and RAG gene expression is observed (93). The pre-TCR, containing the rearranged TCR β and pTα subunits is coupled to the TCR and CD3 signaling subunits (TCRβpTαγεδεζζ) (10, 94). The signals mediated through the pre-TCR, which can occur constitutively and in the absence of extracellular ligands, are required for the survival and further development of thymocytes (95). Signaling through the pre-TCR induces the pre-T cells to enter the cell cycle and undergo a rapid expansion. The pre-T cells transition from a TN to the double positive (DP) stage, where both CD4 and CD8 coreceptors are expressed on the cell surface ($CD4^{+}CD8^{+}$). During the transition from TN to DP, RAG gene expression is induced, initiating the recombination of the TCR α genes (93). RAG gene expression and recombination continues until a complete TCR complex is expressed on the cell surface ($CD3^{+}CD4^{+}CD8^{+}$). The DP thymocytes that express a complete αβ TCR complex on the cell surface are subjected to a rigorous selection process that selects thymocytes with TCRs that are self-recognizing but eliminates thymocytes that are self-reacting.

Although much is understood regarding thymocyte selection events, the precise mechanisms that distinguish positive versus negative selection are largely unknown. Positive thymocyte selection involves the recognition of self-peptide in the context of self-MHC I or -MHC II molecules on the surface of thymic cortical epithelial cells or sometimes hematopoietic cells (95). As a result of positive selection events, RAG gene expression and recombination events are terminated. Only a small proportion of thymocytes (less than 5%) are positively selected to mature into either $CD4^+$ or $CD8^+$ T cells. These cells emigrate from the thymus into peripheral lymphoid compartments (93, 96). In contrast, negative selection of thymocytes involves TCR recognition of self-peptides embedded in self-MHC I or -MHC II molecules expressed on the surface of thymic dendritic cells. The engagement of the costimulatory receptors on T cells also contribute to the signals involved in negative selection (3, 96). The outcome of negative selection is clonal deletion by apoptosis or anergy induction. Several studies have proposed that the specific peptide ligands, the presence of costimulatory signals and the strength and duration of signaling through the TCR can influence selection events (3, 96).

The Peptide Ligands Involved in Positive and Negative Thymocyte Selection

The nature of the peptide embedded within the MHC that is recognized by the TCR contributes to the distinct developmental outcomes of positive and negative selection events. Peptides have been defined based on a hierarchy of responses that they induce in mature T cells following TCR ligation. Agonist peptides can mediate diverse

cellular responses in mature T cells, including protein tyrosine phosphorylation events, intracellular calcium mobilization, cellular proliferation, the acquisition of effector functions and TCR downregulation. Altered peptide ligands (APL), which can vary by only one or two amino acids from the agonist peptide, usually at the TCR contact residues, can induce a range of responses in mature T cells from full T cell activation to partial activation or anergy (86, 96). APLs that are defined as weak agonist peptides can only induce a subset of T cell responses. In contrast, antagonist peptides can actively inhibit mature T cell responses to agonist peptide ligands (86, 97). Thus, mature T cells stimulated with antagonist peptides can be rendered anergic to further stimulation with agonist peptide complexes (86, 97-100). The defined agonist, weak agonist and antagonist peptides for mature T cells have also been proposed to mediate distinct thymocyte selection events. The mechanism that deciphers ligand variations to distinct cellular outcomes, such as positive versus negative thymocyte selection, is not fully understood. However, signal discrimination is proposed to involve either quantitative or qualitative differences in the activation of proximal signaling molecules, including TCR ζ (101-103).

It was reported that APLs, or self-peptides sharing homology with agonist peptides, could promote efficient positive selection (98, 104-106). In addition to the nature of the peptide ligand detected by the TCR, the relative abundance of peptide ligands available can direct thymocyte selection events. During selection, low concentrations of weak agonist or antagonist peptides have been shown to induce positive selection. Increasing the concentration of a single peptide can markedly enhance the

strength of the TCR/peptide/MHC interactions. An increase in signal strength by increased peptide concentrations can shift the outcome of thymocyte selection from positive to negative selection (107). In contrast, strong agonist peptides, even when present in low abundance, were unable to induce positive selection or negative selection (108). Instead, strong agonist ligands promoted the development of anergic cells (108). Since most of the studies using specific peptides during positive and negative selection events used *in vitro* assays, the precise ligands that mediate positive versus negative selection *in vivo* are unclear. In addition, the distinct signals mediated by positively and negatively selecting ligands that dictate the selective outcome remain elusive. Recently, however, some of the peptides mediating positive selection in some systems has been identified, providing a system in which to study the effects of positive and negative selection and the mechanisms involved in these processes (106).

The Roles of the TCR ζζ and CD3 γε/δε Modules during T Cell Development

The elimination of the various TCR/CD3 subunits by gene targeted deletion was undertaken to dissect carefully the individual requirements for each subunit during T cell development *in vivo*. At least four groups reported that gene-targeted disruption of the TCR ζ subunit resulted in a partial block in thymopoeisis at the DP stage (109-112). An overall ten-fold reduction in total thymic cellularity and a decrease in the number of DP cells were observed in TCR ζ-null mice. Very few mature T cells emigrate from the thymus in these mice. The almost complete absence of TCR surface expression in the TCR ζ-null mice confirmed previous data using cell lines that indicated a requirement for

TCR ζ in the proper assembly and transport of the TCR complex to the cell surface (113). The developmental block in TCR ζ-null mice was completely restored by reconstitution with a full-length TCR ζ transgene. Furthermore, the reconstitution of TCR ζ-null mice on a wild-type background with TCR ζ mutant transgenes, either by truncation or amino acid substitution, containing 3, 1 or 0 functional TCR ζ ITAMs completely rescued development (36, 91, 114, 115). These data suggested that T cell development and selection can occur in the absence of functional TCR ζ ITAMs. To more carefully dissect the contributions of the TCR ζ ITAMs to T cell development, these TCR ζ transgenic lines were mated to various TCR transgenic lines. This enabled the analysis of developmental outcomes in specific T cell populations. The TCR ζ transgenic lines were mated to either the P14 or 2C TCR transgenic lines, two TCR transgenic lines containing T cells bearing TCRs with relatively high affinity for their agonist peptides. In these systems, T cell development was normal, even in the absence of all TCR ζ ITAMs (114, 115). It was only when T cell development and selection were analyzed in the TCR ζ transgenic mice mated to TCR transgenic mice bearing relative low affinity TCRs, based on the requirement for costimulation, that a dependence on TCR ζ ITAMs for T cell selection was observed. In fact, a reduction in the efficiency of positive and negative selection events was noted when one or more TCR ζ ITAMs were deleted or mutated. This was observed in at least three TCR transgenic backgrounds, including the HY, DO11.10 and AND TCR transgenic lines (116). Specifically, the reduction in the number of functional ITAMs resulted in a graded decrease in the efficiency of both positive and negative selection in these systems. In fact, in the absence of all six functional ζ ITAMs, a substantial reduction in TCR avidity resulted in a shift from negative to positive

selection of DO11.10 thymocytes (116). It is interesting to note that in the systems used to analyze the functions of TCR ζ in mature cells, which included wild-type and P14 TCR transgenics, both early and late events in TCR-mediated signal transmission were not influenced by the reduction or even complete absence of functional TCR ζ ITAMs (36, 91, 115).

The CD3 ε subunit is required for early T cell development, as its targeted deletion arrests development at the TN3 stage ($CD3^-CD4^-CD8^-CD44^-CD25^+$) (117, 118). T cell development in the CD3 ε-null mice was blocked prior to the completion of TCR β selection, leaving thymocytes unable to express the pre-TCR on the cell surface. Since the CD3 ε subunit associates with the CD3 γ and δ during TCR assembly, it is not surprising that assembly and surface expression of the entire TCR complex would be disrupted by the elimination of CD3 ε. The reconstitution of CD3 ε-null mice with a full length CD3 ε transgene completely restored T cell development. This was observed by normal percentages of the different thymocyte subpopulations (118). Interestingly, mutant CD3 ε subunits (CD3 εM), bearing tyrosine to phenylalanine (YF) substitutions in the CD3 ε ITAM, also restored normal development in wild type and high affinity TCR transgenic systems. When the CD3 εM mice were examined in a low avidity TCR transgenic system (HY), both positive and negative selection processes were slightly impaired (119). These experiments confirmed previous data indicating a requirement for the CD3 ε transmembrane region for proper pre-TCR assembly. In addition, these data indicated a role for the CD3 ε ITAMs for efficient positive and negative selection of thymocytes expressing low affinity TCRs.

The CD3 γ subunit is required for the development of both αβ and γδ T cells in the thymus and disruption of this locus completely blocks thymopoeisis. CD3 γ-null mice exhibited a severe (100-fold) decrease in thymocyte numbers and a block in T cell development at the TN3 stage ($CD3^-CD4^-CD8^-CD44^-CD25^+$) (120). The CD3 γ-null thymocytes had a reduction in TCR surface expression compared to normal thymocytes (120). Despite normal development and germline rearrangement of the TCR β and TCR γ and δ loci, the lack of CD3 γ in thymocytes prohibited surface expression of the pre-TCR and early γδ TCR complexes. Normal development was restored by reconstitution of the CD3 γ-null mice with a CD3 γ subunit bearing YF substitutions in the ITAM. This suggested that CD3 γ is required for pre-TCR assembly. When the CD3 γ ITAM mutant mice were mated to TCR transgenic mice containing T cells with specific TCRs (F5 TCR transgenic) and T cell development was examined, a role for the CD3 γ subunit in mediating T cell positive selection was also noted (114, 121). Specifically, a reduction in the positive selection of F5 TCR transgenic T cells was observed in the absence of a functional CD3 γ ITAM. Notably, the mutation of the CD3 γ ITAM did not affect the binding kinetics of the F5 TCR to agonist peptide/MHC complexes or the initiation of intracellular signaling events that have been linked to negative thymocyte selection. However, the activation of signals involved in positive selection, including the activation of ERK and the phosphorylation of LAT, were impaired in the CD3 γ ITAM mutant mice (122).

An analysis of mice lacking the CD3 δ subunit revealed a unique role for CD3 δ in the development of αβ T cells. CD3 δ is required later during development than CD3 ε and γ, such that only a partial developmental block at the $CD4^+CD8^+$ DP stage was observed in CD3 δ-null mice (123). However, no $CD4^+$ or $CD8^+$ SP T cells were noted, indicating that the CD3 δ subunit is required for positive selection. Surprisingly, in the absence of CD3 δ, the development of γδ T cells occurred normally (123, 124). This indicates a distinct difference in the TCR subunit composition of αβ versus γδ T cells. The development and function of CD3 $\delta^{-/-}$ cells was fully restored when the mice were reconstituted with a wild type CD3 δ transgene. Studies have not yet examined the role of the CD3 δ ITAM during selection events. However, two studies revealed an important role for the extracellular or transmembrane domain of CD3 δ during thymocyte positive selection events (125, 126). This region in CD3 δ associates with a motif in the TCR α chain, termed TCR α-connecting peptide domain (α-CPM). The interaction between CD3 δ and TCR α-CPM facilitates the local concentration and recruitment of signaling molecules, including phospho-ζ, Lck and ZAP-70, into proximity with LAT in the lipid raft-associated "signalosome" during positive selection (125, 126). Blocking this association results in defective TCR ζ phosphorylation within lipid rafts leading to impaired ERK activation, an event required for efficient positive selection. Thus, the CD3 δ subunit appears to play a critical role in the localization of the TCR complex into the T cell "signalosome" during positive selection events.

The studies using gene-targeted deletions of the various components of the TCR ζζ and CD3 γε/δε modules have identified roles for these subunits during αβ T cell

development. Furthermore, these data revealed functional roles for the ITAMs during T cell development and/or signal transmission. During T cell development, the ITAMs of the TCR ζζ and CD3 γε/δε module functioned somewhat redundantly. Most T cells have been shown to be unaffected by the reduction in ITAM number. In contrast, T cells bearing low avidity TCRs, such as the HY TCR transgenics, which are more dependent on coreceptors and costimulation, show a requirement for the ITAMs during positive and negative selection events. These studies had suggested that the TCR ζ and CD3 γ and ε ITAMs function redundantly during positive selection events, in an additive fashion, to enhance the signaling potential of the TCR. Based on these studies, a model for positive and negative thymocyte selection was proposed (114). In this model, individual T cells acquire different signaling thresholds that are directly related to TCR affinity and signaling potential. These thresholds dictate whether thymocytes are positively or negatively selected in the thymus (114). Based on this model, a higher signaling threshold is set for negative selection, while a lower signaling threshold is set for positive selection. Below the positive selection threshold, thymocytes remain unselected and die by neglect. The stronger, rapid signals generated following TCR ligation with negatively selecting pMHC complexes induce a series of signals exceeding the negative selection threshold and induce clonal deletion or anergy. The weaker, more sustained signals that are generated following TCR interactions with positively selecting ligands exceed the threshold for positive selection, but not negative selection, promoting T cell survival and differentiation. Since both T cell affinity and thymocyte signaling potential are related to signal threshold in this model, T cells with low avidity TCRs, as defined by the requirement for costimulation during activation, require an increased signaling potential

for efficient positive selection. In contrast, T cells with relatively high avidity TCRs, which have little or no coreceptor or costimulatory dependence, can be efficiently selected with a reduced signaling potential. This suggests that T cells with low avidity TCRs require a full complement of TCR ITAMs for efficient selection processes. This model also assumes an additive function for the TCR ITAMs during negative selection. Based on this notion, a reduction in the signaling potential of thymocytes with high affinity for self-pMHC that are undergoing negative selection could dramatically alter the developmental outcome. A reduction in signaling potential to levels below that of the negative selection threshold would promote positive selection of thymocytes that, under normal circumstances, undergo negative selection (114). In fact, a reduction in the signaling potential of T cells through TCR ζ ITAM elimination has been shown to result in a developmental shift from negative to positive selection (116, 127). A reduction in thymocyte signaling potential caused by diminish ZAP-70 activity due to a mutation in the COOH-terminal SH2 domain of ZAP-70, facilitated the positive selection of autoreactive T cells. The small numbers of T cells that matured as a result of this developmental shift from negative to positive selection caused spontaneous autoimmunity (49). Thus, the TCR ζ ITAMs can contribute to the fine-tuning of TCR-mediated signaling responses in thymocytes to direct appropriate selective outcomes. What is left unresolved by these studies is whether the particular ITAMs utilized during selection and signaling events is important, since the ITAMs have been shown to associate differentially with distinct adaptor/effector proteins in early studies (68, 128). In addition, while many of these studies analyzed the contributions of the TCR ζ ITAMs during thymocyte selection events, the lack of consistent data regarding the p21 and p23

phospho-ζ intermediates precluded a careful analysis of the role of phospho-ζ during selection. Thus, it was unclear if the physiologically detected phospho-ζ intermediates also contributed to T cell development and selection events.

The Role of the TCR ζζ Module in T Cell Antagonism

The TCR has the amazing capacity to discriminate between peptide/MHC ligands expressed on APCs and alter the T cell response accordingly. The antagonism of T cell responses by altered peptide ligands has been observed *in vivo* in mature T cell populations. Antagonism of T cell responses to pathogens *in* vivo has proven to be detrimental or beneficial to the host. Certain pathogens, including HIV and Hepatitis C Virus, utilize APL to inhibit or downmodulate the immune response, facilitating immune evasion (129, 130). Alternatively, *in vivo* stimulation with virus generated APL has proven beneficial to the host by antagonizing inappropriate and detrimental immune responses (131). The issue of how variable ligands are discriminated and translated into distinct cellular outcomes is important in the understanding of T cell development and activation events.

Although it is unclear how the signal discrimination is translated, it has been proposed that the different phosphorylated TCR ζ intermediates (p21 and p23) may be involved in the discrimination of peptide ligands presented by the MHC molecules on APCs. Notably, the differential induction of p21 and p23 in response to agonist or antagonist peptides has been postulated as a mechanism to transmit distinct intracellular

signals that mediate these responses. Stimulation of cells with agonist peptide complexes induced the tyrosine phosphorylation of both the p21 and p23 TCR ζ intermediates and the subsequent recruitment and activation of ZAP-70 PTK (90, 99, 101, 102). In contrast, a distinct pattern of TCR ζ phosphorylation was observed following stimulation with antagonist peptide complexes, with the preferential induction of p21 and little to no p23 (101-103). Based on these findings, the relative ratios of p23 to p21 were proposed to effectively determine the outcome of TCR engagement; a ratio of p23/p21 nearing one promoting mature T cell activation, while a ratio of p23/p21 closer to zero would antagonize T cell responses and induce T cell anergy. By inference, it was also proposed that the p23/p21 ratios could also dictate thymocyte selection outcomes in the thymus. During development antagonist peptides that induce a ratio closer to zero would promote positive selection, while agonist peptides that induce a ratio nearing one would mediate negative selection events. Although much data has correlated ligand discrimination to signals generated at the TCR complex, the involvement of phospho-ζ in these processes is unclear.

The Role of the TCR ζζ Module in Regulating T Cell Half-Life

The long-term maintenance of both naïve and memory T cells in the periphery is crucial for the preservation of a diverse lymphocyte pool that is capable of responding to a variety of pathogens encountered through a lifetime. Naïve and memory T cells are relatively long lived in the periphery. Naïve T cells have been shown to survive in the periphery for a minimum of eight weeks (132). Although the factors contributing to the

survival of T cells in the periphery are unclear, it is implied that naïve and memory T cells are differentially regulated (133, 134). Multiple factors contribute to the survival of naïve and memory T cells in the periphery. These include cytokines, transcription factors and cell to cell interactions (133, 135, 136). The cell to cell interactions involved in peripheral T cell survival are ill-defined, but a considerable amount of evidence suggests that interactions of the TCR with MHC molecules are necessary for maintenance of both naïve and memory T cells (84, 134, 137). However, the requirement for TCR/MHC interactions for naïve versus memory T cell maintenance is contentious and dependent on the cell type. A specific requirement for selecting MHC II ligands have been shown for the survival of $CD4^+$ T cells in some systems (134, 137, 138). Others have shown that $CD4^+$ T cell survival is MHC II independent (139). In contrast to naïve T cells, the maintenance of memory T cells is independent of selecting MHC ligands and only requires non-specific interactions between the TCR and MHC (134). These interactions are variably coreceptor dependent, with naïve cells relying more heavily on coreceptor signals than memory cells (140).

It has been established previously that p21 is a result of TCR interactions with peptide/MHC complexes in the periphery (82, 84, 139, 141). The constitutive expression of p21, as a result of these TCR/self-pMHC interactions, has been correlated with T cell survival in the periphery (84). Diminished levels of p21 were observed in $CD4^+$ T cells that were isolated from an MHC II-deficient peripheral environment. This loss of p21 correlated with a shortened $CD4^+$ T cell half-life of three and a half weeks (84). A similar pattern of diminished T cell survival was observed in other systems where $CD4^+$

and $CD8^+$ T cells were transferred into mice lacking expression of MHC II and MHC I, respectively (133). Based on these results, it was inferred that the constitutive expression of p21 might be involved in generation of a "survival signal" for peripheral naïve and/or memory T cells.

The TCR ζζ Module in Regulating TCR Stability

In addition to its role in assembly and the initiation of intracellular signals, the TCR ζ subunit is proposed to be involved in regulating the stability of the TCR complex. This function may be mediated by a sequence in the membrane distal cytoplasmic domain of TCR ζ that conceals the dileucine and the tyrosine-based internalization motifs in the cytoplasmic portions of the CD3 γ and δ subunits (142). These signals function in lysosomal targeting and degradation of receptor complexes and are sterically hindered by TCR ζ. It is proposed that following TCR ligation a conformational change in the TCR complex exposes the dileucine internalization motifs, resulting in the downmodulation of both engaged and nonengaged TCRs and thus dampening TCR-mediated signals (143). This function of the TCR ζ subunit in TCR maintenance and downmodulation may involve specific interactions with the actin cytoskeleton, TRIM and/or CTLA-4 (73, 74, 76). The importance of the TCR ζ subunit in the maintenance of a surface expressed TCR and the initiation of TCR mediated signaling is illustrated during chronic infections and some autoimmune diseases. Chronic bacterial infections have been shown to enhance lysosomal degradation of the TCR ζ subunit specifically, causing a downmodulation of TCR ζ, thus rendering T cells less responsive to the pathogen (144).

This downmodulation of the surface expressed TCR ζ occurs in an IFN γ- or TNF α-dependent manner (144, 145). In addition, downmodulation of surface-expressed TCR ζ and T cell hyporesponsiveness has also been identified in the autoimmune disease Systemic Lupus Erythematosis (SLE). The downmodulation of TCR ζ resulted from mutations in either the 3' UTR, which caused an alternative splice variant of TCR ζ with decreased stability, or a mutation in exon 7, which abolished TCR ζ phosphorylation (146, 147). These data indicate a functional role for the TCR ζζ signaling module in regulating the surface expression of TCR for T cell functions.

Altogether, a plethora of studies have identified an important role for the TCR ζζ module in various T cell functions. However, the use of TCR ζ molecules that had been mutated or truncated precluded a complete analysis of the functions of the phosphorylated intermediates that are expressed in vivo. The roles that the p21 and p23 phospho-ζ intermediates contribute to T cell development, T cell activation and T cell antagonism will be addressed.

TCR ζ Transgenic Mice

To address the functions of p21 and p23 in various T cell functions, we generated a series of TCR ζ transgenic mice. These TCR ζ transgenic mice contained select tyrosine to phenylalanine substitutions in the TCR ζ ITAMs (91). These lines were denoted YF1,2 and YF5,6 (containing YF substitutions in the first and second or fifth and sixth tyrosine residues in the TCR ζ ITAMs, respectively) (Figure 4). The patterns of

constitutive and inducible TCR ζ phosphorylation in thymocytes isolated from the various TCR ζ transgenic mice confirmed the previous results from mapping studies. These TCR ζ transgenic mice, containing YF substitutions in the TCR ζ ITAMs, provided a system in which to definitively address the functions of p21 and p23 in T cells. These TCR ζ transgenic lines, in conjunction with a new TCR ζ transgenic line, denoted YF1-6 described herein, were utilized to address the various functional contributions of phospho-ζ, particularly the 21- and 23-kDa intermediates, to T cell development, signal transmission and T cell anergy.

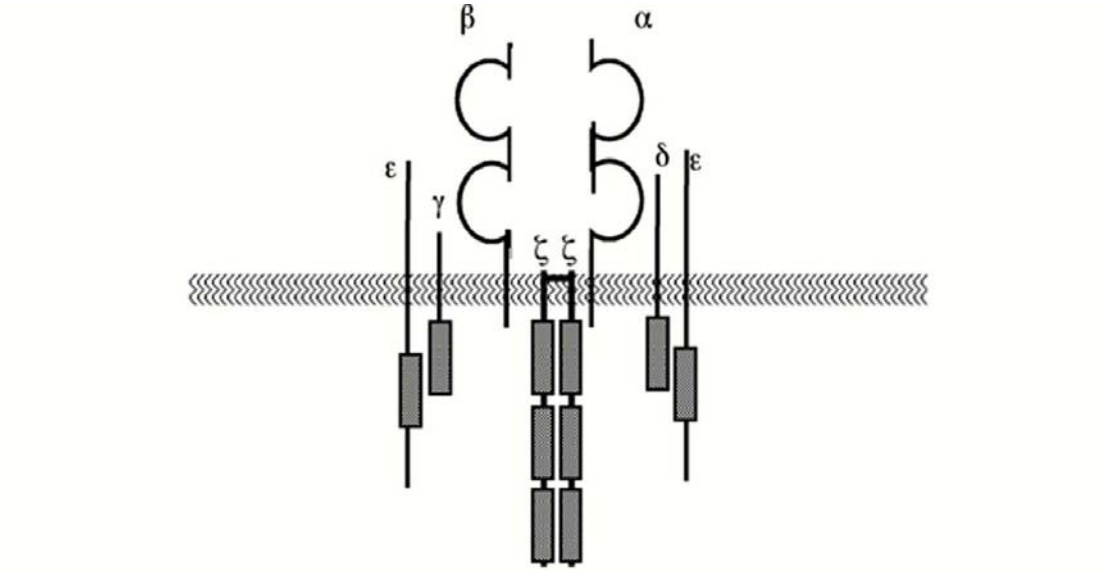


Figure 1. The T Cell Receptor is a Multimeric Complex. The structure of the T cell receptor complex is depicted here. The TCR contains the ligand binding αβ heterodimer in a noncovalent association with the TCR ζ and CD3 γ, δ and ε signaling subunits. The ten ITAMs of the TCR complex are designated by the hatched boxes.

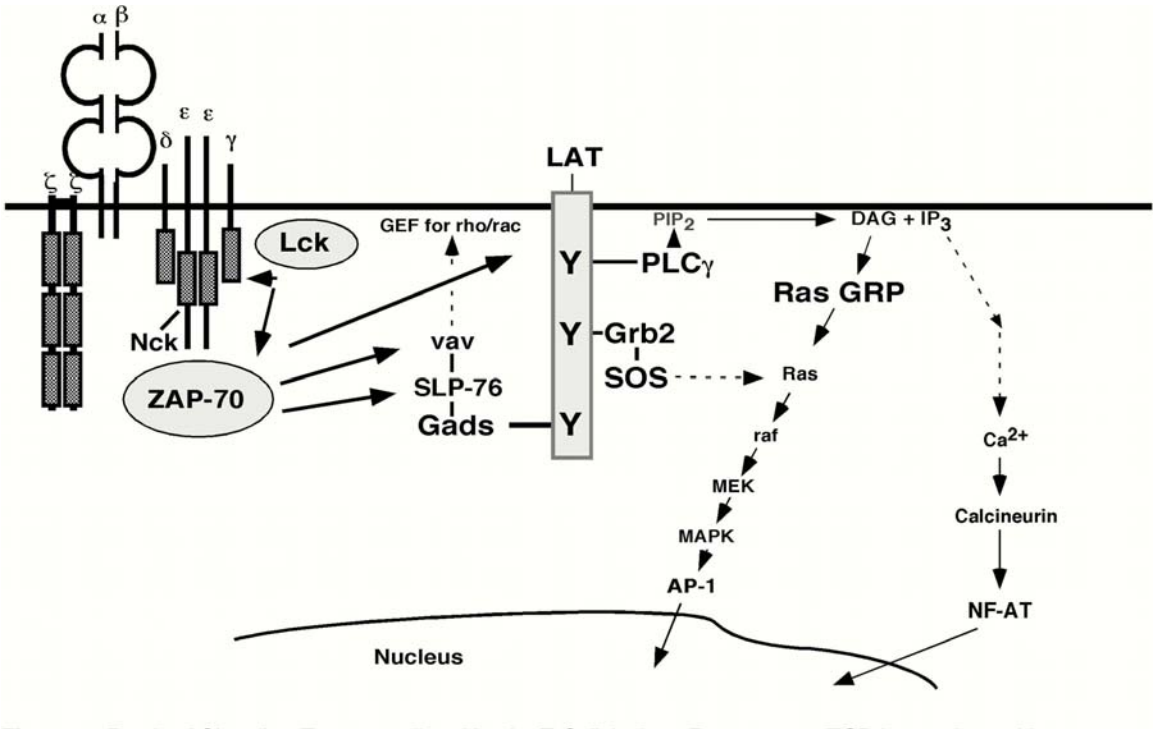


Figure 2. Proximal Signaling Events Mediated by the T Cell Antigen Receptor. αβ TCR interaction with cognate peptide-MHC ligands results in the activation of protein tyrosine kinases and the phosphorylation of the two tyrosine residues within the ITAMs of the TCR ζ and CD3 γ, δ and ε chains. A second PTK, ZAP-70, is recruited to the bi-phosphorylated ITAMs and catalytically activated. ZAP-70 subsequently phosphorylates a number of substrates, which when phosphorylated, recruit adaptor proteins that promote Ras activation, calcium mobilization and actin cytoskeleton rearrangements.

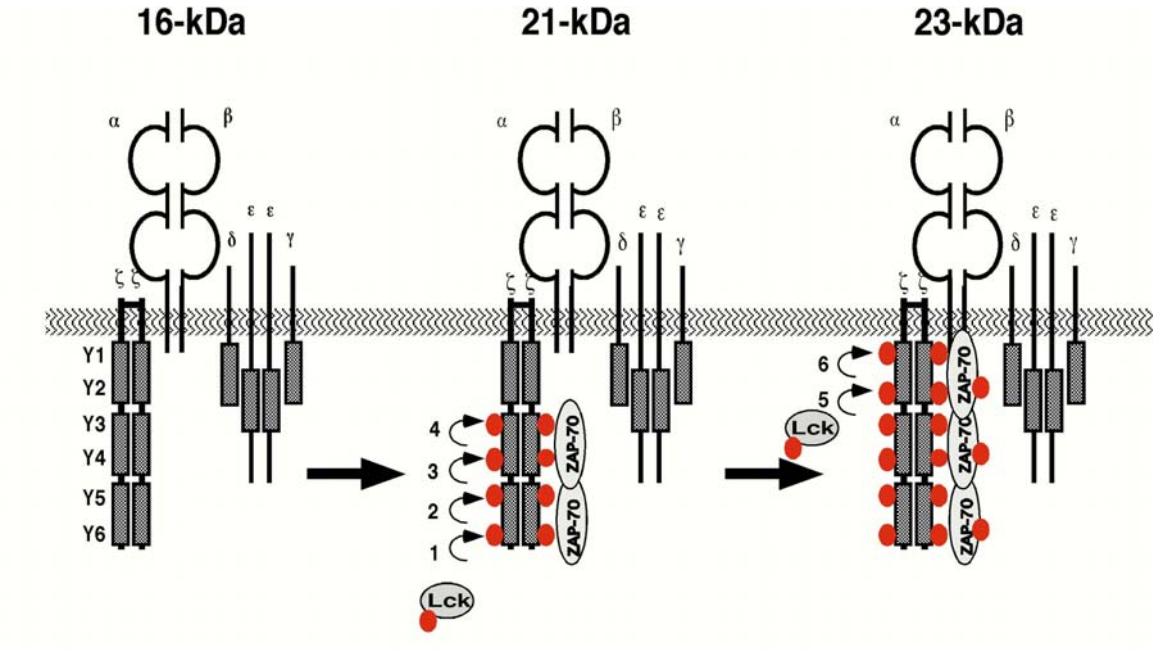


Figure 3. Step-wise model of TCR ζ chain phosphorylation. The Lck or Fyn PTK phosphorylates the TCR ζ subunit in a step-wise manner beginning with the plasma membrane distal tyrosine designated as Y6. Once the third tyrosine residue is phosphorylated, two molecules of ZAP-70 are bound, resulting in the stable formation of the 21-kDa phosphorylated intermediate. Further phosphorylation of the second and first tyrosine residues in the TCR ζ subunit results in the generation of the 23-kDa phosphorylated form. In this model, two and three molecules of ZAP-70 are complexed to the 21- and 23-kDa phosphorylated intermediates, respectively.

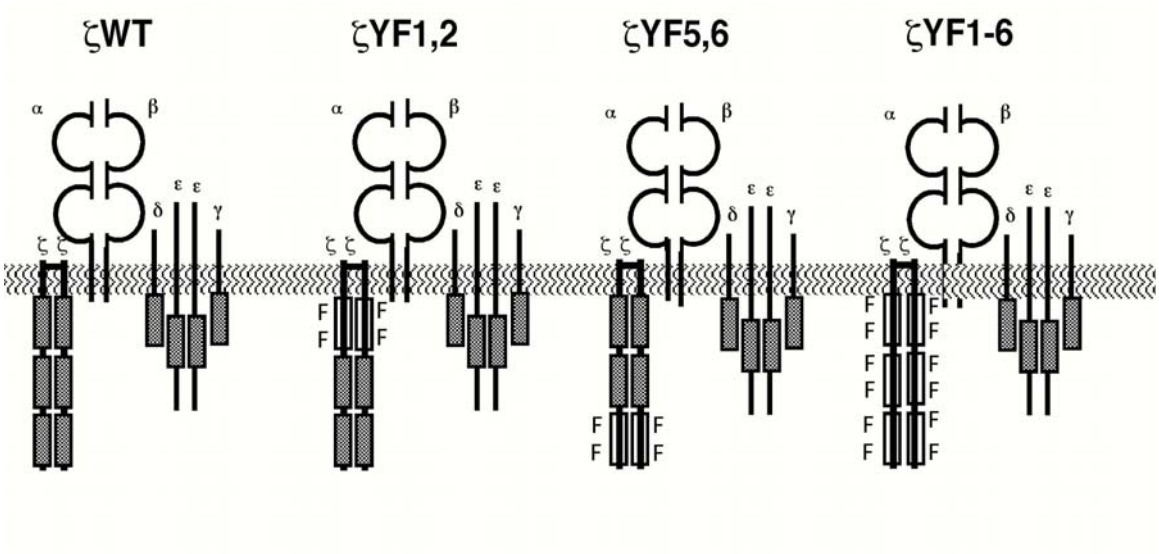


Figure 4. Structure of the TCR complex containing TCR ζ subunits that have selected tyrosine to phenylalanine substitutions in the three ITAMs. TCR ζ transgenic mice were generated and maintained on a TCR ζ-null background.

Chapter II. Materials and Methods

Peptides

T cells from P14 TCR transgenic mice were stimulated with several distinct peptides including an agonist (p33 - KAVYNFATM), a weak agonist (A4Y - KAVANFATM), an antagonist (S4Y - KAVSNFATM) and a control peptide (AV – SGPSNTPPEI) (97). The peptides used for stimulating HY-specific T cells included a strong agonist peptide (Smcy - KCSRNRQYL) and the natural positively selecting antagonist peptide (Ube1x – KSNLNRQFL). The various peptides were purchased from AnaSpec Inc. (San Jose, CA) or synthesized by the Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center (Dallas, TX). The peptides were reconstituted in water and filter sterilized.

Cell Lines

Several different antigen presenting cell lines (APCs) that expressed the MHC Class I $H2^b$ were used to stimulate T cells. These included the dendritic cell line, DC2.4, obtained from Dr. K. Rock (Dana-Farber Cancer Institute, Boston, MA), the macrophage line, IC21 (ATCC, Rockville, MD) and the J774.K^b macrophage cells ($H2^d$ macrophages stably transfected with $H2K^b$) provided by Dr. J. Forman (UTSW, Dallas, TX). The cell lines were grown in DMEM (DC2.4 and J774.K^b) or RPMI 1640 (IC21) supplemented with 10% FBS (Intergen, Purchase, NY), 100 U/ml penicillin, 100 μg/ml streptomycin, 2

mM L-Glutamine and 5 x 10^{-5} M 2-mercaptoethanol. All cell lines were grown at 37ºC in humidified air containing 5-8% CO_2.

Antibodies

The following hybridomas were obtained from ATCC: 145-2C11 (anti-CD3 ε), HB-38 (anti-I-A^b), HB-198 (F4/80), TIB-146 (anti-B220), anti-NK1.1, HB-197 (2.4G2; anti-FcRII), TIB-100 (anti-Thy1.1) and TIB-99 (anti-Thy 1.2). Antibodies were purified from the culture supernatants of the different hybridomas using standard affinity chromatography procedures with either Protein A or Protein G (Amersham, Piscataway, NJ). Purified antibodies specific for phosphotyrosine (4G10) and LAT were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Goat anti-mouse, goat anti-rabbit and rabbit anti-sheep secondary antibodies conjugated to horseradish peroxidase were purchased from Bio-Rad Laboratories (Hercules, CA). The Goat anti-mouse IgG conjugated to horseradish peroxidase, used as a secondary antibody for anti-phosphotyrosine immunoblotting, was purchased from Zymed Laboratories (South San Francisco, CA). Purified antibodies specific for phospho-p42/p44 and p42/p44 MAPK were purchased from Cell Signaling Technologies, Inc. (Beverly, MA). Biotin-, Fluorescein-, Phycoerythrin- or Cychrome-conjugated anti-TCR β, -CD3 ε, -CD4, -CD5, -CD8α, -CD8β, -CD25, -CD44, -CD69, -CD94, -B220, -NK1.1, -Vα2, -Vβ8, and Streptavidin conjugated to Phycoerytherin or Cychrome were purchased from BD Pharmingen (San Diego, CA). Monoclonal antibodies recognizing TCR ζ (6B10.2) or ZAP-70 (1E7.2) have been described previously and were purified from culture

supernatants using Protein A affinity matrices (Amersham, Piscataway, NJ)(33, 148). Polyclonal antisera to ZAP-70 (1225 and 1664) were kindly provided by Dr. A. Weiss (University of California San Francisco, San Francisco, CA). Biotinylated anti-HY TCR α (T3.70) was purchased from eBioscience (San Diego, CA). Polyclonal antiserum to SLP76 was kindly provided by Dr. G. Koretzky (University of Pennsylvania, Philadelphia, PA). Anti-B220 conjugated magnetic beads were purchased from Dynal Biotech (Lake Success, NY). The hybridoma producing a mAb specific for the HY TCR α subunit (T3.70) was kindly provided by Dr. H-S. Teh (University of British Columbia, Vancouver, British Columbia). This antibody was purified from culture supernatants using Protein A affinity matrices (Amersham) and subsequently labeled with NHS-fluorescein (Molecular Probes, Eugene, OR). For the labeling, 0.1 mg of NHS-fluorescein (reconstituted in DMSO at 1 mg/ml) was incubated with 1 mg of purified T3.70 mAb for two hours at 4º C in the dark. Unbound NHS-fluorescein was removed by dialysis. The fluorescein labeled antibody was tested by flow cytometry.

Mice

C57Bl/6 (Thy1.2$^+$), TCR ζ-null, P14 TCR transgenic and Thy1.1$^+$ congenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). The P14 αβ TCR transgenic mice contain αβ T cells (Vα2$^+$Vβ8$^+$) that specifically recognize a peptide (p33) derived from the Lymphochoriomeningitis Virus glycoprotein presented by MHC I H2D^b (149). The HY TCR transgenic line was kindly provided by Dr. H-S. Teh. The HY αβ TCR transgenic mice contain αβ T cells (T3.70$^+$) that are specific for peptide (Smcy)

derived from the male HY protein presented by MHC I $H2D^b$ (150). The CD3 ε mutant mice (CD3 εM) were kindly provided by Dr. P. Love (National Institutes of Health, Bethesda, MD). The CD3 εM mice contain tyrosine to phenylalanine substitutions in the CD3 ε ITAMs (119). The CD3 εM mice were maintained on a CD3 ε-null background.

TCR ζ transgenic mice containing select tyrosine to phenylalanine substitutions in the TCR ζ ITAMs were generated on a C57Bl/6 background using the VA-CD2 transgenic cassette as previously described (91, 151). These included the YF1,2 and YF5,6 TCR ζ transgenic mice. A new YF1-6 TCR ζ transgenic line, containing phenylalanine substitutions at all six tyrosine residues in the TCR ζ ITAMs, was generated by subcloning the previously described TCR ζ YF1-6 DNA into EcoRI/SmaI digested VA-CD2 plasmid (91, 151). The sequence of the TCR ζ YF1-6 DNA fragment was confirmed by sequence analysis by the Molecular Biology Sequencing Core (UTSW, Dallas, TX). Supercoiled VA-CD2/YF1-6 plasmid DNA was digested with KpnI/XbaI and resolved by agarose gel electrophoresis to remove intervening prokaryotic sequences. The 11.05 Kb VA-CD2/YF1-6 linearized DNA was purified using Qiaex II beads (Qiagen, Valencia, CA) and was injected into C57Bl/6 blastocysts at the Transgenic Core Facility at the University of Texas Southwestern Medical Center (Dallas, TX). Transgenic founders were identified by PCR and Southern blotting and backcrossed to TCR ζ-null mice. The YF1,2, YF5,6 and YF1-6 TCR ζ transgenic mice on the TCR ζ-null background were also mated to the P14 and the HY TCR transgenic mice. The various TCR ζ transgenic mice were genotyped by PCR for the presence of the TCR ζ transgene, the P14 or HY αβ TCR transgenes and the absence of endogenous TCR ζ. The

following primers were used for PCR genotyping of the TCR ζ transgenic mice: VA-CD2 5' GCT TTT TAT AGG TGC AGT CTC C; VA-CD2 3' GAG TTT TCT GCT GCC CCA TGG; P14 5' CAT GGA GGC TGC AGT CAC CC; P14 3' GTT TGT TTG CGA GCT CTG TTT TGA TGG CTC; HY 5' ACA AGG TGG CAG TAA CAG GA; HY 3' ACA GTC AGT CTG GTT CCT GA. PCR reactions contained 1.5 mM $MgCl_2$, 0.5 μM primers, 0.2 mM dNTPs and 0.5 U Taq DNA Polymerase (Roche). The PCR conditions were 94°C for 3 minutes, 94°C for 45 seconds, 60°C for 30 seconds, 72°C for 30 seconds and 72°C for 7 minutes. The PCR products were analyzed by agarose gel electrophoresis. All mice were maintained on the C57Bl/6 background. The mice were housed in the specific pathogen free (SPF) animal facility on the North campus at the University of Texas Southwestern Medical Center. A list of the various TCR ζ transgenic lines used and their transgene expression levels relative to wild-type C57Bl/6 mice are presented in Tables II and III.

Table II. TCR ζ transgenic mice maintained on a TCR ζ-null background

TCR ζ Transgene	**Designation**	**TCR Expression (MFI)**	**Relative Expression Level**[a]
Wild-type C57Bl/6	B6	147	Normal (1x)
TCR ζ YF1,2	0563	270	High → Intermediate[b] (4x → 2x)
	2179		Intermediate (2x)
	7831		Intermediate (2x)
	2181	242	Low (1.5x)
TCR ζ YF5,6	7933	229	High (4x)
	2601	247	Low (1.5x)
	YF5,6.22	270	Low (1.5x)
TCR ζ YF1-6	YF1-6.5		Intermediate (2x)
	YF1-6.22	265	Low (1.5x)

[a] TCR expression levels are presented relative to the TCR expression level of wild-type C57Bl/6 mice. These values were determined by flow cytometry and western blot analyses.

[b] The level of TCR surface expression in the 0563 TCR ζ YF1,2 transgenic line were initially high. Over a two year period, the expression levels of this transgenic line were reduced approximately two-fold to an intermediate level, relative to the other TCR ζ transgenic and C57Bl/6 mice.

Table III. Commonly used TCR ζ transgenic mice on a TCR ζ-null background

TCR ζ Transgene	Designation	Lymph Node (MFI)
TCR ζ YF1,2	2181	242
TCR ζ YF5,6	YF5,6.22	246
TCR ζ YF1-6	YF1-6.22	265

Surface and Intracellular Flow Cytometry Analysis

Single cell suspensions were prepared from the thymus, lymph nodes (axillary, inguinal and mesenteric) or spleens of the various wild-type or TCR ζ transgenic lines. The red blood cells were lysed in the splenocyte suspensions. Aliquots of approximately $0.5 – 1.0 \times 10^6$ cells were removed from each sample and used for flow cytometric analyses. The cells were washed with FACS Buffer (PBS containing 1% FBS and 0.1% Sodium Azide) and preincubated with culture supernatant containing the mAb to the Fc receptor (2.4G2) for five minutes at 4ºC. This step prevented the non-specific binding of the various fluorochrome-labeled mAbs by the FcR. The cells were subsequently incubated with a fluorochrome-labeled mAb cocktail for 15-30 minutes at 4ºC. The fluorochrome-labeled cells were washed and resuspended in FACS Buffer.

The levels of IFN γ produced in individual cells were determined by intracellular cytokine staining of unstimulated or stimulated cells. 3 x 10^6 TCR ζ transgenic splenocytes were cultured for four hours in the presence of Brefeldin A (10 μg/ml; Sigma, St. Louis, MO) at 37º C and were washed thoroughly with PBS. The washed cells were fixed with 4% formalin in PBS for 20 minutes at room temperature. The fixed cells were pelleted, washed, and permeabilized with 0.2% saponin (Sigma) in FACS buffer for ten minutes at room temperature. Fixed, permeabilized cells were pelleted and resuspended in culture supernatant containing mAb to the FcR for five minutes at room temperature. Dilutions of antibodies specific to various cell surface markers and IFN γ were prepared in the saponin-containing buffer and incubated with the fixed and permeabilized cells for at least 20 minutes at room temperature. The cells were subsequently washed and analyzed by flow cytometry.

The cells that had been stained with fluorochrome-conjugated mAbs were analyzed on the BD Biosciences FACScan using CELLQuest ™ software (BD Pharmingen, San Jose, CA). Data for analysis were collected from at least 8,000 viable cells, as determined by forward and side light scatter profiles.

Cell Lysis

Cells were lysed in a Tris-based lysis buffer (20 mM Tris-Cl (pH7.6), 150 mM NaCl, 2.0 mM EDTA) containing 1% Triton-X 100 (Sigma). The lysis buffer was supplemented with protease and phosphatase inhibitors (0.5 μM PMSF, 10 μg/ml

Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin, 10 μM Benzamadine, 1 μM Sodium Fluoride, 0.5 μM Sodium Orthovanadate and 0.5 μM Sodium Molybdate). Generally, 10^8 cells were lysed in 1 ml Triton-X lysis buffer for 30 minutes at 4º C. The lysates were then cleared by centrifugation for 10-15 minutes at 14,000 rpm at 4º C. The cleared lysates were used for subsequent immunoblot analyses.

Monoclonal Antibody-Mediated Cell Stimulations

The thymus and lymph nodes (axillary, inguinal and mesenteric) were removed from the various TCR ζ transgenic mice and single cell suspensions were prepared. Cells were enumerated and the percentage of $CD3^+$ T cells in each sample was determined by flow cytometry. 10^8 thymocytes or 10^7 $CD3^+$ peripheral T cells from each of the various TCR ζ transgenic mice were left untreated or incubated with 10 μg/ml 145-2C11 (anti-CD3 ε) for 10 minutes at 37º C. The untreated and mAb-stimulated cells were pelleted, lysed in 1% Triton-X lysis buffer and subsequently used for immunoblot analyses.

Peptide-Mediated Cell Stimulations

Thymus or lymph nodes were isolated from the various P14/TCR ζ transgenic mice and single cell suspensions were prepared. The cells were counted and the percentages of P14-specific thymocytes or peripheral T cells ($V\alpha2^+V\beta8^+$) were determined by flow cytometry. The DC2.4 APCs had been preincubated with 3 μM of the P14-specific agonist, weak agonist, antagonist or control peptides for two hours at

37°C. The APCs were washed to remove excess unbound peptide. 10^8 thymocytes or 2 x 10^7 LCMV-specific peripheral T cells were left untreated or stimulated with 3 x 10^6 peptide-loaded APCs for five minutes at 37°C. After the five minute stimulation, the P14 responder cells and APCs were lysed in the 1% Triton X lysis buffer. To compare the kinetics of ZAP-70 and SLP76 phosphorylation in the P14/TCR ζ and HY/TCR ζ transgenic thymocytes following peptide-induced TCR stimulation, 3.5x 10^7 P14-specific thymocytes from the various P14/TCR ζ transgenic mice were incubated with APCs that had been preincubated with agonist or control peptides at 37ºC. Following a time course of stimulation for 0, 5, 15, 45 or 90 minutes, the cells were lysed in 1% Triton-X lysis buffer. The lysates used for subsequent immunoblot analyses.

HY-specific thymocytes or T cells ($T3.70^+$) from the HY and HY/TCR ζ transgenic mice were stimulated with 3 x 10^6 APCs that had been preincubated with control or the HY agonist peptide, Smcy. The HY-specific cells were stimulated for 0, 3, 10, 30, 90 or 180 minutes. The cells were lysed in 1% Triton X-100 lysis buffer and used for subsequent immunoblot analyses. For analyses of ERK activation in the various HY and HY/TCR ζ transgenic thymocytes, the peptide-loaded APCs were fixed prior to incubation with HY-specific cells. 4.3 x 10^5 DC2.4 were plated into 96 well plates and pulsed for 2 hours with 3 μM control or HY agonist peptide at 37 º C. Unbound peptide was removed by washing and the APCs were fixed with a glutaraldehyde solution (PBS containing 0.1% glutaraldehyde (Sigma)) for one minute. The fixing reaction was stopped by the addition of PBS containing 0.2M lysine (Sigma) for one minute. The peptide-pulsed, fixed APCs were washed and used to stimulate 5 x 10^6 total thymocytes

isolated from the various HY/TCR ζ transgenic mice at 37º C. After a time course stimulation of 0, 3, 10, 30, 90 or 180 minutes, the cells were lysed in 1% Triton-X lysis buffer.

Immunoprecipitation and Western blotting

Cleared cell lysates from thymocytes or peripheral T cells were incubated with 2-4 μg of mAb against TCR ζ, CD3 ε, or ZAP-70 or 2-4 μl SLP76 antisera and 20 μl Gammabind G Sepharose or Protein A Sepharose (Amersham, Piscataway, NJ) for 2-4 hours at 4°C. In some experiments, ZAP-70 was immunoprecipitated using rabbit antisera against ZAP-70 (1664 and 1222) and goat-anti rabbit agarose (Sigma). After the 2-4 hour incubation period, the immunoprecipitated proteins were washed three times in 1% Triton-X lysis buffer and boiled in reducing SDS-sample buffer. The TCR ζ and CD3 ε immunoprecipitates were resolved by 12.5% SDS-PAGE while the ZAP-70 and SLP76 immunoprecipitates were resolved by 10% SDS-PAGE. The proteins resolved by SDS-PAGE were transferred to polyvinylidene fluoride membranes (PVDF; Millipore Limited, Bedford, MA) for Western blotting. The PVDF membranes were incubated with 4% BSA (Serologicals, Norcross, GA) in a Tris-buffered saline solution containing 0.5 % Tween-20. The membranes were then incubated with anti-phosphotyrosine (4G10) followed by washing and incubation with a goat anti-mouse IgG secondary antibody conjugated to horseradish-peroxidase. Detection of tyrosine-phosphorylated proteins was completed using enhanced chemiluminescence procedures (Amersham). In certain cases, the blots were stripped in Tris buffered solution (62.5 mM Tris, pH 6.7) containing

2% SDS and 100 mM β-mercaptoethanol and re-blotted with either mAb or antisera specific for TCR ζ, ZAP-70 or SLP76. To assess the kinetics of ERK activation, cleared total cell lysates from the HY/TCR ζ transgenic thymocytes were resolved by 10% SDS-PAGE, transferred to PVDF membranes and subsequently immunoblotted with antibodies against phospho-p42/p44 or total p42/p44 ERK. The membranes were subsequently incubated with horseradish peroxidase secondary antibodies and detection of phospho-ERK or total ERK was accomplished using enhanced chemiluminescence (Amersham).

Intracellular Calcium Flux

The various TCR ζ transgenic mice were compared for the ability to release intracellular calcium stores following mAb-mediated stimulation of the TCR. The lymph nodes were isolated from wild type C57Bl/6 or the TCR ζ transgenic mice and single cell suspensions were prepared. The B cells present in the lymph node suspensions were negatively depleted using magnetic beads conjugated with the mAb to the B cell surface marker B220 (Dynal Biotech, Lake Success, NY). The anti-B220 Dynabeads were used following manufacturers instructions. The B cell-depleted lymph node preparations were then incubated with Fluo-3-AM (Molecular Probes, Eugene, OR) as described (152). Fluo-3-AM is fluorescent probe that binds calcium. The binding of Fluo-3-AM to calcium results in an increase in fluorescence intensity, which can be measured by flow cytometry. Thus, an increase in Fluo-3-AM fluorescence intensity can be used to assess the release of intracellular calcium stores following cell stimulation. The kinetics and

magnitude of intracellular calcium release, as an indication of cellular activation, were compared in the TCR ζ transgenic mice following mAb-mediated stimulation of the TCR and subsequent flow cytometric. During these analyses, the Fluo-3 labeled cells were maintained at 25-37º C. Baseline levels of intracellular calcium were determined before the addition of mAbs in the Fluo-3 labeled T cells by flow cytometry. The cells were then stimulated with a mixture of biotinylated mAbs against CD4, CD8 and TCR β for one minute and changes in intracellular calcium levels were assessed by flow cytometry. The mAbs bound to the TCR then were crosslinked using streptavidin (>2 U/ml) and the increase in intracellular calcium was analyzed by flow cytometry. Following four to five minutes of mAb-mediated TCR stimulation, saturating levels of intracellular calcium were obtained by the addition of Ionomycin (Calbiochem, San Diego CA) to the cells. The fluorescence intensity of Fluo-3 in the absence of calcium was obtained by the addition of $MnCl_2$ to the cells.

T Cell Proliferation Assays

Peripheral T cells were isolated from the lymph nodes (axillary, inguinal and mesenteric) of wild type and TCR ζ transgenic mice and single cell suspensions were made. The cells were counted and the percentages of $CD3^+$ cells in each sample were determined by flow cytometry. Equivalent numbers of $CD3^+$ cells from the TCR ζ transgenic mice (2.5×10^5/well) were cultured in flat bottom 96 well plates. These cells were stimulated with varying concentrations of either plate bound anti-CD3 ε (145-2C11), Concanavalin A (Sigma) or superantigen SEB (kindly provided by Dr. M. Racke,

The University of Texas Southwestern Medical Center, Dallas, TX) for 72 hours at 37º C. For anti-CD3 ε stimulation, 96 well flat bottom plates were coated overnight at 4º C with goat anti-hamster Ig at 10 μg/ml (Accurate Chemical Co., Westbury, NY), washed twice in PBS and coated with increasing concentrations of 145-2C11 for four hours at 37º C. Excess antibody was removed and the 145-2C11 pre-coated plates were used for mAb-mediated stimulation of T cells. 2.5×10^5 $CD3^+$ cells from the various TCR ζ transgenic mice were added to each well. For all proliferation assays, each sample condition was triplicated. T cell proliferation was measured by the uptake of ^{3}H-thymidine (ICN Biochemicals, Irvine, CA), which was added at 1 μCi/well for the last 16 hours of culture. The cells were harvested using a Tomtec Harvester 96™ MachII Automatic Cell Harvester and the DNA was retained on glass fiber mats (Wallac, Turku, Finland). The glass fiber mats were air-dried and counted in a Wallac 1205 Betaplate™ Liquid Scintillation Counter. T cell proliferation was assessed by the amount of ^{3}H-thymidine incorporated into each sample.

Peptide-Induced Proliferation

Antigen presenting cells were prepared by generating a single cell suspension of TCR ζ-null splenocytes, depleting red blood cells and irradiating the splenocytes at 1500 Rads. The irradiated APCs were plated at 5×10^4 cells/well in 96 well flat bottom plates and incubated with increasing concentrations agonist peptides (10^{-10}M to 10^{-4}M) for 1 hour at 37^{o}C. Excess peptide was removed from the APCs. The peptide-pulsed APCs were plated at 5×10^4 cells per well and used to stimulate the various TCR ζ transgenic T

cells. The lymph nodes of P14 or P14/TCR ζ transgenic mice were isolated and single cell suspensions were prepared. The percentages of P14-specific T cells in each sample were determined by flow cytometry. 1 x 10^5 P14-specific T cells from the P14 and P14/TCR ζ transgenic mice were plated into the 96 well plates containing the peptide pulsed APCs. The responder cells and APCs were incubated at 37º C for 72 hours. 1 μCi of ^{3}H-thymidine was added to each well for the last 16 hours of culture. The cells were harvested as described and T cell proliferation was determined by the amount ^{3}H-thymidine incorporated into each sample.

For proliferation assays using the HY/TCR ζ transgenic T cells as responder cells, irradiated splenocytes from female TCR ζ-null mice were prepared as the APCs. The use of female TCR ζ-null splenocytes ensured that only exogenous HY male peptide was presented to the HY-specific T cells. The APCs were plated into round bottom 96 well plates at a concentration of 5 x 10^4 cells per well and pulsed with increasing concentrations (10^{-10}M to 10^{-4}M) of Smcy peptide for one hour at 37º C. Unbound peptide was removed by washing. Single cell suspensions were prepared from the thymus and lymph nodes of the various HY and HY/TCR ζ transgenic mice. The lymph node cell suspensions were counted and the percentage of HY-specific T cells in each sample was determined by flow cytometry. To assess T cell proliferation in response to graded doses of agonist peptide, 1 x 10^4 HY-specific peripheral T cells or 1 x 10^5 total thymocytes were cultured into wells containing 5 x 10^4 peptide-pulsed APCs for 72 hours at 37º C. Exogenous IL-2 (20 U/ml; obtained from the National Institutes of Health, Bethesda, MD) was only added to the cultures when the proliferation of thymocytes and

peripheral T cells from HY and HY/YF male mice was analyzed. Proliferation was measured by the uptake of 3H-thymidine as described above.

T Cell Antagonist Assays

The irradiated TCR ζ null splenocytes described above were also used as APCs in T cell antagonist assays. The APCs were plated into 96 well round bottom plates at a concentration of 5 x 10^4 cells per well. The APCs were prepulsed with a suboptimal concentration (10^{-8} M) of the P14 agonist peptide, p33, for two hours at 37°C. Excess peptide was removed by washing in PBS. The APCs were then incubated with increasing concentrations (10^{-7}M to 10^{-4}M) of the antagonist peptide, S4Y for one hour. The peptide-pulsed APCs were washed and transferred to a 96 well flat bottom plates. Lymph nodes were removed from the P14 and P14/TCR ζ transgenic mice and single cell suspensions were made. The percentages of P14-specific T cells in each sample were determined by flow cytometry. Equivalent numbers of P14-specific T cells (10^5 per well) were plated into individual wells of a 96 well plate containing the agonist and antagonist peptide-pulsed APCs. The mixed cells were incubated for 72 hours at 37°C. To measure T cell antagonism in the mixed cultures, 1 μCi/well ^{3}H-thymidine was added for the last 16 hours of culture. The cells were harvested and the amount of ^{3}H-thymidine incorporation determined as described. T cell antagonism was measured as a reduction in the amount of ^{3}H-thymidine incorporated into the samples containing antagonist peptide compared to control samples, where the APCs were prepulsed only with suboptimal doses of agonist peptide.

Upregulation of CD69 in Thymocytes

Thymocytes were isolated from the HY/TCR ζ transgenic lines and single cell suspensions were made. Irradiated $\zeta^{-/-}$ female splenocytes were used as APCs. The APCs were plated at a concentration of 10^5 cells per well into 96 well plates with increasing doses of either an HY TCR specific antagonist peptide (Smcy) or an HY TCR specific antagonist peptide (Ube1x). The APCs were incubated with peptide for one hour at 37º C. The peptide-pulsed APCs were washed to remove excess peptide. Equivalent numbers (1×10^5) of total thymocytes from the various HY and HY/TCR ζ transgenic mice were cultured for 19 hours with the peptide-pulsed APCs at 37º C. After 19 hours, the mixed cultures were thoroughly washed and the upregulation of CD69 on the cell surface of the thymocytes was determined by flow cytometry. The mixed cultures were stained with fluorochrome-conjugated mAb against CD4, CD8α and CD69 and analyzed by flow cytometry. CD69 upregulation in the various HY and HY/TCR ζ transgenic thymocytes was represented as the fold increase in CD69 expression in peptide-stimulated cultures compared to no-peptide control cultures. The fold increase reflects the average and standard deviation of three individual cultures for each condition.

T Cell Survival Assays

Pools of lymph nodes and spleens from the various TCR ζ transgenic mice were used to make single cell suspensions. Red blood cells were depleted and the lymphocyte pools were washed. To generate a T cell enriched population of cells, B cells were negatively depleted using magnetic beads conjugated to the B cell surface marker, B220 (Dynal). Alternatively, the T cells were enriched by negative depletion of B cells, macrophages and NK cells using Low-Tox-M Rabbit Complement (Cedarlane Laboratories, Hornby, Ontaria, Canada). The bulk lymphocyte pool was incubated with culture supernatants containing antibodies specific for B cells (anti-B220), macrophages (F4.80) and NK cells (anti-NK1.1) for 30 minutes at 4º C. The lymphocytes were washed to remove unbound antibodies and counted. The mAb labeled lymphocytes were incubated with rabbit complement for 30 minutes at 37º C. The complement depleted lymphocytes were washed thoroughly and counted. T cell purity following Dynabead or complement depletions was determined by flow cytometry to be >85%. The purified T cells were labeled on intracellular proteins with the fluorescent dye CFSE (Carboxy-fluorescein diacetate succinimidyl ester; Molecular Probes, Eugene, OR). The CFSE was reconstituted as a stock solution concentration of 5 mM in DMSO. For T cell labeling, the T cell-enriched lymphocytes were resuspended at 10^7 cells/ml in PBS. The T cells were incubated with 2.5 μM CFSE at room temperature in the dark for 10 minutes. Excess CFSE was bound by the addition of FBS and the CFSE labeled cells were washed thoroughly. It was determined by flow cytometry that all of the lymphocytes were labeled with high levels of CFSE. 10^7 of the CFSE-labeled purified TCR ζ transgenic T cells were injected intravenously into unirradiated littermate mice. At various timepoints from one to five weeks post transfer, the recipient mice were analyzed for the presence of

and proliferative history of the adoptively transferred TCR ζ transgenic T cells. CFSE was used as a label for the T cells to monitor the survival of the T cells as well as the number of rounds of division that the T cells had undergone. Following each round of division, CFSE is divided equally between daughter cells. Thus, a two-fold reduction in CFSE intensity is indicative of one round of cell division. The recipient mice were sacrificed, the lymph nodes and spleens were removed and single cell suspensions were made. Red blood cells were depleted from the splenocyte suspensions. The lymph node and spleen suspensions were washed and labeled with fluorochrome antibodies against CD3 ε. The presence of $CFSE^+CD3^+$ T cells in the lymph nodes and spleens of the different TCR ζ transgenic mice and the amount of T cell proliferation was analyzed by flow cytometry. The number of cell divisions that the CFSE labeled cells had undergone was determined by counting the number of peaks with decreasing CFSE intensity in the $CD3^+$ gated lymph node or splenocytes populations.

Listeria monocytogenes Infections

Listeria monocytogenes 10403 serotype I (LM) was kindly provided by Dr. J. Forman (University of Texas Southwestern Medical Center, Dallas, TX). The bacteria were grown on Brain Heart Infusion agar plates. To maintain virulent stocks, the bacteria were passaged monthly through C57Bl/6 mice. For infections, cultures of LM were grown in BHI medium at room temperature for 16-20 hours to a density of approximately 1.5×10^9/ml. The bacterial cultures were washed and serial dilutions were made in PBS. The LD_{50} of LM is 2×10^4 for C57Bl/6 mice. Wild-type B6 and TCR ζ transgenic mice

were initially infected intravenously with a low dose of LM (0.1x LD_{50}) in the tail vein. The mice were rested for four to six weeks. The immunized mice were rechallenged with a high dose of LM (10x LD_{50}) intravenously. Four to six days after the secondary challenge, the infected and control animals were sacrificed. The livers and spleens were removed and Dounce homogenized in either HBSS or sterile water. Serial dilutions of the liver and spleen cell suspensions were plated in duplicate onto BHI agar plates. The BHI plates were incubated at 37º C for 24 hours. The number of colonies on each plate was counted and the CFU per organ was calculated by averaging colony counts on the duplicate plates and adjusting for the dilution.

To assess the production of IFN γ by T cells in response to a secondary challenge with Listeria, cultured splenocytes from LM-infected mice were compared by intracellular cytokine flow cytometry. The J774.K^b macrophages were used as APCs in these assays. The APCs were cultured overnight in antibiotic-free media and subsequently infected with LM at a multiplicity of infection of 5:1 for one hour at 37º C. The LM-infected APCs were then washed and incubated for three hours in complete DMEM containing 100mg/ml Gentamycin at 37º C. 3 x 10^5 of the LM-infected J774.K^b were subsequently cultured with 3 x 10^6 TCR ζ transgenic splenocytes at 37º C for 16-20 hours. Brefeldin A was added for the last four hours of culture. The mixed cells were then washed and prepared for flow cytometric analysis.

Chapter III. T Cell Antagonism is Functionally Uncoupled from the 21- and 23-kDa Tyrosine Phosphorylated TCR ζ Subunits

Introduction

The αβ T cell receptor complex (TCR) is a multisubunit complex consisting of the antigen specific αβ heterodimer that is non-covalently associated with the TCR ζ homodimer and CD3 invariant chains (δε, γε). Ligation of the αβ TCR with its cognate peptide/MHC ligand expressed on antigen presenting cells is translated into a series of intracellular signals through specific sequences in the cytoplasmic portions of the TCR ζ and CD3 chains (153). These sequences, termed ITAMs or immunoreceptor based tyrosine activation motifs, are present in one or more copies in the CD3 and TCR ζ chains and contain a conserved signaling sequence, YxxL x_{6-8} YxxL (17). TCR interactions with appropriate ligands result in the rapid phosphorylation of the two tyrosine residues present in the ITAMs of the TCR ζ and CD3 chains. These bi-phosphorylated ITAMs complex to the Syk/ZAP-70 family of PTKs, resulting in the subsequent activation of downstream effector molecules (50). The ultimate outcome of these signaling events is the induction of cellular responses such as differentiation, proliferation, cytokine secretion, or cytolytic functions (153).

The TCR complex has an amazing capacity to discriminate between small differences in the peptide ligands bound to the MHC. In fact, single amino acid substitutions in particular peptides can dramatically alter the biological responses of T cells, resulting in either positive or negative selection for thymocytes, and T cell activation or T cell anergy for peripheral T cells (86, 154). Several studies have suggested that the distinct phosphorylated derivatives of the TCR ζ subunit can directly affect these developmental and functional decisions (155). The TCR ζ chain is proposed to represent one of two independent signaling modules in the TCR complex, contributing six of the ten possible ITAMs in the complex (61, 85). TCR ζ is one of the first and more heavily tyrosine phosphorylated proteins following receptor ligation and has been detected as two stable phosphorylated forms of 21- and 23-kDa (p21 and p23, respectively)(reviewed in (37)). A portion of TCR ζ is constitutively phosphorylated as the p21 phospho-ζ intermediate in thymocytes and peripheral T cells as a consequence of TCR interactions with self-peptide/MHC complexes (82-84). This phosphorylated form of TCR ζ (p21) is complexed to an inactive population of ZAP-70 molecules (82). Though many studies have focused on the functions of the individual ITAMs of the TCR ζ subunit, the specific contribution of p21 and/or p23 to T cell development and signaling have remained elusive (85). The TCR ζ ITAMs clearly contribute to T cell development in TCR transgenic mice expressing low avidity TCRs (114). It has also been suggested that the phosphorylated TCR ζ subunits function as molecular sensors, capable of discriminating between agonist and antagonist peptide ligands complexed to the same MHC molecule (101, 102). This notion began with the characterization of altered peptide ligands (APL), variants of agonist peptides that specifically inhibit T cell responses (86).

The interaction between T cells and antigen presenting cells expressing an agonist peptide/MHC complex results in the initiation of TCR-mediated intracellular signals. Consequently, the TCR ζ subunit develops into two heavily phosphorylated intermediates of 21- and 23- kDa and the ZAP-70 PTK is subsequently recruited to these phosphorylated subunits and becomes catalytically active (101, 102). In contrast, the stimulation of T cells with antagonist peptide results in a very weak induction of p23 relative to p21. This contributes to the recruitment and association of an inactive population of ZAP-70 molecules (101-103). Based on these findings, it was hypothesized that the relative ratios of p23 to p21 could determine whether T cells were activated or antagonized (101-103). In fact, the constitutively phosphorylated p21 expressed in peripheral T cells was proposed to contribute an inhibitory signaling environment (156).

Antagonist peptides may also be required for positive selection processes in the thymus (reviewed in (96)). By inference, the induction of p21would be necessary for thymocyte positive selection while the induction of p23 would lead to negative selection. In spite of these dramatic conclusions, several studies have reported that T cell selection and T cell antagonism are not dependent on the phosphorylation states of the TCR ζ subunits (36, 104, 114, 115, 157). However, in many of these studies, the constitutively tyrosine phosphorylated p21 was not maintained and the induction of p23 was difficult to assess. These issues leave unresolved the functional contribution of the two predominant phosphorylated forms of ζ on T cell development and T cell antagonism.

We have previously mapped the tyrosine residues that are phosphorylated in both p21 and p23 (91). Using these phosphorylation maps, we have generated a unique series of TCR ζ transgenic mice in which selected tyrosine residues were substituted with phenylalanine. The tyrosine substitutions result in the selective elimination of just p23, or both p21 and p23 in the transgenic mice (37, 91). It is important to note that one of these lines (YF1,2) is the only TCR ζ substituted line reported to date that maintains the constitutive phosphorylation of p21 without any evident p23 before or after receptor ligation. Each transgenic line was mated to the P14 LCMV-specific αβ TCR transgenic mice. The various P14/TCR ζ transgenic lines allowed a definitive assessment of the functional contribution of both p21 and p23 on T cell development and T cell antagonism. In this report, we provide evidence that the positive selection of the P14 transgenic line is functionally uncoupled from the 21- and 23-kDa ζ chains. Furthermore, using a series of agonist, weak agonist and antagonist peptides, we show that T cell antagonism is independent of p21 and/or p23. Significantly, these results provide the first direct demonstration refuting a dominant role for p21 in inhibiting T cell functions.

Results

T Cell Development is Independent of Phospho-ζ Intermediates

To directly study the contributions of both the 21- and 23- kDa forms of TCR ζ on T cell development, we utilized a new series of TCR ζ transgenic mice, in which select tyrosine residues in the TCR ζ ITAMs were substituted with phenylalanine. These substitutions were based on previous mapping studies and resulted in the elimination of either the 2- kDa (YF1,2) or both the 21- and 23- kDa forms of TCR ζ (YF5,6) (91). An additional TCR ζ transgenic line, denoted YF1-6, contained tyrosine to phenylalanine substitutions in all six tyrosine residues in the TCR ζ subunit. The substitution of all six tyrosine in the TCR ζ ITAMs prevented the formation of any phosphorylated TCR ζ intermediates. The structures of the TCR complex containing the tyrosine substitutions in the various TCR ζ transgenic mice are designated YF1,2; YF5,6 and YF1-6 (Figure 4). Importantly, all of these lines were maintained on a TCR ζ-null background.

T cell development in the three distinct TCR ζ transgenic lines were compared to those containing the wild type TCR ζ subunit by staining thymocytes and peripheral T cells with mAbs detecting the surface molecules CD4 and CD8, as well as CD3 and B220. As mentioned previously, the TCR ζ subunit is required for efficient assembly of the TCR complex and a deficiency in TCR ζ results in a developmental block at the $CD4^+CD8^+$ stage (Figure 5) (reviewed in(85)). Interestingly, the expression of the YF1,2, YF5,6 and YF1-6 constructs as transgenes in TCR ζ null mice completely restored thymocyte development. The numbers and

percentages of $CD4^+CD8^+$ thymocytes were similar to wild type mice. Thymocytes from the three transgenic lines were also capable of developing into mature $CD4^+CD8^-$ or $CD4^-CD8^+$ cells. This is further confirmed by an analysis of the peripheral lymphoid organs. Lymph node cells from the various mice were stained with mAbs against CD4 and CD8 or B220 and CD3 (Figure 6). Again, the various transgenes restored the peripheral T cell compartments as similar numbers and percentages of $CD4^+$ and $CD8^+$ T cells were detected. In addition, our results indicate that the $CD3^+$ T cells constitute approximately 50% of the lymph node compartment, showing no skewing of T of B cell ($B220^+$) numbers. Thymocytes were also stained with mAbs to CD3 and B220 to detect levels of transgene expression and examine the possibility of contaminating B cells (Figure 5). The introduction of the transgenic constructs elevated the levels of CD3 expression slightly in the transgenic mice compared to wild type but this did not impact the development of T cells. The YF1-6 construct appears to express the transgene at increased levels compared to the YF1,2 and YF5,6. In spite of these slight variations, thymocyte development proceeds normally in mice that only express the constitutively phosphorylated form of TCR ζ (YF1,2) and is fully retained in mice containing no phosphorylated TCR ζ subunits (YF1-6). These results clearly show that the constitutively phosphorylated 21-kDa form of TCR ζ does not attenuate T cell development. Second, the 23-kDa form of TCR ζ is not required for positive or negative selection.

Although we had previously analyzed the TCR ζ phosphorylation patterns in thymocytes isolated from the YF1,2 and YF5,6 transgenic lines, we extended these experiments by examining the consequences of TCR cross-linking on TCR ζ phosphorylation. As described previously, the 21-kDa form of TCR ζ is constitutively expressed in thymocytes (Figure 7).

Upon TCR ligation, the second 23-kDa form of TCR ζ is detected in thymocytes from wild-type mice (Figure 7, lane 2). The substitution of the first two tyrosines in TCR ζ (YF1,2) permits the formation of the 21-kDa species. However, there is no 23-kDa form of TCR ζ evident after receptor engagement (Figure 7, lanes 3 and 4). In the YF5,6 line, we have eliminated the constitutively expressed form of TCR ζ, as well as the inducible 23-kDa form. Interestingly, TCR stimulation of these thymocytes yielded several faster migrating species that ranged in molecular mass from 19-20-kDa (Figure 7, lanes 5 and 6). Notably, the levels of these phosphorylated species are substantially less than the 21-kDa form of phospho-ζ and were they were only detected following prolonged chemiluminescence exposures. Finally, an analysis of the YF1-6 lines indicated that there are no phosphorylated TCR ζ species in the absence of tyrosines one through six (Figure 7, lanes 7 and 8). Interestingly, there are some very weakly phosphorylated proteins that likely represent the CD3 subunits.

Overall, every TCR ζ transgenic construct restored T cell development in a non-TCR transgenic system, suggesting a minimal role for the phosphorylated TCR ζ species in T cell development (114, 115).

P14 αβ TCR Transgenic T Cells Develop Normally in the Presence of the Constitutively Tyrosine Phosphorylated TCR ζ Subunit

To definitively address the functional contribution of the 21- and 23-kDa tyrosine phosphorylated forms of TCR ζ on P14 T cell development, the TCR ζ transgenic mice were bred onto the P14 TCR transgenic mice (maintained on a ζ-null background). The

P14 mice contain αβ T cells that specifically recognize a peptide derived from LCMV, termed p33, which is presented by the MHC class I molecule $H2D^b$ (149).

We characterized these different mice for the development of LCMV-specific αβ T cells. Thymus, lymph nodes, and the spleen were isolated from the different mice. Single cell suspensions were prepared and an aliquot of cells was stained with fluorochrome labeled mAbs to CD4 and CD8, Vα2 and Vβ8, or CD3 and B220, and analyzed by flow cytometry. Consistent with previous reports, the development of $CD4^-$ $CD8^+$ thymocytes cells expressing the transgenic αβ TCR is severely impaired in TCR ζ-deficient animals (Figure 8A and B) (114, 115).

Reconstitution of the P14/TCR ζ-null mice with the YF1,2 and the YF5,6 TCR ζ subunits completely restored development of normal numbers and percentages of $CD4^-$ $CD8^+$ P14 (12 and 11 %, respectively) thymocytes expressing the αβ transgene (Figure 8A and B). On the basis of seven independent experiments, we found no significant differences in the numbers and percentages of thymocyte subsets in the P14, P14/YF1,2 and P14/YF5,6 lines. We did, however, not reduced numbers of cells in the P14/YF1-6 line. Several groups had previously reported that the development of mature $P14^+$ $CD8^+$ T cells proceeds without the involvement of the TCR ζ ITAMs (114, 115). Importantly, the YF1,2 line is the only TCR ζ transgenic line generated to date that selectively maintains the constitutively tyrosine phosphorylated p21. This form was never maintained in any of the TCR ζ mutant transgenic lines described in those studies. Our results clearly demonstrate that αβ T cell development can proceed normally in the

presence of the constitutively tyrosine phosphorylated p21 form and in the complete absence of p21 and p23. The YF1-6 TCR ζ molecule also facilitated the development of $CD4^-CD8^+$ P14 TCR transgenic thymocytes. Taken together, these results clearly demonstrate that the development of P14 LCMV-specific TCR transgenic thymocytes is independent of the phosphorylation state of the TCR ζ subunit although the efficiency is reduced slightly in the YF1-6 line.

We subsequently analyzed the lymph nodes for the appearance of the $CD4^-CD8^+$ P14 TCR transgenic T cells. Single cell suspensions were stained for various cell surface markers and analyzed by flow cytometry. As shown in Figure 9, the $CD4^-CD8^+$ T cells from P14 TCR transgenic mice comprise approximately 50-55% of the lymph node cells (Figure 9A and B). Almost no $CD4^-CD8^+$ T cells are detected in the absence of the TCR ζ subunit. Consistent with the findings in the thymus, the appearance of $CD4^-CD8^+$ TCR transgenic T cells are re-established once the YF1,2 or YF5,6 subunits are expressed. Although the YF1-6 construct restored the appearance of P14 TCR transgenic $CD4^-CD8^+$ T cells in the peripheral lymphoid organs, the numbers of these cells was always reduced1.5-2.0 fold relative to wild-type mice. In fact, the number of $CD4^+CD8^-$ T cells was enhanced in the P14/YF1-6 transgenic line (22% versus 8% in a wild-type P14 mouse). Overall, these results indicate that the P14 mice that maintain a constitutive tyrosine phosphorylated p21 develop as efficiently as mice without any evidence of constitutive TCR ζ phosphorylation, and that positive selection is not affected or antagonized by the constitutively phosphorylated p21.

The 21- and 23-kDa Forms of TCR ζ Can Be Elicited by Antagonist Peptides

Two peptides related to p33, termed A4Y and S4Y, have been described based on their ability to partially activate or antagonize T cell effector functions to p33, respectively (97, 98). To carefully examine the effect of these peptides on the induction of different phosphorylated forms of TCR ζ in the P14 TCR transgenic system, thymocytes or peripheral T cells isolated from the P14 TCR transgenic mice were stimulated with p33, S4Y, A4Y or a control peptide. Briefly, DC2.4 dendritic cells ($H2D^b$) were prepulsed with 3 μM of the indicated peptides and then co-cultured with T cells for five minutes. The T cells were subsequently lysed and the phosphorylated TCR ζ subunits associating with the ZAP-70 PTK were co-immunoprecipitated with anti-ZAP-70 polyclonal antisera. In the absence of peptide stimulation, p21 was constitutively expressed and associated with ZAP-70 (Fig. 10, lane 1). In addition, a small amount of p23was detected in these cells (Figure 10, lane 1). Stimulation of the P14 thymocytes with a control peptide, AV, caused a slight increase in the levels of p23 (Figure 10, lane 2). Stimulation with the strong agonist peptide, p33, induced a 10-fold increase in p23 (Figure 10, lane 3). The antagonist peptide, S4Y, also induced an increase in p23, but at levels markedly less than the wild-type agonist peptide (Figure 10, lane 4). Identical stimulation experiments were repeated with T cells isolated from spleen and lymph node. Again, p21 was constitutively expressed in peripheral P14 T cells (Figure 10, lane 1). Stimulation of these cells with agonist peptide also resulted in a reproducible increase in p23 (Figure 10, lane 3). This fully phosphorylated TCR ζ subunit was not as readily

detected when the cells were stimulated with the control or antagonist peptides (lanes 2 and 5). Taken together, these results demonstrated that the p23 form of TCR ζ is easily detected following agonist peptide stimulations. However, the ability of weak agonist and antagonists to induce p23 is more difficult to assess, particularly in the peripheral T cells. As such, these experiments leave unresolved the contribution of p21 and/or p23 on T cell proliferation and T cell antagonism.

The Two Membrane Distal ITAMs in TCR ζ are Essential for Maintaining the Constitutive Tyrosine Phosphorylated 21-kDa Form in P14 TCR Transgenic Mice

To rigorously confirm that the various YF transgenic lines resulted in the selective elimination of p23 alone or both p21 and p23 in the P14 system, we examined the phosphorylation state of ζ before and after agonist stimulation. Thus, thymocytes or peripheral T cells from the P14, P14/YF1,2; P14/YF5,6; and P14/YF1-6 transgenic lines were isolated and incubated with unloaded or p33 peptide-loaded dendritic cells. Five minutes after stimulation, the cells were lysed and the TCR ζ subunit was directly immunoprecipitated with an anti-ζ mAb in the presence of 0.1 % SDS. These experiments were performed in the presence of SDS in order to ensure that the TCR ζ subunit was isolated in the absence of any associating proteins or co-precipitating CD3 subunits. The ζ immunoprecipitates were resolved on a 13.5% SDS PAGE gel and subsequently immunoblotted with an anti-phosphotyrosine antibody (4G10).

Thymocytes from P14 TCR transgenic mice constitutively express p21 and, following stimulation, can be induced to express p23 (Figure 11A, lanes 1 and 2). Although the constitutively expressed p21 was maintained in the P14/YF1,2 double transgenic line, there was no detectable p23 following p33 stimulation (Figure 11 A, lanes 3-4). In the P14/YF5,6 line, there was no evidence of any constitutive or inducible p21 or p23(Figure 11A, lanes 5-6). Instead, several very weakly phosphorylated species were detected with molecular masses ranging from 19- to 20-kDa following peptide stimulation (Figure 11A, lanes 5 and 6). As expected, there were no detectable phosphorylated intermediates in the P14/YF1-6 line (Figure 11A, lanes 7 and 8). Despite the selective elimination of p21 and p23 subunit, there is no alteration in the non-phosphorylated 16-kDa form, as it was readily detected in all the transgenic lines characterized (Figure 11B). It should be noted that treatment of the YF1,2 cells with the general phosphatase inhibitor, pervanadate, also fails to elicit any p23 (91). Moreover, stimulation of the YF5,6 line with pervanadate results in the formation of several heavily phosphorylated TCR ζ intermediates, all with molecular masses less than 20-kDa. Similar results were found with lymph node T cells (data not shown). For the YF1-6 line, there is some evidence of TCR ζ degradation that we have been unable to prevent with protease inhibitors (data not shown). These results clearly indicate that we have effectively eliminated the p23 or both p21 and p23 phosphorylated forms of TCR ζ in the various P14/TCR ζ transgenic lines.

The Constitutively Phosphorylated 21-kDa TCR ζ Subunit Promotes Full Activation of T Cells by Agonist Peptide

Using the P14/TCR ζ transgenic lines described above, we were in a unique position to examine the effects of p21 and p23 on T cell activation and T cell antagonism. Transgenic T cells from the various mice were isolated and incubated with varying doses of peptide pulsed antigen presenting cells. Proliferative responses were measured by ^{3}H-thymidine incorporation during the last sixteen hours of culture. In response to increasing doses of agonist peptide (10^{-10}M to 10^{-4}M), the P14 TCR transgenic T cells exhibited a nearly five fold increase in proliferation (Figure 12A). This dose response curve was nearly identical when the P14/YF1,2 T cells were stimulated in an identical manner. These results indicate that p21, expressed independent of p23, does not inhibit proliferative responses. A very similar dose response curve was revealed when the P14/YF5,6 transgenic line was compared to wild-type cells (Figure 12B). These results suggest neither p21 or p23 are contributing to proliferative responses. The ability of the YF1-6 T cells, which lack all tyrosine phosphorylated TCR ζ subunits, was slightly impaired versus wild type P14 T cells in response to agonist p33 peptide, with only a three-fold increase in proliferation over the dose range used (Figure 12C). The response to the weak agonist peptide, A4Y, was equivalent in the YF1,2 and YF5,6 transgenic lines compared to the P14 wild-type cells (data not shown). When the various P14/TCR ζ thymocytes were compared to the P14 line for the induction of phosphoproteins, equivalent patterns of ZAP-70, SLP-76 and LAT phosphorylation were detected following peptide/MHC stimulation (data not shown). Taken together, these results

directly demonstrate that the phosphorylated 21- and 23-kDa forms of TCR ζ are uncoupled from activation of membrane proximal signals and the proliferative response of the P14 T cells. More specifically, the constitutive 21-kDa form of TCR ζ doesn't interfere with or antagonize T cell activation by agonist peptides in this system or in a non-TCR transgenic system.

T Cell Antagonism is Uncoupled from the 21- and 23-kDa Tyrosine Phosphorylated Forms of TCR ζ

T cell proliferative responses versus an agonist peptide can be effectively inhibited by the addition of select antagonist peptides (98, 156). The various P14/TCR ζ transgenic lines which selectively express either p21 or both p21 and p23, were used to directly analyze whether the 21-kDa form of TCR ζ correlated with T cell antagonism. The predominant expression of p21 was previously reported to actively inhibit T cell responses and induce anergy (156). This finding would suggest that the YF1,2 cells in our system should have enhanced antagonist responses. T cells isolated from lymph nodes of P14/TCR ζ mice were co-cultured with antigen presenting cells that had been prepulsed with a suboptimal (10^{-8}M) concentration of the p33 agonist peptide, followed by increasing concentrations of antagonist peptides in the range from 10^{-7} to 10^{-4} M. P14 T cells were effectively inhibited by the addition of high concentrations of the antagonist peptide, S4Y, with a three-fold decrease in thymidine uptake compared to the suboptimal concentration of agonist peptide (Figure 13A). Notably, the P14/ζYF1,2 transgenic T cells which only express the constitutively phosphorylated p21 exhibited an equivalent

magnitude of antagonism over the entire dose of peptide used (Figure 13 A). Likewise, the P14/ζYF5,6 transgenic T cells that lack p21 and p23 also show an equivalent level of T cell antagonism compared to wild type P14 T cells (Figure 13 B). These results provide direct evidence that the 21- and/or 23-kDa tyrosine phosphorylated TCR ζ subunits are completely uncoupled from T cell antagonism. Interestingly, we were unable to detect T cell antagonism in the absence of any tyrosine phosphorylated TCR ζ intermediates in the YF1-6 line (Figure 13 C). The implications of this finding are discussed below.

Discussion

The TCR ζ subunit is one of the earliest and the most heavily tyrosine phosphorylated proteins detected in T cells following TCR ligation (reviewed in (26)). In fact, two distinct tyrosine phosphorylated derivatives of 21- and 23-kDa are commonly revealed upon agonist peptide or antibody mediated activation of T cells (37). P21 is specifically phosphorylated on the four tyrosine residues in the two membrane distal ITAMs, while p23 is fully phosphorylated on the six tyrosines dispersed among all three ITAMs (91). Notably, p21 appears as a constitutively phosphorylated protein in thymocytes and peripheral T cells and is associated with an inactive pool of ZAP-70 molecules (82). The constitutive phosphorylation of p21 results from TCR interactions with self-peptide/MHC complexes in the thymus and peripheral lymphoid organs (43, 82-84, 139). A plethora of publications have supported a functional role for both p21 and p23 in predicating the outcomes of T cell selection ,T cell antagonism, and peripheral T cell survival (for reviews, see (37, 85, 86)). Yet, an equally impressive number of publications have refuted roles for these phosphorylated derivatives in regulating these biological events (for reviews, see (37, 85, 86)). In spite of these publications, not one study has examined the consequences of maintaining p21 in the absence of p23.

In this report, we definitively demonstrate that positive selection and T cell antagonism in the P14 TCR transgenic system are completely uncoupled from the presence of p23 or both p21 and p23. First, we used a series of TCR ζ transgenic mice that contain substitutions at several distinct tyrosine residues that result in the selective elimination of just p23, or both p21 and p23 (91). Importantly, the YF1,2 line is the only transgenic line

developed to date that maintains p21 without being able to form p23. When introduced onto the P14 TCR transgenic system, we determined that positive selection was independent of p23 and was unaffected by the presence of the constitutively tyrosine phosphorylated p21 form. Second, we used a series of peptide analogs previously defined as agonist, weak agonist, and antagonist peptides in the P14 system to show that T cell antagonism is not influenced by p21 and/or p23. One prediction of the earlier model concerning T cell antagonism is that the level of antagonism would increase if p21 were the only phosphorylated intermediate. In fact, we find an equivalent dose response curve for antagonist peptide-mediated inhibition of T cell proliferation when comparing P14 and the P14/YF1,2 mice. Moreover, we determined that the magnitude of T cell antagonism is equivalent in wild-type mice and in mice lacking both the 21- and 23-kDa derivatives of TCR ζ. It had been proposed that the constitutively phosphorylated p21 present in resting T cells provides a predominant inhibitory signal for T cells, and that this may explain T cell anergy (156) (61, 99, 102, 155) (101). Our results clearly refute a role for p21 in dominantly affecting T cell antagonism. An earlier report suggested that monophosphorylated ITAMs were the basis of the 21-kDa structure (90). Importantly, our mapping studies demonstrated that the 21-kDa form of TCR ζ consists solely of two biphosphorylated ITAMs (91). We cannot rule out the possibility that monophosphorylated ITAMs, distinct from the 21-kDa form and not detected in our system, may actually contribute to T cell antagonism. For example, Allen and co-workers have demonstrated, using CD8/ζ chimeric molecules, that monophosphorylated ITAMs can promote T cell antagonism (156). We have found that the YF1-6 TCR ζ molecule was unable to support antagonism and this molecule remains completely non-

phosphorylated prior to and following TCR cross-linking. As reported elsewhere, phosphorylated TCR ζ molecules can contribute to T cell selection with low avidity TCRs (114). It is conceivable that the high avidity P14 line used in our study requires some phosphorylated ζ molecules distinct from p21 and/or p23 for effective antagonism. For example, a previous study using the P14 TCR transgenic system in which TCR ζ was selectively substituted at the first, third, and fifth tyrosine residues (called $a1^{-}b1^{-}c1^{-}$) revealed that antagonism could only be established when using weak agonists such as AV, and not p33 (115). Yet, the proliferative responses to p33 were equivalent in those mice when compared to wild-type P14 mice. Since the YF5,6 line used in our study is weakly phosphorylated in the P14 system following agonist stimulation, a single tyrosine within the second ITAM of TCR ζ could potentially mediate antagonism. However, these possibilities seem unlikely for the following reasons. First, we have shown that T cell proliferation occurs in the YF1-6 line, which lack any functional ITAMs in the ζ chain. Second, we have previously used a TCR ζ construct in which tyrosines three and four were substituted with phenylalanine (YF3,4)(91). T cell lines expressing this TCR ζ construct exhibit T cell response identical to wild-type T cells, eliminating the third and fourth tyrosine residues as potential inhibitory phosphorylation sites (91). Third, Malissen and co-workers have generated TCR ζ transgenic lines with specific substitutions at the first, third, and fifth tyrosine residues (115). Notably, T cells expressing this TCR ζ molecule could be antagonized again suggesting that no specific individual tyrosine residue is mediating antagonism. In an other report, T cell antagonism was evident with fully-truncated TCR ζ molecules expressed in TCR ζ-null T cell hybridomas (157). However, no constitutive tyrosine phosphorylated p21 was

evident in this system, precluding an assessment of p21. Since this hybridoma system involves a TCR distinct from the P14 system, the avidity of the receptor for particular peptides may be sufficiently high to preclude any phosphorylated TCR ζ involvement. Current experiments are underway to address these issues.

It remains unclear what signals are involved in the antagonism of T cells responses. One possibility is that a threshold of signaling must be reached for cells to be fully activated. We have some preliminary evidence suggesting that the CD3 subunits may form the predominant signaling module in T cells (Pitcher et al., submitted). Furthermore, there is some correlative data indicating that the degree of CD3ε phosphorylation may regulate T cell responsiveness versus antagonism (101). We are also exploring the possibility that the level of ZAP-70 PTK activation may be the primary determinant in regulating T cell antagonism as ZAP-70 is generally not detected as a phosphoprotein following the stimulation of T cells with antagonist peptides. Other mechanisms have been proposed for the different T cell responses to agonist versus antagonist peptides (reviewed in (86)). These include conformational changes in the TCR complex upon ligand binding, the relative affinity of the receptor for the peptide/MHC molecule ligand and/or the kinetics of activation. Alternatively, a negative regulator may be involved in generating an antagonist response (158). A paradigm for this model has been established with the identification of the SH2-domain containing SOCS family of inhibitors for cytokine/cytokine receptor signaling (159). Notably, the expression of several viral-ITAM containing proteins in lymphocytes can directly attenuate subsequent signal transduction through the antigen receptors (21, 160-162).

Similar mechanisms may operate to maintain T cell antagonism. However, several studies have again refuted this possibility, since cross-antagonism is not seen with T cells expressing two distinct T cell receptors (163, 164).

Overall, our studies have shown that the development and function of T cells from the P14 TCR transgenic line are separate from the two major tyrosine phosphorylated derivatives of the TCR ζ subunit. Current experiments are focusing on the functional contribution of p21 to peripheral T cell survival.

In summary, we have developed a number of TCR ζ transgenic lines, one of which is the only TCR ζ transgenic line ever described that successfully permits expression of the constitutively tyrosine phosphorylated p21 form of TCR ζ without any available p23. This YF1,2 line has uniquely enabled us to provide definitive and unequivocal results pertaining to the p21 form of TCR ζ. With these mice as the basis for our investigations, we definitively show that p21, on its own, does not attenuate T cell development nor contribute to T cell antagonism, refuting the generally accepted theory that p21 plays a dominant inhibitory role.

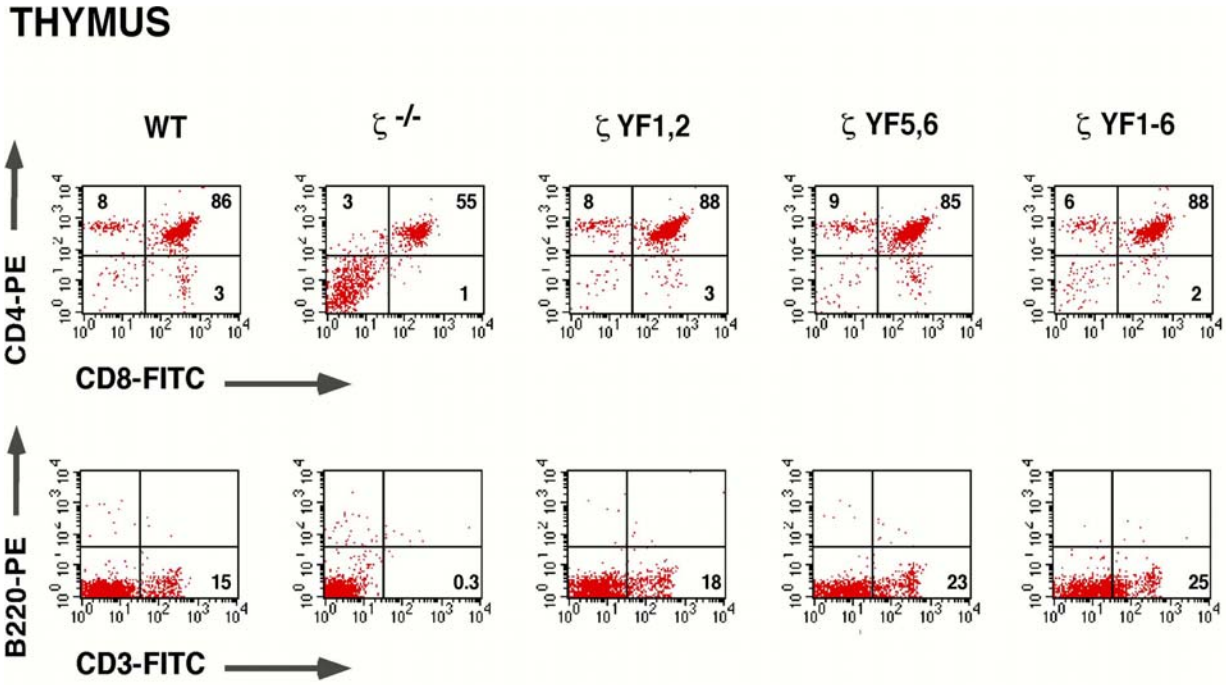


Figure 5. T Cell Development Proceeds Normally in the TCR ζ Transgenic Mice Bearing Selected Tyrosine to Phenylalanine Substitutions in the ITAMs. Thymocytes were isolated from wild-type mice and the TCR ζ transgenic mice that contained substitutions in the first ITAM tyrosine (YF1,2) the third ITAM tyrosines (YF5,6) or all six tyrosine residues in the TCR ζ ITAMs (YF1-6). Single cell suspensions were prepared and stained with mAbs to CD4 and CD8 or CD3 and B220. The cells were analyzed by flow cytometry. These results are representative of three independent experiments.

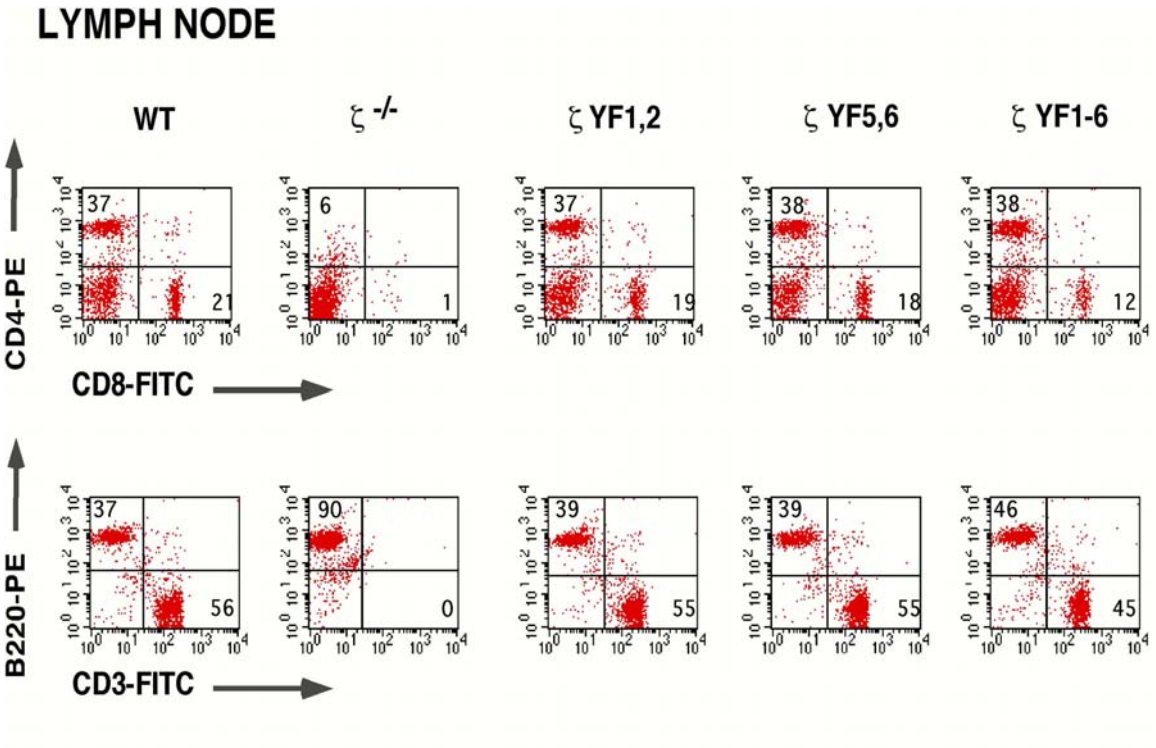


Figure 6. T Cell Development Proceeds Normally in the TCR ζ Transgenic Mice Bearing Selected Tyrosine to Phenylalanine Substitutions in the ITAMs. Lymph node cells were isolated from wild-type mice and the TCR ζ transgenic mice that contained substitutions in the first ITAM tyrosine (YF1,2) the third ITAM tyrosines (YF5,6) or all six tyrosine residues in the TCR ζ ITAMs (YF1-6). Single cell suspensions were prepared and stained with mAbs to CD4 and CD8 or CD3 and B220. The cells were analyzed by flow cytometry. These results are representative of three independent experiments.

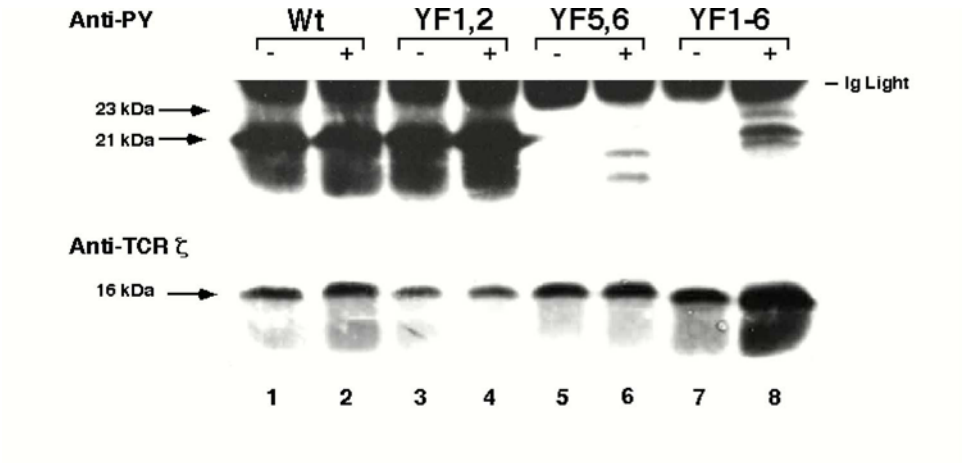


Figure 7. The Constitutive and Inducible Phosphorylation of TCR ζ Depends on the Presence of Particular ITAM Tyrosines. The TCR ζ phosphorylation patterns in *ex vivo* thymocytes were compared before and after TCR cross-linking for five minutes. Cell lysates were prepared and the TCR ζ subunit was directly immunoprecipitated with anti-TCR ζ mAbs. The precipitates were subsequently Western immunoblotted with anti-phosphotyrosine followed by anti-TCR ζ mAbs. Thymocytes from wild-type mice (*lanes 1 and 2*) were compared to the YF1,2 (*lanes 3 and 4*), YF5,6 (*lanes 5 and 6*) and YF1-6 (*lanes 7 and 8*) TCR ζ transgenic lines prior to (*lanes 1, 3, 5 and 7*) or following TCR cross-linking (*lanes 2, 4, 6 and 8*).

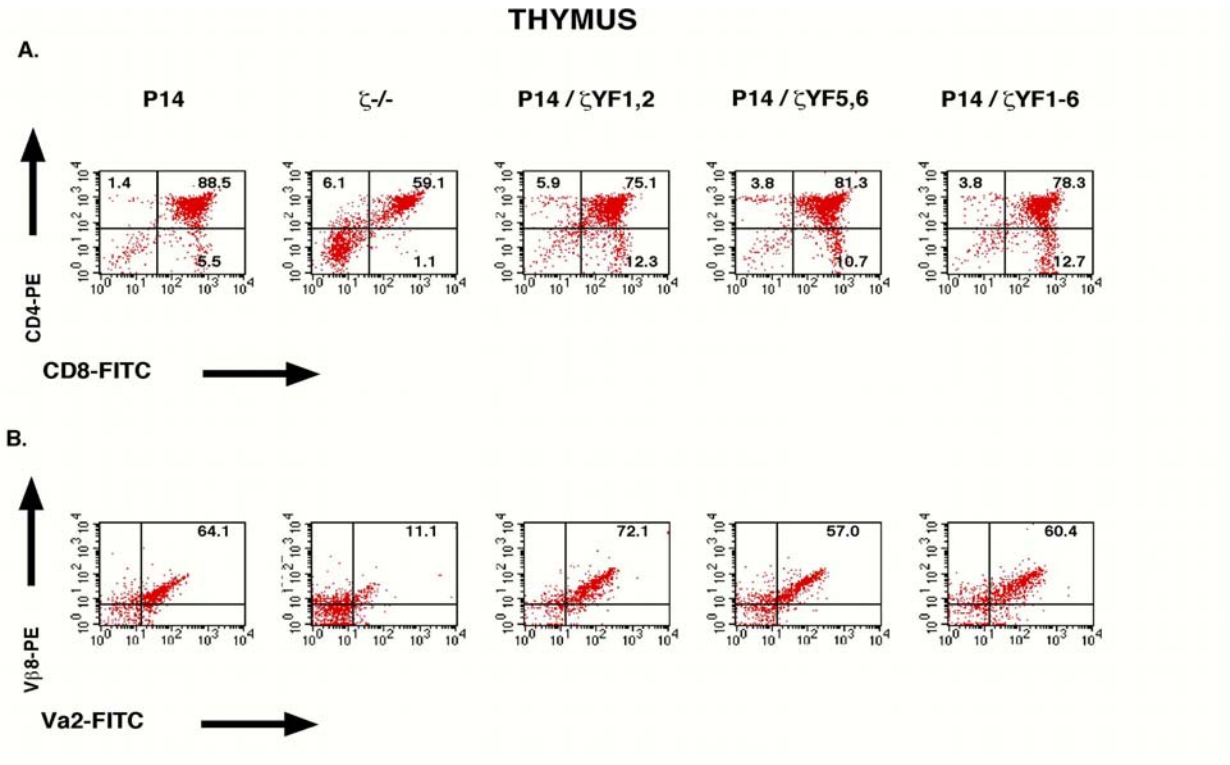


Figure 8. The Development of P14 TCR Transgenic Thymocytes is Normal in the Presence of the 16-, or 21-, or 21- and 23-kDa Tyrosine-Phosphorylated Forms of the TCR ζ Subunit. Thymocytes from P14 and P14/TCR ζ-null mice were compared with mice expressing the YF1,2; YF5,6; or YF1-6 TCR ζ constructs on the P14/TCR ζ-null background. Single cell suspensions were prepared and stained with mAbs to CD4 and CD8 (A) or Vβ8 and Vα2 (B) and analyzed by flow cytometry. Results are representative of eight independent experiments.

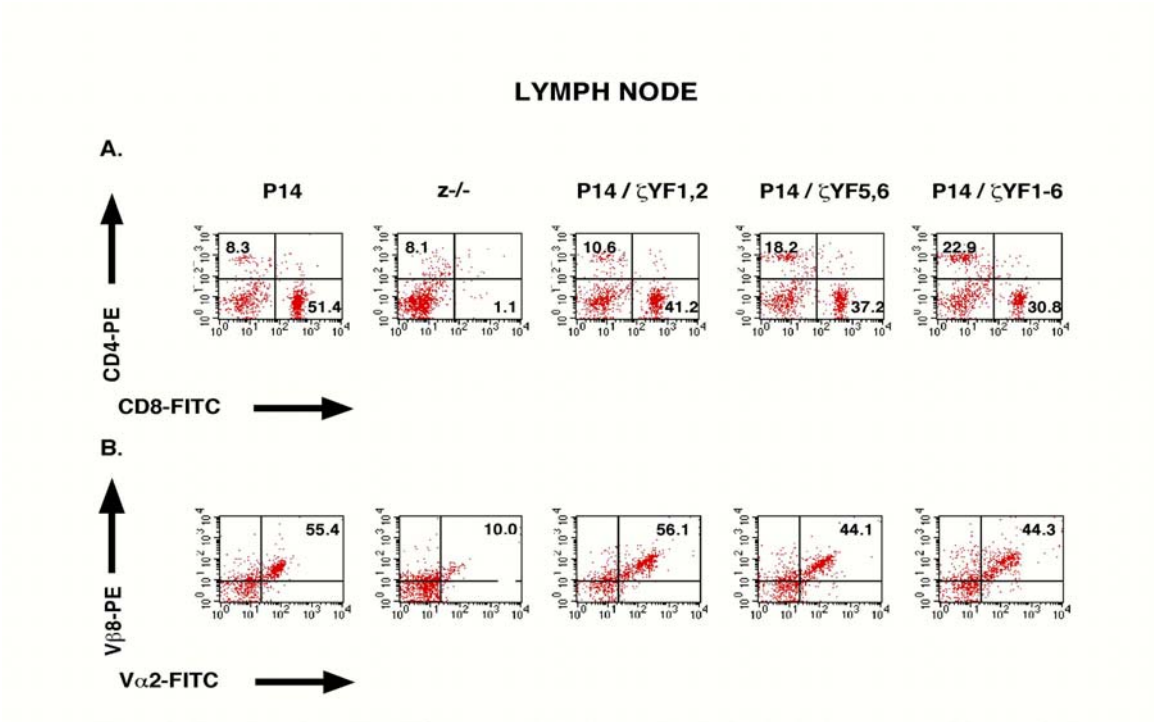


Figure 9. Normal Numbers of P14 TCR Transgenic Lymph Node T Cells Develop in the Presence of the Constitutively Tyrosine-Phosphorylated TCR ζ Subunit. Lymph node cells were isolated from P14; P14/TCR ζ-null; P14/TCR ζ YF1,2; P14/TCR ζ YF5,6; and P14/TCR ζ YF1-6 mice. Single cell suspensions were prepared and stained with mAbs against CD4 and CD8 (A) or Vβ8 and Vα2 (B) and analyzed by flow cytometry. Results are representative of six independent experiments.

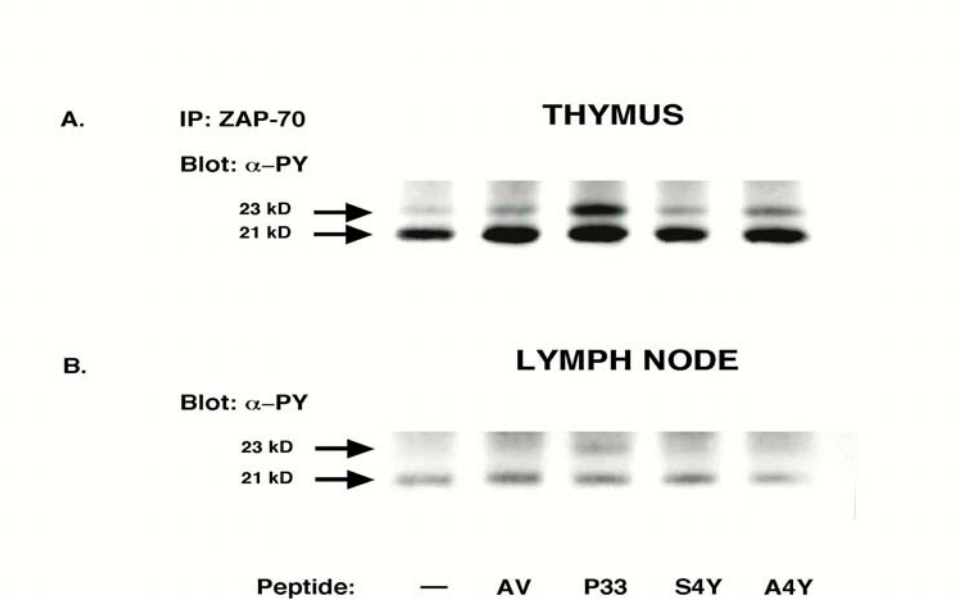


Figure 10. The 21- and 23-kDa Tyrosine-Phosphorylated Forms of TCR ζ are Induced Upon Stimulation with Antagonist Peptides. Dendritic cells ($H2D^b$) were pulsed with 3 micromolar of a control peptide (AV, *lane 2*), that agonist peptide (p33, *lane 3*), an antagonist peptide (S4Y, *lane 4*), or a weak agonist peptide (A4Y, *lane 5*). These peptide loaded dendritic cells were incubated with thymocytes (A) or peripheral T cells (B) from P14 TCR transgenic mice for a five minute period. Following the incubation period, the cells were lysed and the ZAP-70 PTK was immunoprecipitated. The precipitates were resolved by 12.5% SDS-PAGE and immunoblotted with an anti-phosphotyrosine mAb.

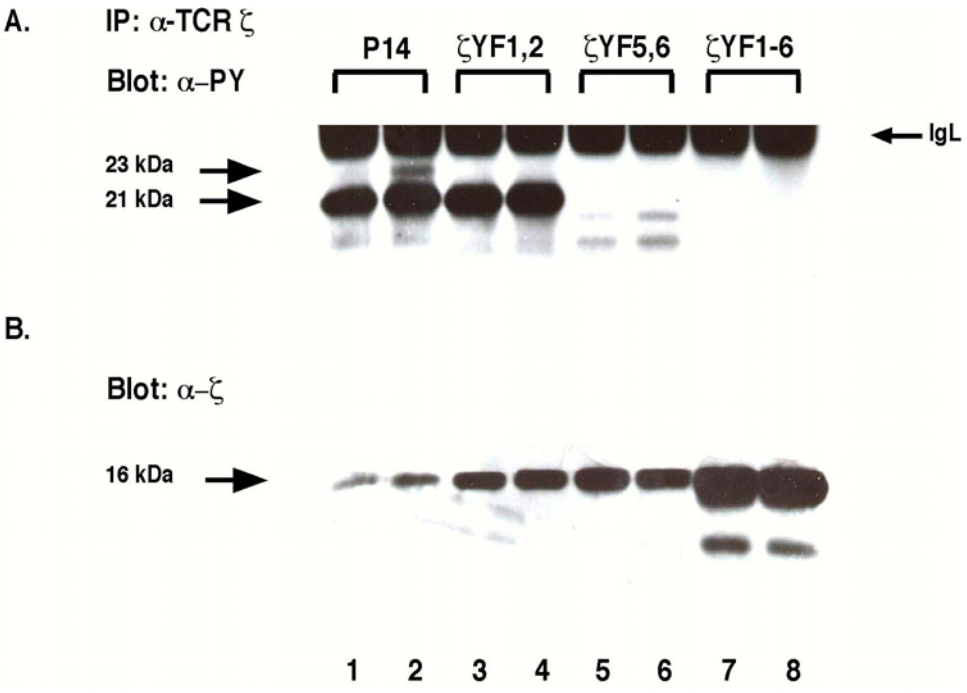


Figure 11. The Selective Elimination of the 21- and/or 21- and 23-kDa Tyrosine-Phosphorylated Forms of TCR ζ in the P14/TCR ζ Transgenic Mice Stimulated with Agonist Peptides. Thymocytes were isolated from P14 mice expressing a wild-type TCR ζ subunit (*lanes 1 and 2*) or TCR ζ chains containing various tyrosine to phenylalanine substitutions designated as YF1,2 (*lanes 3 and 4*), YF5,6 (*lanes 5 and 6*), or YF1-6 (*lanes 7 and 8*). These cells were incubated with dendritic cells ($H2D^b$) that had been untreated or pre-pulsed with 3 micromolar of the strong agonist peptide, p33. Following a five minute incubation period, the cells were lysed and the TCR ζ subunit was directly immunoprecipitated in the presence of 0.1% SDS. The precipitates were resolved by 12.5% SDS-PAGE and immunoblotted with an anti-phosphotyrosine mAb (A) followed by an anti-TCR ζ mAb (B).

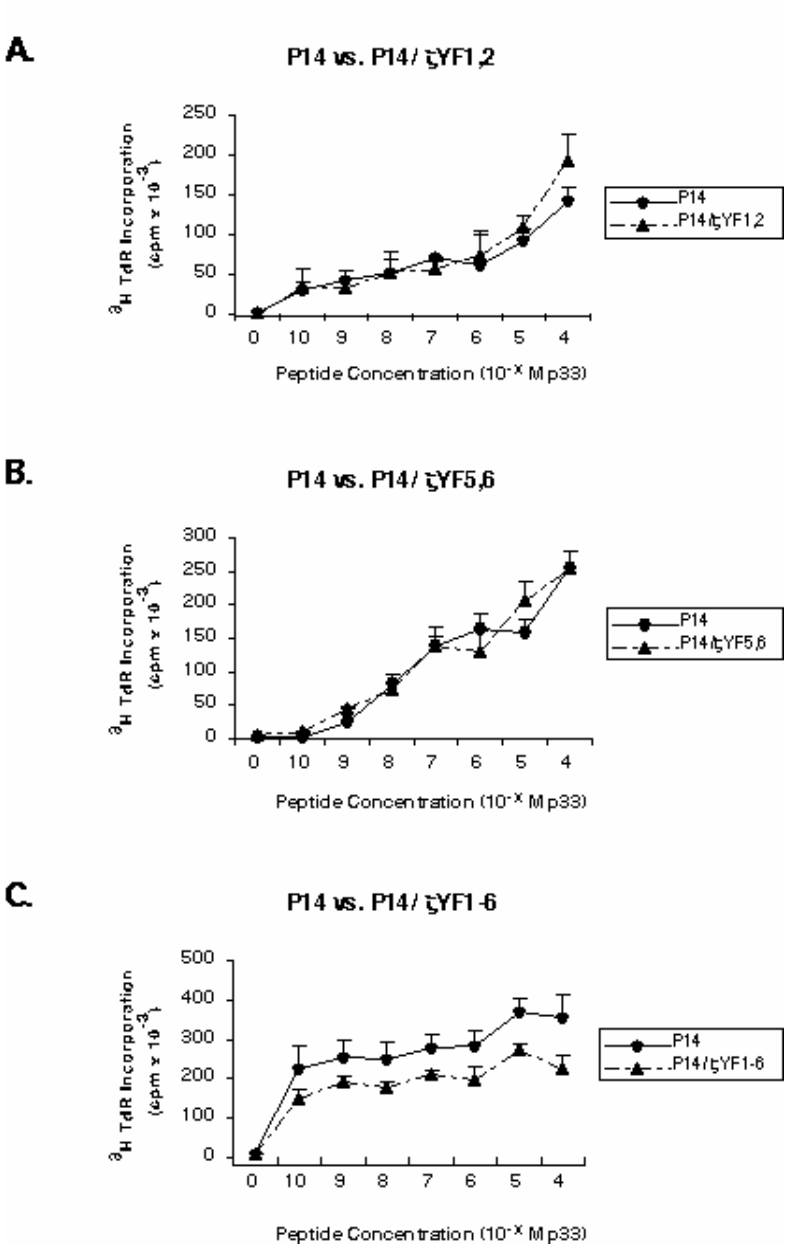
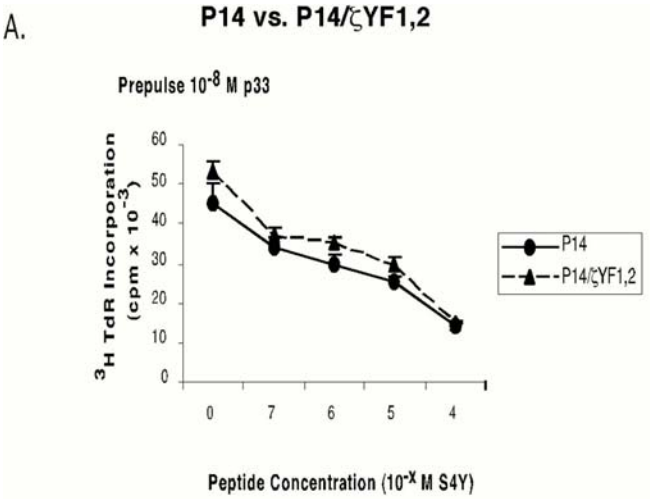
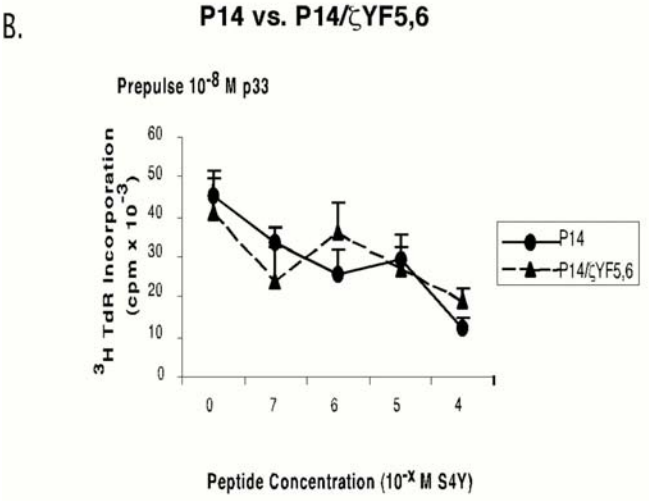


Figure 12. Agonist Peptide-Induced Proliferation in the P14 T Cells is Independent of the Phosphorylated TCR ζ Subunits. Lymph node cells (10^5) were isolated from wild-type P14 mice or P14 mice bearing selected tyrosine to phenylalanine substitutions in the TCR ζ chain designated as P14/ζ YF1,2 (A); P14/ζ YF5,6 (B); and P14/ζ YF1-6 (C). These cells were cultured with varying concentrations of agonist peptide (p33) pulsed antigen presenting cells (5 x 10^4) for 48 hours. Proliferation was measured by adding 1 mCi ^{3}H-thymidine for the last 16 hours of culture. These results are representative of three independent experiments.

A.


P14 vs. P14/ζYF1,2
Prepulse 10-8 M p33
3H TdR Incorporation (cpm x 10-3)
60
50
40
30
20
10
0
0
7
6
5
4
Peptide Concentration (10-X M S4Y)
P14
P14/ζYF1,2

B.


P14 vs. P14/ζYF5,6
Prepulse 10-8 M p33
3H TdR Incorporation (cpm x 10-3)
60
50
40
30
20
10
0
0
7
6
5
4
Peptide Concentration (10-X M S4Y)
P14
P14/ζYF5,6

C.

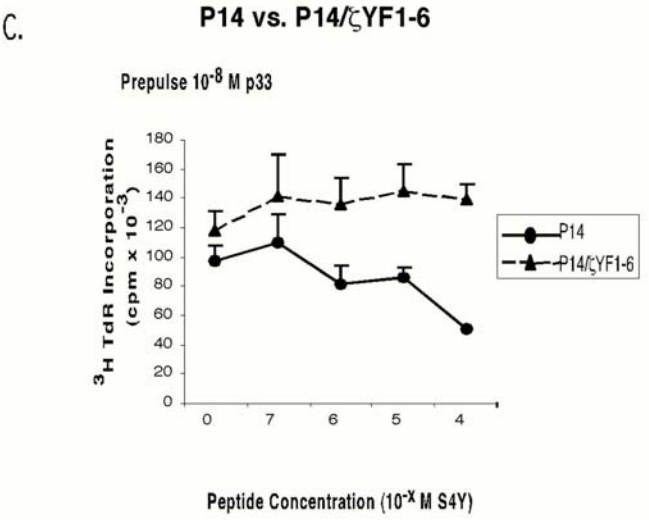

P14 vs. P14/ζYF1-6
Prepulse 10-8 M p33
3H TdR Incorporation (cpm x 10-3)
180
160
140
120
100
80
60
40
20
0
0
7
6
5
4
Peptide Concentration (10-X M S4Y)
P14
P14/ζYF1-6

Figure 13. T Cell Antagonism Does Not Involve the 21- and 23-kDa Tyrosine-Phosphorylated TCR ζ Subunit. Lymph node cells (10^5) from the various P14 single and double transgenic lines were cultured with antigen presenting cells that had been prepulsed with suboptimal concentrations of agonist peptide (10^{-8} M p33) followed by increasing concentrations of antagonist peptide (10^{-7} to 10^{-4} M S4Y). P14 and P14/ζ YF1,2 (A); or P14 and P14/ζ YF5,6 (B); or P14 and P14/ζ YF1-6 (C) were compared. After 48 hours, proliferation of T cells was measured by pulsing cultures with 1 mCi ^{3}H-thymidine for an additional 16 hours of culture. These results are representative of three independent experiments. The magnitude of proliferation was less in the YF1-6 line in an independent experiment.

Chapter IV. The CD3 γε/δε Signaling Module is the Principal Regulator of TCR Signal Transmission

Introduction

The T cell receptor (TCR) is a multimeric complex comprised of the ligand binding αβ heterodimer and the associated signaling subunits, TCR ζ and CD3 γ, δ and ε (2, 14). These signaling subunits contain a semi-conserved cytoplasmic amino acid sequence ($YxxLx_{6-8}YxxL$) termed the ITAM, or immunoreceptor-based tyrosine activation motif (17, 18). Upon receptor ligation, two tyrosine residues within each ITAM are rapidly phosphorylated by a member of the Src-family protein tyrosine kinases (PTKs), transforming them into high affinity ligands for the Syk-PTKs (reviewed in (37)). The coordinated actions of the Src- and Syk-PTKs initiate a cascade of signals that ultimately lead to T cell proliferation, cytokine secretion and effector functions (54).

An analysis of the intact TCR complex has suggested a structural design with two independent signaling modules involving either the TCR ζζ (6/10 ITAMs) or the CD3 γε/δε subunits (4/10 ITAMs) (4, 11, 61). All of the ITAM-containing subunits can activate similar signaling processes, suggesting additive or synergistic functions for the ITAMs (reviewed in (37, 85)). In contrast, biochemical studies revealing a hierarchy of distinct protein interactions with phospho-ζ versus phospho-CD3 γ, δ and ε suggest distinct functions for the TCR ζζ- and CD3 γε/δε-modules (35, 68, 70).

A common premise is that the TCR ζζ-module forms the predominant signaling component of the TCR complex. First, TCR engagement results in the rapid tyrosine phosphorylation of the 16-kDa TCR ζζ homodimer such that it appears as two distinct phosphoproteins of 21- and 23-kDa (p21 and p23)(91). This contrasts the CD3 γ, δ and ε subunits, which are difficult to detect as tyrosine-phosphorylated proteins (31, 32, 34). Second, p21 is constitutively phosphorylated when isolated from thymocytes and peripheral T cells, complexing with an inactive pool of ZAP-70 PTK (82). Given these observations, it was somewhat surprising to find that T cells are fully functional in their ability to respond to TCR stimulation even when all of the signaling capabilities of TCR ζ are eliminated by truncations or ITAM substitutions (36, 58, 61, 114-116, 165). These experiments suggested one of two possibilities. First, the CD3 γε/δε-module could be the principal signaling module in T cells. This is supported by a recent report that identified a unique and essential role for CD3 ε in the initiation of TCR-mediated signaling through a TCR-induced interaction with the adaptor protein Nck (79). A second possibility is that maximal T cell receptor signal transmission requires only a minimal number of ITAMs from the TCR ζζ- or CD3 γε/δε-modules. Yet, all of the experiments supporting these two interpretations were performed with peripheral T cells that had undergone a normal development in the thymus.

To precisely determine the functional roles of the TCR ζζ- and CD3 γε/δε-modules in TCR-mediated signal transmission and development, we analyzed mice that had a partial or complete elimination of the TCR ζζ-module while leaving the CD3 γε/δε-

module intact (37, 91). These lines were backcrossed to the HY TCR transgenic line, wherein a direct dependence on ζ ITAMs for positive selection has been noted previously (114, 116). Using these TCR ζ transgenic lines, each containing selected tyrosine to phenylalanine (YF) substitutions in the TCR ζ ITAMs, we have identified a critical role for the phospho-ζ ITAMs in the positive selection of HY-restricted cells. In spite of this direct dependence, the activation of traditional signaling pathways in thymocytes and T cell proliferative responses to peptide/MHC complexes were independent of any phospho-ζ intermediates. These findings strongly implicate a principal role for the CD3 γε/δε-module in TCR signal transmission in primary T cell populations.

Results

The CD3 γε/δε-Module is Sufficient for Normal Proliferative Responses of T cells

To examine how TCR-mediated intracellular signals that lead to T cell proliferation are influenced by particular phospho-ζ intermediates, we used a series of TCR ζ transgenic mice with selected tyrosine to phenylalanine substitutions in the ITAMs of the TCR ζ subunit. These result in the specific elimination of p23 alone (YF1,2), both p21 and p23 (YF5,6) or all tyrosine-phosphorylated TCR ζ intermediates (YF1-6) (37, 91). The YF1,2 and YF5,6 substitutions leave intact 8/10 ITAMs in the TCR complex, while the YF1-6 line contains only the four ITAMs in the CD3 γε/δε-module. We compared the proliferative responses of T cells from wild-type mice and the TCR ζ transgenic lines following various TCR stimulations. T cells from a wild-type mouse exhibited a dose-dependent increase in proliferation following stimulation with a mAb against CD3 ε (Figure 14 A). T cells that maintain expression of just p21, without p23, had a slightly augmented proliferative response (YF1,2, Figure 14 A). T cells expressing only weakly phosphorylated species of TCR ζ (YF5,6) had a comparable proliferative response to wild-type T cells. Interestingly, T cells with only the CD3 γε/δε-module intact (YF1-6) also had equivalent proliferative responses when compared to wild type T cells (Figure 14 A). Peripheral T cells from the various TCR ζ transgenic lines were also stimulated with Con A, a T cell mitogen or superantigen (Figure 14 B and C). Similar findings were revealed with these stimulations when compared to anti-CD3-mediated responses (Figure 14 A, B, C; data not shown). In fact, equivalent early activation

events, such as protein phosphorylation and intracellular calcium mobilization were revealed when comparing the wild type and YF series of mice (data not shown)((165). These data strongly suggest that the signals generated through the TCR complex in response to mAb-, mitogen-, superantigen- or peptide/MHC stimulation are independent of phospho-ζ and primarily involve the CD3 γε/δε-module (36, 91, 114, 115).

Phosphorylated ζ Intermediates are Essential for the Development of HY TCR Transgenic T Cells

In all of the TCR ζ transgenic systems where signal transmission was analyzed, including those described above, T cell development was independent of phospho-ζ (37, 114, 115, 165). The HY αβ TCR transgenic system, which is specific for the male-derived Smcy peptide in the context of $H2D^b$, is one TCR transgenic line that shows a direct dependence on phospho-ζ ITAMs for positive and negative selection (114, 116, 166). However, the contributions of p21 and p23 had not been examined in this system. To assess the involvement of the distinct phospho-ζ intermediates in positive selection, we compared T cell development in the HY and HY/TCR ζ transgenic females (Figure 15). Positive selection of male-specific T cells in the HY TCR transgenic female mice results in the development of $CD4^-CD8^+$ thymocytes expressing the HY TCR, detected by the clonotypic mAb T3.70 (167)(Figure 15). In age-matched females that express p21 in the absence of p23, the efficiency of positive selection of thymocytes is reduced nearly two-fold, observed as a decrease in the numbers of $CD4^-CD8^+T3.70^+$ thymocytes compared to wild-type HY (8% vs. 16%)(HY/YF1,2; Figure 15). The elimination of both

p21 and p23 also resulted in a two-three-fold reduction in $CD4^-CD8^+T3.70^+$ thymocytes from wild type (7% vs. 16%)(HY/YF5,6; Figure 15). Finally, the absence of all phospho-ζ intermediates almost completely abolished the development of mature HY-specific thymocytes (2% vs. 16%)(HY/YF1-6; Figure 15). These results are summarized in Table IV. Importantly, the experiments described herein rule out a dominant inhibitory role proposed for p21 and are consistent with previous studies using truncated TCR ζ subunits (85, 115, 116, 156).

The reduced efficiency of positive selection in the thymus of the HY/YF lines is reflected as fewer HY-specific T cells in the peripheral lymphoid organs. In the HY female mice, the male-reactive $CD8^+$ T cells represent approximately 6.4% of total T cells in the lymph node and this is reduced about 3-fold in age-matched HY/YF1,2 and HY/YF5,6 female mice (Figure 16). Less than 0.5% of these cells are detected in the HY/YF1-6 female mice, indicating that positive selection of thymocytes in the low avidity HY TCR system is directly dependent on the number of ζ ITAMs (Figure 16). In addition, the similar reduction in both the HY/YF1,2 and HY/YF5,6 lines (8/10 functional ITAMs) suggests that the TCR ζ ITAMs function additively rather than p21 and p23 contributing differentially to positive selection.

The CD3 γε/δε Signaling Module is Primarily Responsible for TCR Signal Transmission

The HY/TCR ζ transgenic mice represent the best experimental system to determine the contributions of the TCR ζζ- and CD3 γε/δε-modules on TCR signal

transmission since the TCR ζ ITAMs were directly involved in positive selection, implying they were coupled to the strength of signal transmission. To address this, we initially assessed the phosphorylation state of the TCR ζ subunit in the various HY/YF lines elicited by the strong agonist peptide, Smcy, to confirm the presence or absence of particular phospho-ζ intermediates. In wild-type HY thymocytes, p21 was constitutively expressed while p23 was elicited following agonist peptide stimulation (Figure 17). The HY/YF1,2 thymocytes also constitutively expressed p21, but were unable to generate p23 after stimulation. Thymocytes from the HY/YF5,6 line did not constitutively or inducibly express p21 or p23, but expressed weakly phosphorylated TCR ζ intermediates of p19/p20 following stimulation (Figure 17). In the HY/YF1-6 line, the substitution of all six tyrosine residues in the TCR ζ subunit completely eliminated the constitutive or inducible expression of any phospho-ζ (Figure 17). Each of these transgenic lines maintained the unphosphorylated 16-kDa form of TCR ζ. We also assessed the phosphorylation state of the CD3 subunits following TCR stimulation of the various HY/TCR ζ thymocytes. Notably, the CD3 subunits were difficult to detect as phosphoproteins, even in the HY/YF1-6 line, indicating that the CD3 subunits do not over-compensate for the lack of TCR ζ ITAMs (data not shown).

We next compared the classic downstream biochemical signals induced following TCR interactions with peptide-MHC. The kinetics of ZAP-70 tyrosine phosphorylation in thymocytes was compared in the distinct HY/TCR ζ transgenic lines following a time course of agonist peptide stimulation. Prior to stimulation, little phosphorylation of ZAP-70 was detected. In wild-type HY females, ZAP-70 appeared as a phospho-protein

within three minutes (Figure 18). A slight decrease in ZAP-70 phosphorylation was detected by 90 minutes. Equivalent kinetics of ZAP-70 phosphorylation were detected in the HY/YF1,2, HY/YF5,6 and HY/YF1-6 lines (Figure 18).

We compared next the tyrosine phosphorylation kinetics of SLP76, a substrate for ZAP-70 PTK. In wild-type thymocytes stimulated with the agonist peptide, Smcy, SLP76 is inducibly tyrosine-phosphorylated within three minutes of stimulation (HY; Figure 19). Similar kinetics of SLP76 phosphorylation was observed in all the HY/YF series mice. Some diminished phosphorylation of SLP76 was revealed in the HY/YF1-6 line at very late time points (90 minutes), but this was not consistently revealed. Altogether, these data indicate that immediate early activation events are roughly equivalent in the HY/TCR ζ transgenic thymocytes, suggesting a predominant role for the CD3 γε/δε-module.

Multiple signaling pathways, involving Ras-GRP, SLP76-SOS and Shc, contribute to ERK activation in T cells. A prolonged activation of ERK has previously been correlated with positive selection of thymocytes (125, 126). We compared the kinetics of ERK activation in the various HY/TCR ζ transgenic lines following agonist peptide stimulation to assess whether differences in ERK activation were responsible for the impaired positive selection in the HY/YF1-6 transgenic line. In all the HY/YF mice, an increase in the phosphorylation of ERK (p42) was detected within three minutes following TCR stimulation, with maximal levels of phospho-p42 detected at 30 minutes, and a return to baseline occurring by 180 minutes (Figure 20). These data indicate that

the impaired positive selection observed in the HY/YF1-6 transgenic line is not a direct result of altered activation of ERK, suggesting that phospho-ζ may contribute to positive selection through alternative pathways distinct from ZAP-70, SLP76 and ERK.

Since these studies were undertaken with whole thymocyte preparations and fixed agonist peptide concentrations, signaling distinctions may have been obscured. To address this concern, we analyzed the signaling properties of the $CD4^+CD8^+$ thymocytes using different doses of both agonist and antagonist peptides. We compared the late signaling events, including upregulation of CD69 and downregulation of TCR, following peptide stimulation in the HY/TCR ζ transgenic thymocytes using a range of agonist peptide doses. In the absence of stimulation, few (less than 10%) of the wild-type HY thymocytes express CD69 (Figure 21). At high doses of the agonist peptide (Smcy), CD69 expression is increased approximately 11-fold over control cultures without peptide (11.2 ± 5.5). A similar dose-responsive increase in CD69-expressing cells was detected in the HY/YF1,2, HY/YF5,6 and HY/YF1-6 lines (9.3 ± 2.0; 9.6 ± 2.9; 10.9 ± 0.3; Fig. 21).

These experiments were repeated with the natural positively selecting antagonist peptide Ube1x (105). In all of the HY/YF lines, the number of CD69 expressing cells increased approximately two-fold in the presence of Ube1x-pulsed APCs (Figure 22). Similar patterns of TCR-downregulation were also detected in the distinct HY/TCR ζ transgenic lines following agonist peptide stimulation (data not shown). We did not detect differences in CD5 expression, indicating that modifications in the signaling

threshold could not account for our findings (data not shown)(168, 169). Collectively, these results indicate that both early and late signaling events are roughly equivalent in the HY/TCR ζ transgenic lines bearing both TCR ζζ- and CD3 γε/δε- or just the CD3 γε/δε-module.

Proliferative Responses of Peripheral T Cells are Controlled by the CD3 γε/δε-Module

We next examined the functionality of T cells in the peripheral lymphoid organs from the HY and HY/YF mice. Small numbers of $T3.70^{+}CD8^{+}$ cells can develop without any functional ζ ITAMs in the HY/YF1-6 female mice, enabling us to directly examine the proliferative responses of these T cells independent of phospho-ζ. Similar dose dependent proliferative responses were revealed when we compared the HY, HY/YF1,2, HY/YF5,6 and HY/YF1-6 lines (Figure 23). In fact, a slightly augmented proliferation was noted in the HY/YF1-6 line (Figure 23). These data suggest that the major traditional signal transmission pathways leading to T cell effector functions are predominantly mediated by the CD3 γε/δε-module.

Discussion

The TCR complex encompasses ten ITAMs that are distributed in two distinct signaling modules (TCR ζζ and CD3 γε/δε) (Figure 24). We used a series of mice containing selected tyrosine to phenylalanine (YF) substitutions in the ζ ITAMs, resulting in a partial or complete elimination of the TCR ζζ-module, while leaving intact the CD3 γε/δε-module.

A comparative analysis of these various TCR ζ transgenic lines revealed a direct role for the TCR ζζ-module in the positive selection of HY TCR transgenic T cells. Thus, the efficiency of positive selection was directly regulated by the number of functional TCR ζ ITAMs present in the TCR complex. The HY/YF1,2 and HY/YF5,6 female mice (8/10 ITAMs) had a partial reduction in the positive selection of HY-restricted $CD8^+$ T cells relative to the wild-type HY mice, while the HY/YF1-6 line (4/10 ITAMs) had an almost complete absence of mature HY^+CD8^+ T cells. The ability of the TCR ζ ITAMs to support positive selection has been previously reported in TCR transgenic mice containing low avidity TCRs, such as the HY, DO11.10 and AND TCR transgenic lines (114, 116).

We extended these initial finding by determining that the efficiency of positive selection does not directly correlate with the patterns of phospho-ζ intermediates that are detected immediately ex-vivo. For example, the YF1,2 line maintains a high level of the

constitutively tyrosine-phosphorylated 21-kDa form of TCR ζ, while the YF5,6 line expresses only very weakly inducibly phosphorylated ζ intermediates of 19- and 20-kDa. These findings suggest that the p21 occurs as a consequence of multiple self-peptide/MHC interactions that are not necessarily involved in positive selection (43, 82). At face value, the data strongly imply that TCR ζ ITAMs are providing an enhanced signaling strength following TCR interactions with positively selecting peptide/MHC complexes.

Astonishingly, the induction of TCR-mediated signaling pathways was equivalent whether or not the TCR ζζ-module was functional. First, the induction and duration of ZAP-70, SLP-76 and ERK phosphorylation was almost identical when comparing agonist-stimulated thymocytes from the different HY and HY/YF sets of mice bearing altered numbers of functional TCR ζ ITAMs. Second, CD69 expression following TCR stimulation with either the strong agonist peptide or the natural positively selecting peptide was roughly equivalent in $CD4^+CD8^+$ T cells from all the HY/YF mice. Third, proliferative responses to different doses of agonist peptide were nearly superimposible in the various HY and HY/YF mice. These findings differ from previous reports showing that p21 can either enhance or inhibit T cell responses, respectively (140, 156, 170). Our data do concur with previous studies where signal transmission was independent of phospho-ζ, although none of these earlier studies analyzed mice where a phospho-ζ dependence for positive selection was evident (36, 61, 114-116, 165). We have also identified a ζ-independence for signal transmission in the P14 system, with equivalent dose-responsive early and late T cell activation events ((165); unpublished data). Taken

together, these data support a model where the CD3 γε/δε subunits form the predominant signaling module responsible for the activation of major TCR-induced signaling pathways involving ZAP-70, SLP76, intracellular calcium mobilization and ERK (Figure 19). The concept of two unique TCR-regulated signaling modules originated from studies showing that the TCR complex comprises two independent signaling modules involving TCR ζζ or CD3 γε/δε (61). Our experiments extend this original hypothesis by showing that the CD3 γε/δε-module predominates in this capacity. It is clear that TCR ζ is capable of facilitating normal TCR-mediated intracellular signals, as evidenced by the use of chimeric receptors containing one or more ζ-ITAMs (59). We propose that the phospho-ζ ITAMs perform a secondary role in signal transduction when examined in the context of the intact TCR complex. This may involve particular phospho-ζ intermediates, as p21 is constitutively expressed in thymocytes and peripheral T cells due to self-peptide/MHC interactions *in situ* (43, 82). Some reports have indicated that endogenous peptides/MHC interactions with the TCR can facilitate responses to an agonist peptide (170, 171). Supporting our interpretation is a recent report which identified an essential interaction between CD3 ε and the adaptor protein Nck (79). In addition, earlier studies comparing the signaling capacity of the TCR ζ subunit and the CD3 ε subunit revealed both overlapping functions and a unique signaling role of CD3 ε in the induction of particular phosphoproteins (60). Finally, the CD3 δ subunit has important, ITAM-independent roles in the activation of ERK (125, 126).

Interestingly, we have identified a role for phospho-ζ in the positive selection of T cells, suggesting that some form of signal transmission is regulated by phospho-ζ.

Although signal transmission was the same in the various HY/YF lines, the assays employed herein might not mimic the contribution of phospho-ζ during positive selection *in vivo*, which may utilize alternate, non-traditional signaling pathways involving Shc or other molecules (71). Given our clear demonstration that phospho-ζ is uncoupled from the predominant TCR signaling processes in T cells, what are the phosphorylated forms of TCR ζ involved in (Figure 24)? One possibility is that phospho-ζ may function in maintaining T cell homeostasis by regulating T cell half-life, although this remains contentious (84, 139). A second possibility is that phospho-ζ may have distinct contributions during acquired immune responses, resulting in ζ turnover during chronic infections and cancer (38, 143, 144, 172, 173).

In summary, we propose that the TCR ζζ-and CD3 γε/δε-modules contribute both redundant and non-redundant functions to T cells. Within this model, the CD3 γε/δε-module is primarily responsible for signal transmission, while both the CD3 γε/δε- and TCR ζζ-modules contribute to positive selection (Figure 24)(174). In view of the fact that the classic TCR-mediated signaling pathways functioned normally, even in the absence of all six TCR ζ ITAMs, a predominant role for the CD3 γε/δε module in signal transmission is proposed. This may be achieved through unique protein interactions with the CD3 γε/δε module. However, it is also possible that most selected TCRs depend on just two or three phosphorylated ITAMs from any of the TCR/CD3 subunits for efficient T cell activation. We are currently addressing the mechanisms contributing to the dominant signaling of the CD3 γε/δε-module. To definitively address the notion of a predominant role for the CD3 γε/δε signaling module requires an extensive analysis of

combinations of mice bearing combinations of ITAM and non-ITAM sequence mutations in the CD3 γε/δε and TCR ζζ modules. The findings from these studies may yield important insights into the evolutionary design of the TCR complex with two independent and only partially redundant signaling modules.

Table IV. Positive Selection of Thymocytes in HY/TCR ζ Transgenic Mice

	Thymocytes (x 10^7)	**% $CD4^+CD8^+$**	**% $CD4^+CD8^-$**	**% $CD4^-CD8^+$**
HY	9.3 ± 2.8	57.0 ± 5.4	7.7 ± 2.5	15.4 ± 6.3
HY/YF1,2	9.1 ± 5.3	72.0 ± 4.9	9.7 ± 1.6	8.4 ±3.2 *
HY/YF5,6	13.0 ± 5	72.0 ± 4.0	9.4 ± 2.3	7.4 ± 2.4 *
HY/YF1-6.22	9.3 ± 3.4	72.7 ± 4.9	12.1 ± 1.8	2.9 ± 1.3 *

Numbers are determined from 5 or more mice

* p values < 0.001 compared to HY female

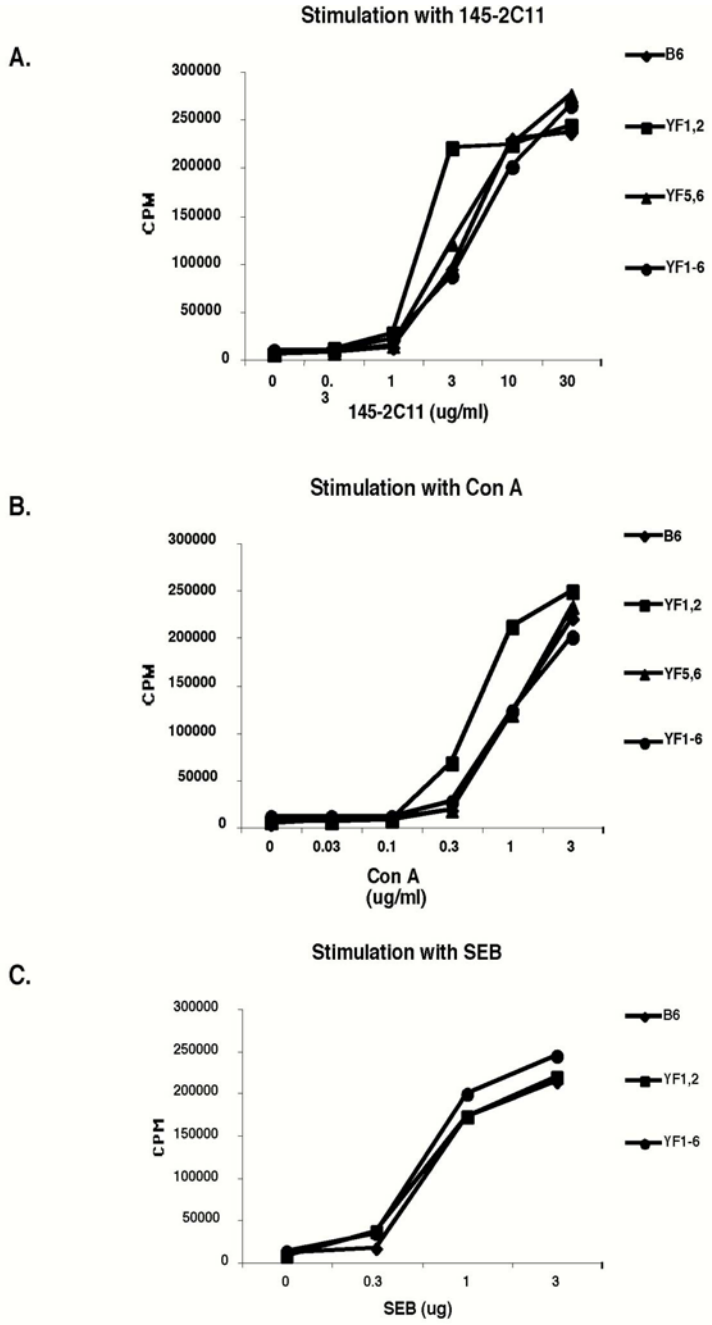


Figure 14. T Cell Proliferation is Independent of TCR ζ ITAMs. Peripheral T cells (2.5 x 10^5) from wild-type and TCR ζ transgenic mice with select tyrosine to phenylalanine substitutions in the first (YF1,2), third (YF5,6) or all three (YF1-6) TCR ζ ITAMs were incubated with increasing concentrations of anti-CD3 ε (A), Con A (B) or SEB (C) for 72 hours. Proliferation was assessed by ^{3}H-thymidine uptake.

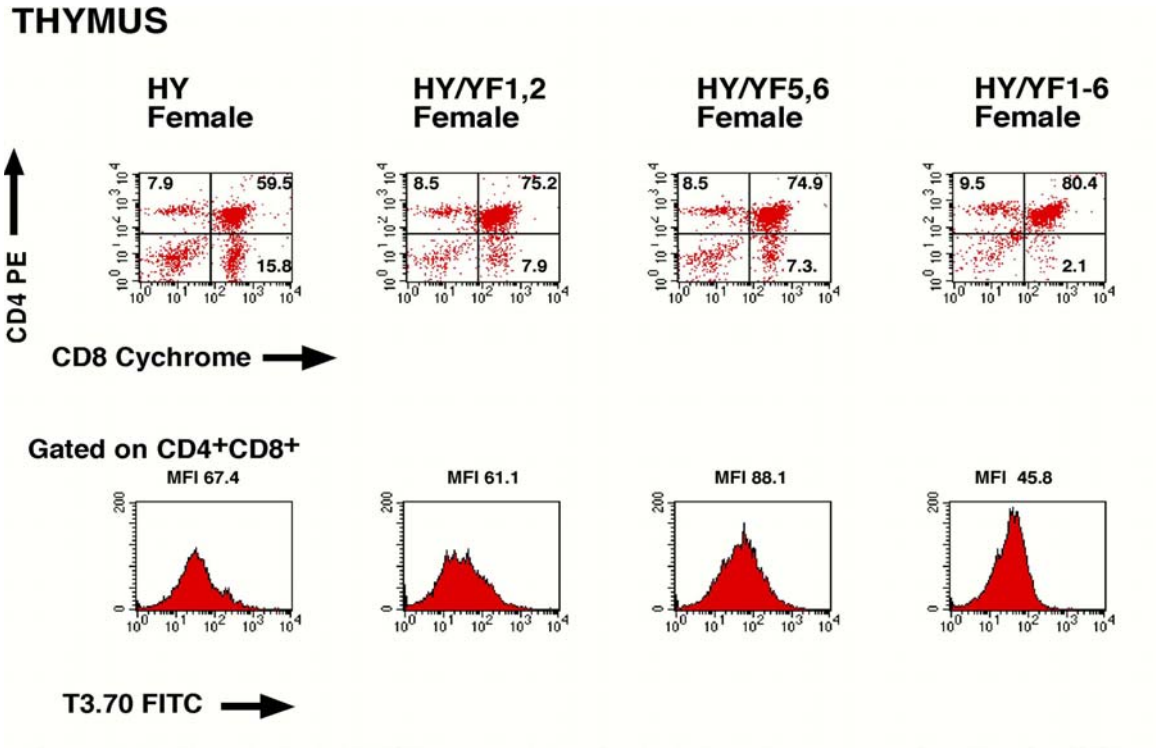


Figure 15. Positive Selection in HY/TCR ζ Transgenic Female Mice is Dependent on the Number of Functional TCR ζ ITAMs. Thymocytes were isolated from six week-old HY/YF female mice, labeled with mAb against CD4, CD8 and T3.70 and analyzed by flow cytometry.

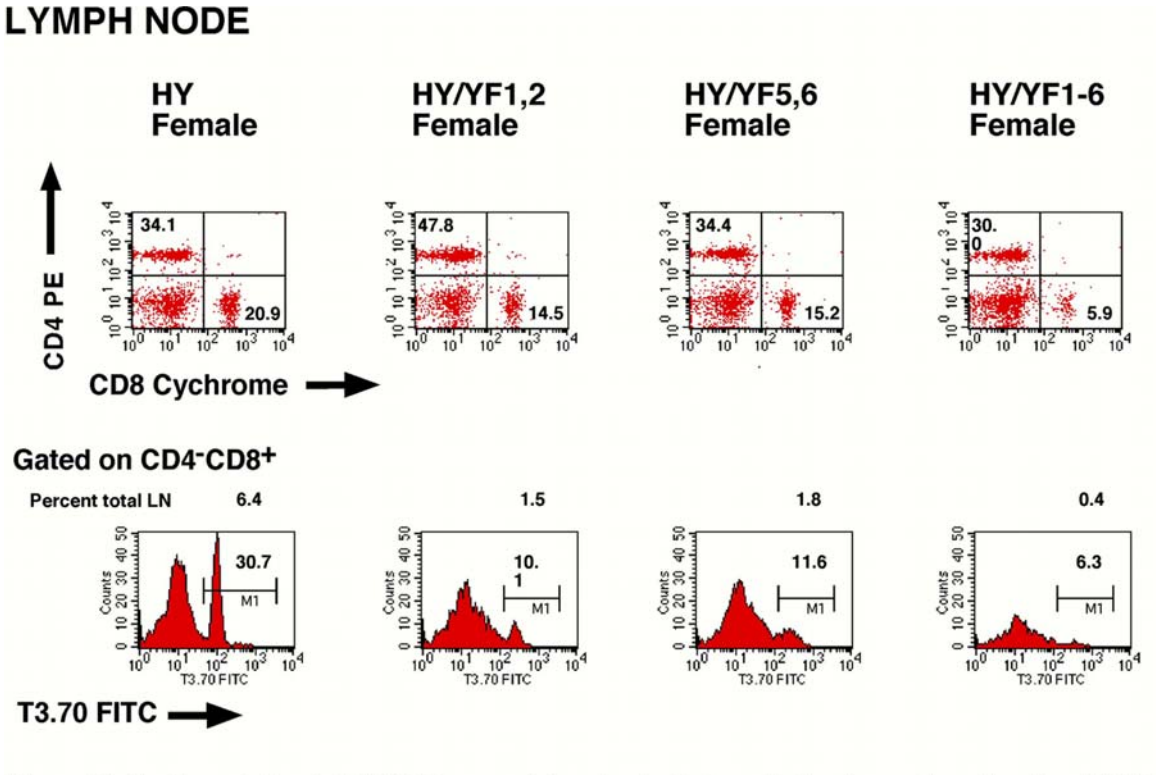


Figure 16. Positive Selection in HY/TCR ζ Transgenic Female Mice is Dependent on the Number of Functional TCR ζ ITAMs. Lymph node cells were isolated from six week-old HY/YF female mice, labeled with mAb against CD4, CD8 and T3.70 and analyzed by flow cytometry.

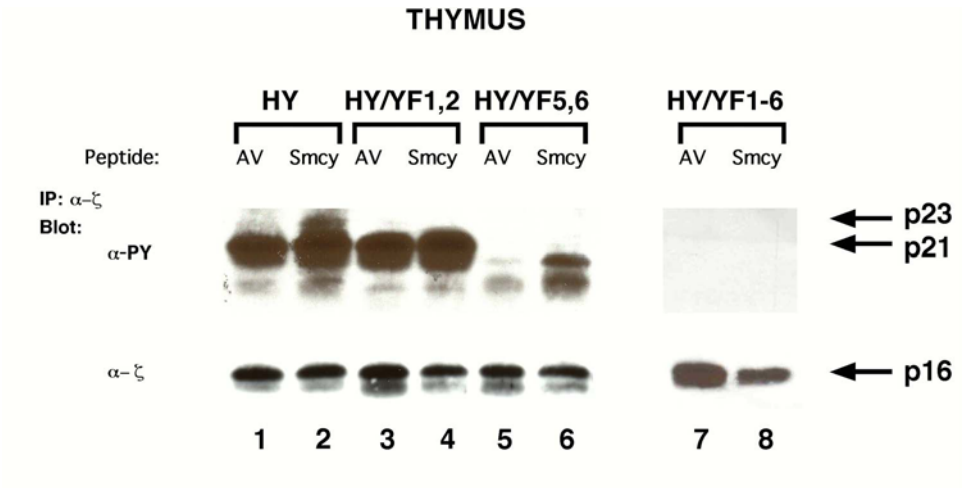


Figure 17. The Patterns of TCR ζ Phosphorylation in HY/YF Female Thymocytes. To compare the phosphorylated intermediates elicited in the various HY/TCR ζ transgenic lines following stimulation, thymocytes were stimulated for five minutes with control (AV) or agonist (Smcy) peptide at 37ºC, lysed and the TCR ζ subunit was directly immunoprecipitated. The lysates were resolved by 12.5% SDS-PAGE and immunoblotted with anti-phosphotyrosine followed by anti-ζ.

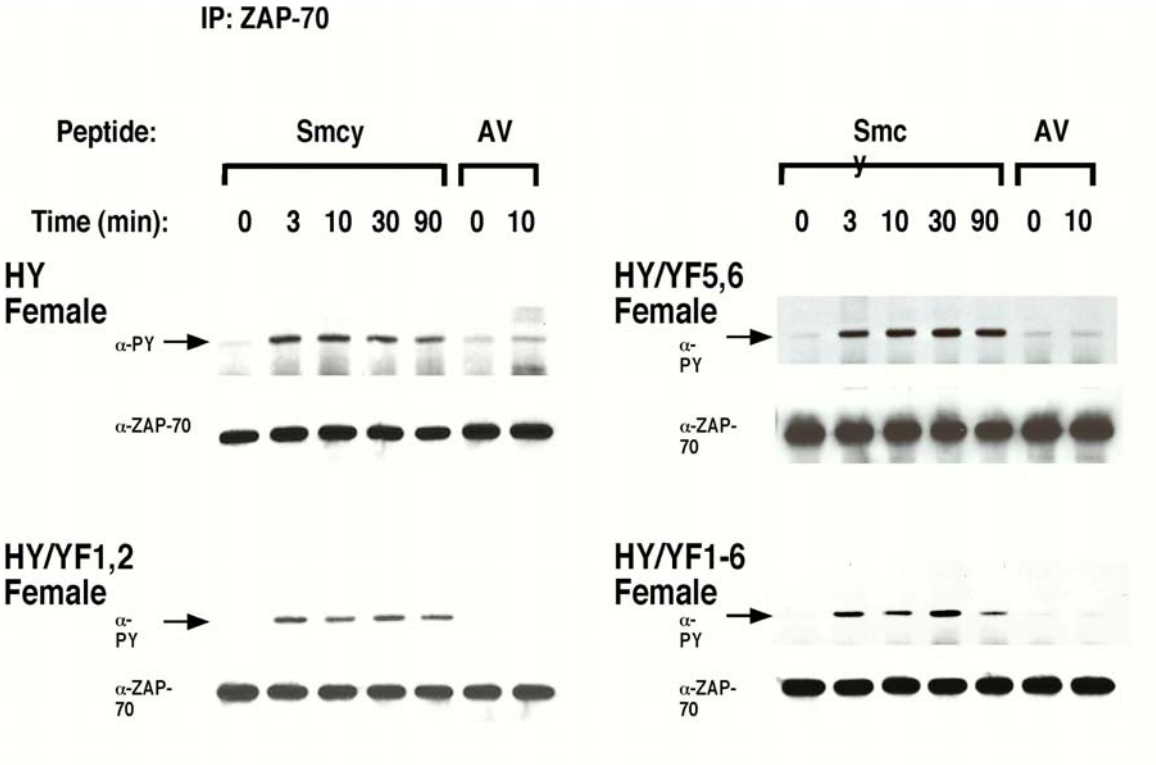


Figure 18. Protein Phosphorylation in HY/YF Thymocytes is Primarily Regulated by the CD3 γε/δε-Module. The tyrosine phosphorylation of ZAP-70 PTK in the HY/TCR ζ transgenic thymocytes bearing select tyrosine to phenylalanine substitutions in the TCR ζ subunit was compared following peptide/MHC stimulation. Thymocytes were stimulated with control or agonist peptide of 0, 3, 10, 30 or 90 minutes at 37ºC, lysed and ZAP-70 was directly immunoprecipitated and resolved by 10% SDS-PAGE. The samples were immunoblotted with mAb against anti-phosphotyrosine followed by anti-ZAP-70.

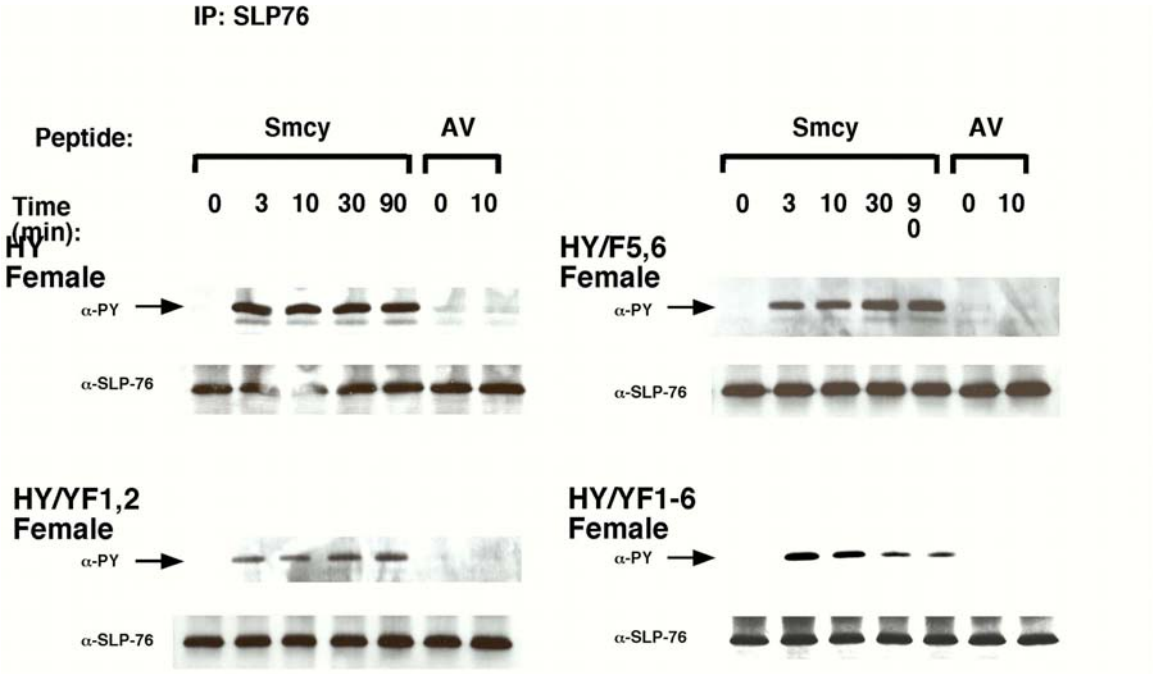


Figure 19. Protein Phosphorylation in HY/YF Thymocytes is Primarily Regulated by the CD3 γε/δε-Module. They tyrosine phosphorylation of SLP76 in the HY/TCR ζ transgenic thymocytes bearing select tyrosine to phenylalanine substitutions in the TCR ζ subunit was compared following peptide/MHC stimulation. Thymocytes were stimulated with control or agonist peptide for 0, 3, 10, 30 or 90 minutes at 37º C, lysed and SLP76 were directly immunoprecipitated and resolved on 10% SDS-PAGE. Samples were immunoblotted with anti-phosphotyrosine followed by SLP76.

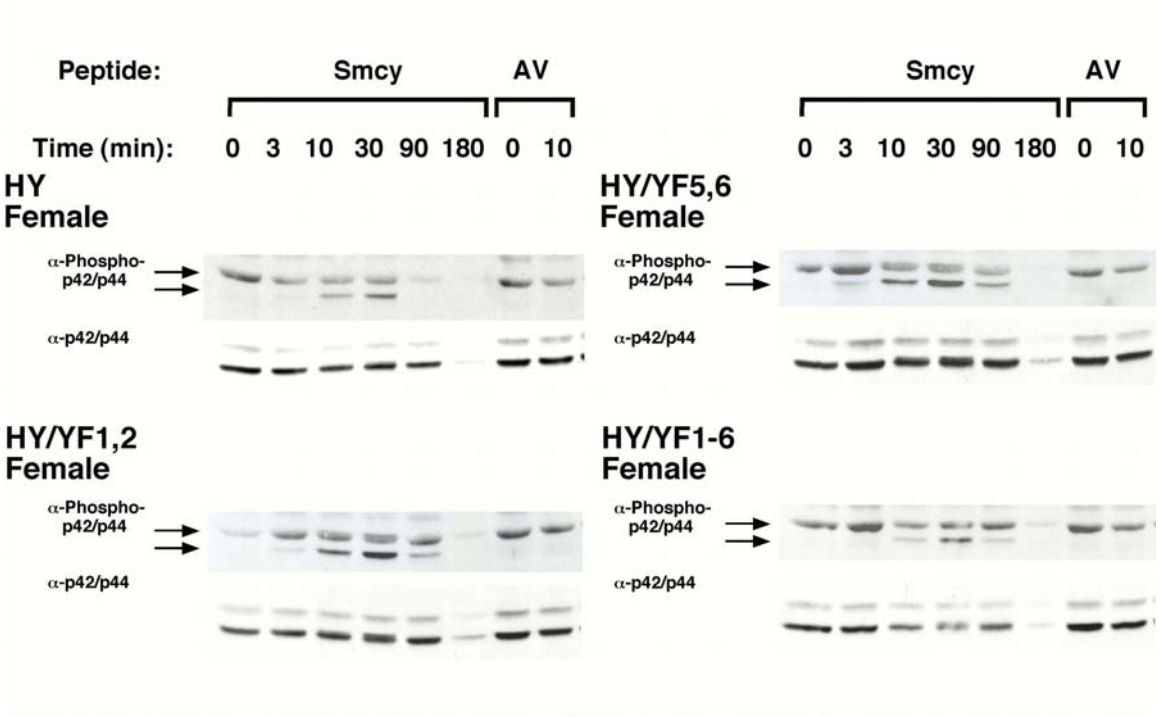


Figure 20. The Phosphorylation of ERK in HY/TCR ζ Thymocytes is Mediated by the CD3 γε/δε-Module. Thymocytes were stimulated with control or agonist peptide-pulsed, fixed APCs for 0, 3, 10, 30, 90 or 180 minutes, lysed and whole cell lysates were immunoblotted with anti-phospo-p42/p44 (ERK) followed by anti-p42/p44.

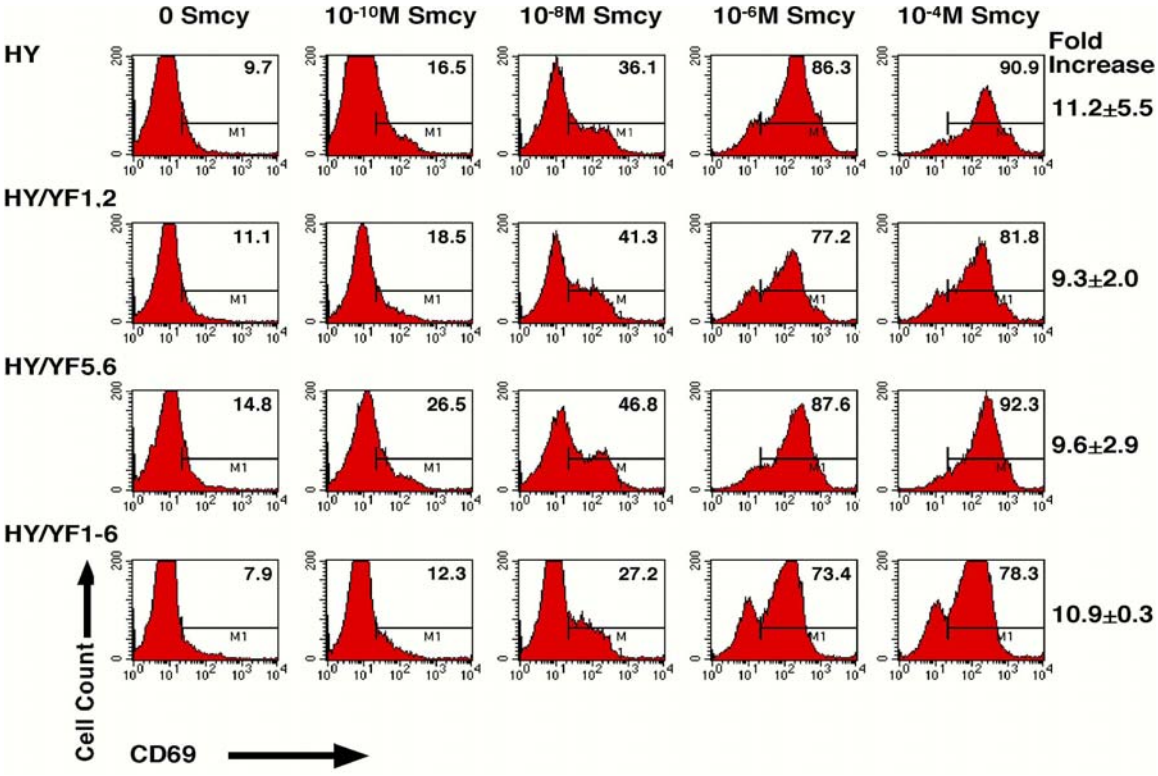


Figure 21. The Induction of CD69 by Strong Agonist Peptides is Regulated by the CD3 γε/δε Signaling Module. Thymocytes were cultured for 19 hours with agonist-pulsed antigen presenting cells and CD69 upregulation was assessed on the $CD4^+CD8^+$ subset by flow cytometry. The percent of $CD4^+CD8^+$ thymocytes expressing CD60 and the fold induction over control cultures is indicated.

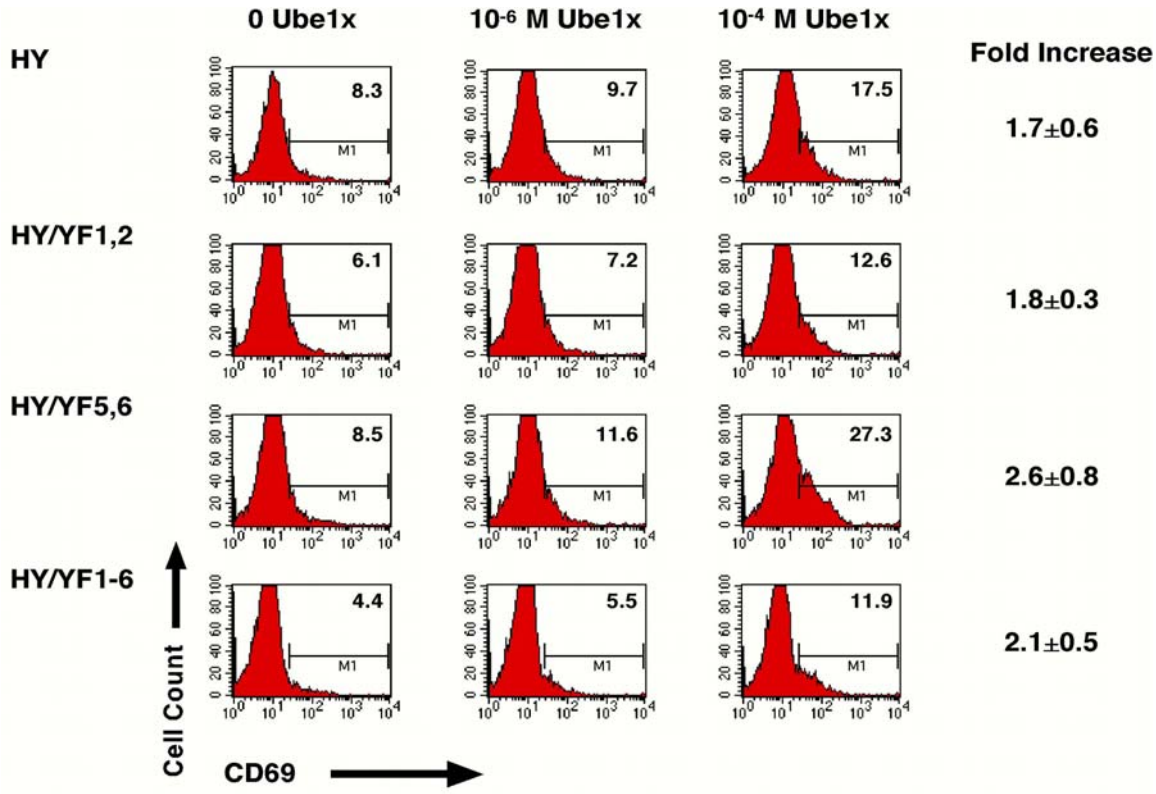


Figure 22. The Induction of CD69 by Antagonist Peptides is Regulated by the CD3 γε/δε Signaling Module. Thymocytes were cultured for 19 hours with antagonist peptide-pulsed antigen presenting cells and CD69 upregulation was assessed on the $CD4^+CD8^+$ subset by flow cytometry. The percent of $CD4^+CD8^+$ thymocytes expressing CD69 and the fold induction over control cultures is indicated.

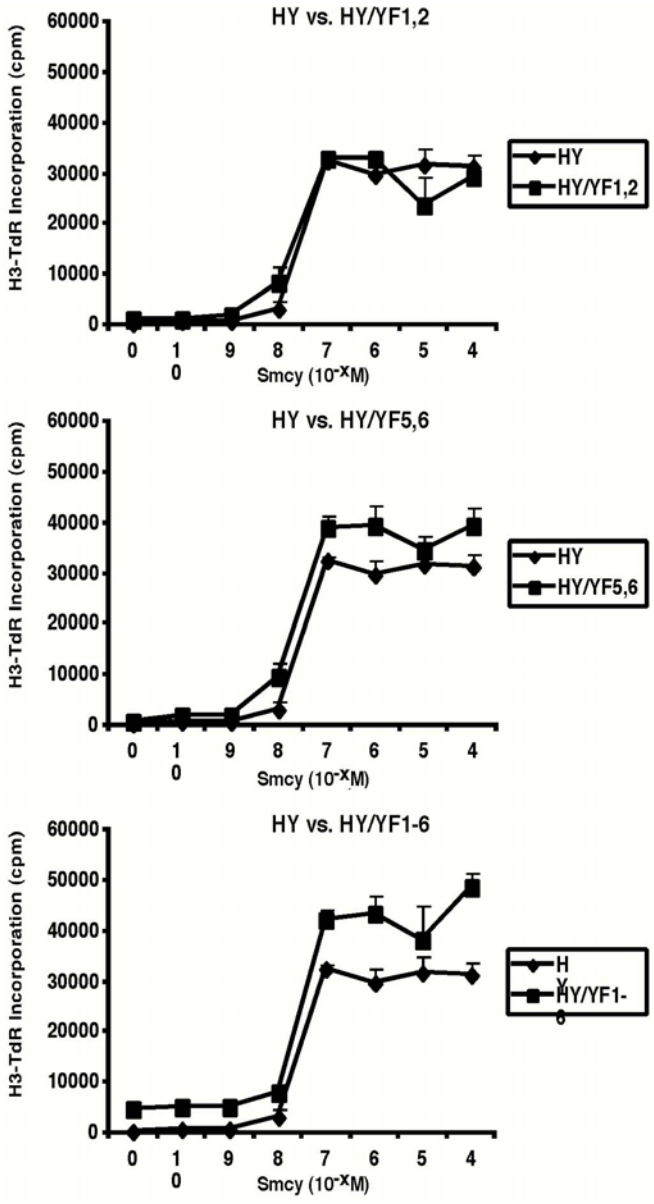


Figure 23. The Proliferative Responses of HY/YF T Cells is Mediated by the CD3 γε/δε-Module. T cells were isolated from the lymph nodes of the female HY; HY/YF1,2; HY/YF5,6; and HY/YF1-6 TCR ζ transgenic mice and were cultured for 72 hours with increasing concentrations of agonist peptide (10^{-4} M – 10^{-7} M). Proliferation was measured by the addition of ^{3}H-thymidine for the last 16 hours of culture.

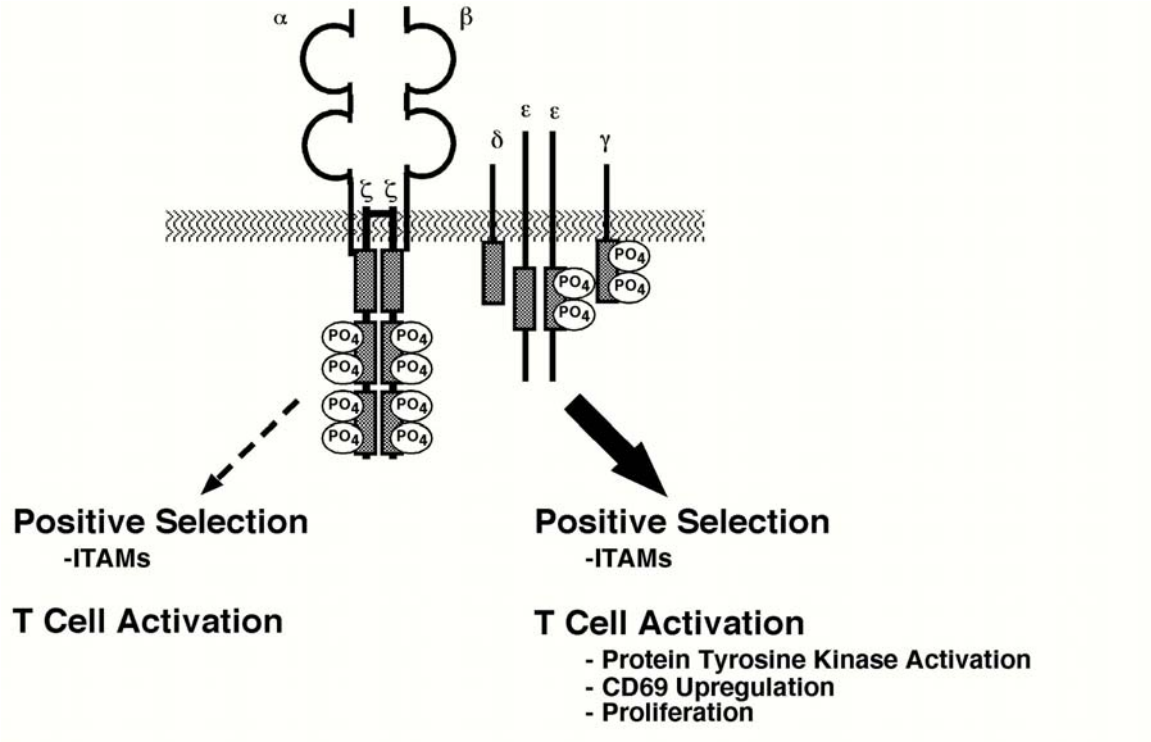


Figure 24. The TCR ζζ- and CD3 γε/δε-Modules Contribute Redundant and Non-redundant Functions to T Cells. Depicted here is a model of TCR development and signal transmission in which the CD3 γε/δε-module is primarily responsible for activation of protein tyrosine kinases, CD69 upregulation and T cell proliferation. The TCR ζζ-module functions during positive selection and may contribute a secondary role during T cell activation events.

Chapter V. Selective Expression of the 21-kDa Tyrosine Phosphorylated Form of TCR ζ Promotes the Emergence of T cells with Autoreactive Potential

Introduction

The processes of positive and negative selection are shaped by the ability of the αβ T cell receptor (TCR) to recognize self-peptide/self-MHC molecules expressed in the thymus (3). These developmental decisions are largely determined by the intracellular signals initiated by the multiple immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic tails of the TCR invariant chains (reviewed in (17, 85, 153)). The ITAMs are signaling motifs present in three copies in the TCR ζ subunit and one copy in each of the CD3 γ, δ, and ε subunits (reviewed in (14, 85, 174). Once the TCR engages a self-peptide/self-MHC complex, the ITAMs become transiently phosphorylated on two tyrosine residues. This results in the recruitment and activation of the Syk family of PTKs, with each bi-phosphorylated ITAM associating with one molecule of ZAP-70 (69, 85). The current structural model for the TCR complex provides for a total of ten ITAMs (αβ TCR with TCR ζζ and CD3 γε/δε) (14). These ten ITAMs can function in an additive capacity in during both positive and negative selection (114, 116, 175). For αβ TCR transgenic mice expressing high avidity TCRs (P14 and 2C), only a few ITAMs are required for efficient selection (reviewed in (37, 85, 165)).

Upwards of six to ten ITAMs are necessary for positive and negative selection in TCR transgenic lines with low avidity TCRs (HY, DO11.10 and AND). In fact, the loss of two ITAMs in the HY system can reduce the efficiency of both positive and negative selection (116, 175).

The 16-kDa TCR ζ subunit (p16), which contains three ITAMs per subunit, can form two predominant tyrosine phosphorylated derivatives with distinct molecular masses of 21- (p21) and 23-kDa (p23), respectively (reviewed in (37)). In thymocytes and peripheral T cells, a percentage of TCR ζ exists in a constitutively tyrosine-phosphorylated state, characterized by a specific molecular mass of 21-kDa (p21) due to the complete phosphorylation of the two membrane distal ITAMs (43, 91). P21 associates with an inactive pool of ZAP-70, and primarily results from TCR interactions with self-peptide/self-MHC complexes (82-84). The function of p21 has been somewhat of a conundrum (139, 156). First, it has been suggested that p21 serves to enhance T cell responses to foreign antigens (141, 170). Second, studies using T cell clones have indicated that p21 can actively inhibit T cell responses to agonist peptides (156). In addition, the preferential expression of p21 in response to antagonist peptides has been both linked and unconnected to the induction of anergy in T cells (99, 101, 104, 157). We have previously demonstrated that the select expression of p21, in the absence of p23, has no discernable negative impact on T cell signal transmission (165, 175). In fact, TCR-mediated signaling is completely normal in the absence of all phospho-ζ intermediates (175).

The constitutive expression of p21 results from T cell receptor interactions with peptide/MHC complexes in the thymus and periphery (36, 141, 170). High levels of p21 in the periphery have also been found in autoimmune strains of mice, suggesting a role for p21 in the generation of autoimmunity (176, 177). To more carefully assess the functions of the phosphorylated forms of TCR ζ to negative selection and the role of p21 in autoimmunity, we used a series of TCR ζ transgenic mice in which specific tyrosine residues in the TCR ζ ITAMs were substituted with phenylalanine. *In vivo*, thymocytes and peripheral T cells from these different mice selectively expressed just p21 in the absence of p23 (YF1,2 line), weakly phosphorylated intermediates of 19- and 20-kDa (YF5,6 line), or no tyrosine-phosphorylated forms of TCR ζ (YF1-6 line) (37, 91, 165). Our analyses also included a CD3ε ITAM mutant (CD3 εM), which bears tyrosine to phenylalanine substitutions in the CD3 ε ITAM while maintaining all TCR ζ ITAMs and phospho-ζ intermediates (119). Notably, the YF1,2, YF5,6, and CD3 εM mutant lines all retain eight out of ten ITAMs within the TCR complex (8/10). These lines were mated to the HY TCR transgenic mice that express a αβ TCR specific for the male HY peptide, Smcy. The male HY TCR mice are routinely used as a model system for studying negative selection (119, 166).

In this report, we provide direct evidence that the selective expression of p21, in the absence of p23, modifies negative selection in the HY/YF1,2 male mice, facilitating the development of potentially autoreactive T cells. Thus, the HY/YF1,2 male mice maintain expression of T cells bearing the autoreactive TCR with increased levels of the CD8 coreceptor and contain an expanded population of $CD11b^+$ $B220^+$ B cells in the

spleen, a phenotype very distinct from that characterized in the HY/YF5,6, HY/CD3 εM T cells and HY/YF1-6 lines. These results provide the first direct demonstration of important functional distinctions between different ITAMs in the TCR ζ subunit during T cell development and reveal a link between p21 and autoreactive potential.

Results

The 21-kDa Tyrosine Phosphorylated Form of TCRζ Selectively Attenuates Negative Selection in Male HY TCR Transgenic Mice.

The HY TCR transgenic male mice are routinely used as a model for studying negative selection, since the agonist peptide, Smcy, for the HY TCR is expressed in the thymus (166). Previous studies with this model system have shown that the efficiency of negative selection is directly correlated with the number of ITAMs present in the TCR complex (114, 116). Since these studies were undertaken with particular TCR ζ truncations preventing the formation p21 and/or p23, we wanted to carefully examine how p21 and other phospho-ζ intermediates contributed to negative selection (156). For this purpose, we bred the HY TCR transgenic mice to distinct TCR ζ transgenic lines that selectively expressed only the constitutively phosphorylated 21-kDa form of TCR ζ (p21; YF1,2), two weak inducibly phosphorylated forms of TCR ζ (p19/p20; YF5,6), or no tyrosine-phosphorylated forms of TCR ζ (YF1-6)(37, 91, 165). Notably, the YF1,2 and YF5,6 lines contain an equivalent number of ITAMs (8/10) in the TCR, while expressing distinct phosphorylated forms of ζ. An independent set of mice with specific mutations in the CD3 ε ITAMs (CD3 εM), which contain 8/10 ITAMs and express both p21 and p23, were also included in these analyses (119).

In wild-type HY TCR transgenic male mice, the majority of thymocytes are deleted, resulting in a residual population of $CD4^-CD8^-$ thymocytes that represent over 75% of total thymocytes (Figure 1A). Notably, there is an almost complete absence of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells, consistent with all previous publications (166, 167, 178). In contrast, the introduction of the YF1,2 ζ transgene into HY male mice resulted in less efficient negative selection, with a significant increase in the percent of $CD4^+CD8^+$ thymocytes (HY/YF1,2; Figure 25, Table V). Small numbers of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells were also observed in the thymus. Interestingly, there was a decrease in the TCR density of the HY/YF1,2 thymocytes compared to HY male mice. When the $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocyte populations were analyzed separately, it was determined that the TCR density in the $CD4^-CD8^-$ population was substantially decreased, while normal levels of TCR were detected in the $CD4^+CD8^+$ T cells. This contrasts the HY male mice, which express high levels of the male-specific TCR in the $CD4^-CD8^-$ population. Thus, the select expression of p21 in the HY/YF1,2 males results in a distinct down modulation in TCR expression in the $CD4^-CD8^-$ thymocytes and reduces negative selection. Since the YF1,2 line has only 8/10 ITAMs, the reduced efficiency of negative selection may have been caused by the loss of ITAMs. We analyzed next the HY/YF5,6 line, which maintains an equivalent number of ITAMs (8/10) but expresses distinct phospho-ζ intermediates of p19 and p20, forms not normally seen in wild type mice. There was also a significant increase in the number of $CD4^+CD8^+$ thymocytes relative to HY male mice (Figure 25, Table V). Despite the increase in DP thymocytes, the number of $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells expressing normal coreceptor levels in the HY/YF5,6 male mice was also extremely low. In

addition, the thymocytes from the HY/YF5,6 line did not exhibit the reduction in TCR density noted in the HY/YF1,2 line in the $CD4^-CD8^-$ population. Taken together, these results strongly support the contention that expression of p21, in the absence of p23, attenuates negative selection in a distinct manner. As an extension of these findings we also analyzed the HY/YF1-6 male mice, which lack all phosphorylated TCR ζ intermediates. In these male mice, the reduced number of ITAMs dramatically reduces the efficiency of negative selection, as indicated by the large percentage of $CD4^+CD8^+$ thymocytes and high expression levels of the HY-specific TCR (Figure 25). These results are consistent with early studies with ζ-0 ITAM male mice (Figure 25, Table V) (116). The HY/$\zeta^{-/-}$ thymocytes were included as controls revealing that the absence of ζ expression results in the appearance of a high percentage of $CD4^+CD8^+$ thymocytes that lack TCR expression. An analysis of the HY/CD3 ε ITAM mutant mice (CD3 εM) was included, since they contain an equivalent number of ITAMs as the HY/YF1,2 and HY/YF5,6 lines (8/10) but can express both p21 and p23. Notably, these mice exhibit only a slight increase in the number of $CD4^+CD8^+$ thymocytes (21.9%) and maintained high expression levels of the HY TCR (Figure 28). Overall, these results suggest that a reduction in the number of TCR ζ ITAMs reduces the efficiency of negative selection in the thymus. In addition, the select expression of p21, in the absence of p23, delays the expression of the HY-specific TCR in a manner distinct from that observed in the HY, HY/YF5,6 and HY/YF1-6 male mice.

To assess whether the impaired negative selection specifically in the HY/YF1,2 male mice resulted in alterations in TCR signal strength, both the $CD4^-CD8^-$ and

$CD4^+CD8^+$ thymocytes were compared for the expression of CD5 and CD69 on the cell surface. Similar levels of CD5 were expressed in the HY, HY/YF1,2 and HY/YF5,6 male mice in both the $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocyte populations (data not shown). In contrast, thymocytes from the HY/YF1-6 male mice expressed diminished levels of CD5 on the cell surface, likely as a compensatory mechanism for the decreased signal strength due to the loss of all six TCR ζ ITAMs (data not shown). When the expression patterns of the early T cell activation marker CD69 were compared, the HY and HY/YF1,2 male mice contained similar percentages of CD69+ cells in the thymus (data not shown). The HY/YF5,6 male mice had slightly elevated percentages of CD69+ cells in the CD4+CD8+ populations, while the HY/YF1-6 males had slightly decreased percentages of CD69+ cells in the thymus (data not shown). These data indicate that the distinctive impairment of negative selection in the HY/YF1,2 line does not result in a change of TCR signal strength by altered patterns of CD5 and CD69 expression.

To determine how the attenuated negative selection processes in the various HY/YF mice affected peripheral T cell development, we analyzed lymph node T cells. In HY male mice, an unusual lineage of $CD4^-CD8^-$ and $CD4^-CD8^{lo/-}$ T cells that express the autoreactive TCR emerge in the periphery (Figure 26)(data not shown)(178). The down modulation of CD8 on the cell surface renders them unresponsive to antigenic stimulation (167)(Figure 26 and 27). These cells represent a unique population of cells, exhibiting a memory-like phenotype with more innate cell functions (179, 180). In contrast to the HY male mice, the HY/YF1,2 male line contained an increased percentage of mature $CD4^+CD8^-$ (15.0%) (Figure 26 and 27)(Table VI). Unexpectedly, the $CD4^-CD8^+$ T cells

that emerged in the HY/YF1,2 line maintained expression of the autoreactive TCR, albeit at reduced levels, and contained an intermediate level of CD8 coreceptor ($CD8^{int}$) when compared to HY male ($CD8^{lo}$) and female mice ($CD8^{hi}$). The levels are similar to the HY-specific cells that develop in a partially selecting background (179). The HY/YF5,6 male mice also contained normal percentages of $CD4^-CD8^+$ cells and an increased percentage of $CD4^+CD8^-$ (7.9%) T cells, similar to the percentages in the HY/YF1,2 line (Figure 26)(Table VI). However, in contrast to the HY/YF1,2 $CD8^+$ T cells, these cells expressed a reduced level of CD8 ($CD8^{lo}$), comparable to the wild-type HY males (Figure 26 and 27). These results reveal a very critical distinction in the ability of p21 (in the absence of p23) to facilitate the emergence of T cells that are potentially autoreactive. When the lymph nodes of the HY/YF1-6 male mice were examined, there was an increase in the percentage of $CD4^+CD8^-$ T cells (14.8%) and a reduction in the $CD4^-CD8^+$ T cells (8.1%) compared to the HY and other HY/YF lines (Figure 26). The $CD4^+CD8^-$ T cells appear to arise from endogenous TCR α gene rearrangements. These data are entirely consistent with previous results showing an additive effect for ITAM numbers in the efficiency of negative selection (116). Our data, however, reveal a critical and unique functional role for p21. This notion was further addressed by analyzing the HY/CD3 εM male mice. We found similar numbers of $CD4^-CD8^+$ (33.0%) and $CD4^+CD8^-$ (8.5%) T cells as the HY and HY/YF males (Figure 28). Yet, the $CD8^+$ T cells in these mice were $CD8^{lo}T3.70^+$, akin to those in the HY and HY/YF5,6 male mice but distinct from those in the HY/YF1,2 set (Figure 28). Of particular interest with the HY/YF1,2 line is the slight downregulation of the transgenic TCR, suggesting that ongoing receptor/self-ligand interaction may have been occurring (Figure 27). Taken

together, these data clearly demonstrate a distinct functional role for p21 in regulating negative selection.

Antigen Reactivity of HY/TCR ζ Transgenic Male Thymocytes

We next compared the T cell responses to cognate male antigen in the different HY and HY/YF lines. We initially analyzed the capacity of these cells to upregulate CD69, an early marker of T cell activation. Whole thymocyte preparations from male HY or male HY/YF series mice were cultured for 19 hours with APCs presenting either no peptide or increasing concentrations of the agonist peptide Smcy and subsequently analyzed by flow cytometry. HY-specific T cells from female mice were included for comparative purposes. The inducible expression of CD69 was represented as fold-increase in the percentage of the cells expressing CD69 following incubation with high agonist peptide concentrations (Smcy) compared to those incubated with no peptide. In the wild-type HY female thymocytes, the percent of cells expressing CD69 is increased eleven-fold (10.77 ± 5.7) following stimulation with agonist peptide (Figure 29)(175). In contrast, there was only a three-fold increase in percent of $CD69^{+}$ cells in the HY male thymocytes (3.0 ± 1.0) (Figure 29). In the HY/YF1,2 male thymocytes, the frequency of cells that upregulate CD69 was increased 5-fold (5.2 ± 2.8) (Figure 29). Analysis of the HY/YF5,6 and HY/YF1-6 male thymocytes following agonist peptide stimulation revealed 10-fold (9.97 ± 4.3) and 15-fold (14.9 ± 7.9) increases in the percent of cells expressing CD69, respectively (Figure 29). Although these differences were interesting, they were not statistically significant and there were no obvious differences in the

capacity of the $CD4^-CD8^-$ and $CD4^+CD8^+$ cells to respond (data not shown). It was also noted that the degree of CD4 and CD8 coreceptor downmodulation in response to agonist peptide stimulation was comparable in the various HY and HY/YF lines (data not shown). It is interesting to note from these results that the elevated expression of CD8 in the HY/YF1,2 thymocytes did not correlate with enhanced signaling capacity.

Proliferative Responses of the HY TCR ζ Transgenics

We next evaluated the proliferative capacity of the HY and HY/TCR ζ transgenic male thymocytes and lymph node T cells in response to Smcy peptide-pulsed antigen presenting cells. The wild-type HY male thymocytes exhibited a high basal rate of proliferation and responded only marginally, with approximately a 1.5 to 2-fold increase in proliferation with increasing Smcy concentrations (Figure 30 A). When comparing the HY/YF1,2 and HY/YF5,6 lines, a similar 3-5-fold increase in dose-responsive proliferation to the agonist peptide was detected (Figure 30 A). The HY/YF1-6 line responded with a 5-6-fold increase in proliferation at high doses of agonist peptide (Figure 30 A).

The proliferative responses of the mature $T3.70^+CD8^+$ cells isolated from the lymph nodes were then compared in the various HY/YF male mice and to HY female and male mice. In the absence of exogenous IL-2, the HY male cells were significantly

impaired in their ability to proliferate to male peptide when compared to HY female cells (data not shown). Only in the presence of IL-2 did the HY male cells proliferate to high doses of agonist peptide (Figure 30 B). When the peripheral T cells from the HY/YF1,2, HY/YF5,6 and HY/YF1-6 male mice were examined, nearly identical dose-response curves were observed (Figure 30 B). Notably, all of the HY and HY/YF lines appear to be less responsive to low agonist peptide doses when compared to HY females (Figure 30). We also compared the proliferative responses to the T cell mitogen, Con A. All of the HY and HY/YF male T cells isolated from the lymph nodes proliferated equally to mitogenic stimulation (data not shown). These data indicate that despite distinct phenotypic differences in the T cells from the HY/YF males, these cells all retain a similar capacity to respond to their cognate male peptide, Smcy, in *in vitro* assays. This is consistent with our previous findings in the HY/YF female lines (175).

Autoimmune Phenotype in Aged Mice

Previous studies have drawn correlations between enhanced expression of p21 and autoimmune disease (176, 177). To ascertain whether autoimmunity is induced by the selective expression of p21 in the HY/YF1,2 line, whereby less efficient negative selection occurs enabling the generation of a population of potentially autoreactive cells, we analyzed HY versus HY/TCR ζ transgenic male mice that had been aged 8-12 months. The various HY and HY/TCR ζ transgenic mice were compared for signs of autoimmunity, including changes in T and B cell populations, activation markers and the generation of autoantibodies. In the spleens of the HY/YF1,2 males, an increased

percentage of $CD11b^{+}B220^{+}$ cells were noted compared to control HY males and the HY/YF5,6 and HY/YF1-6 lines ($p<0.01$)(Figure 31 A). These cells were analyzed further and it was determined that the $CD11b^{+}B220^{+}$ cells expressed CD19, indicating that they represent a subset of B cells (data not shown). When we assayed the sera for the presence of antibodies against total histone/dsDNA as an indication of autoimmunity, in the various HY and HY/YF male mice, the HY/YF1,2 male mice exhibited only a slight increase in the presence of autoantibodies compared to the HY, HY/YF5,6 and HY/YF1-6 male mice (Figure 31 B). The levels of autoantibody present in the HY/YF1,2 males were below the threshold considered to be autoimmune (>200 AU), which is represented by the B6.*Sle1b* mice (181).

Although the HY/YF1,2 male mice did not exhibit severe autoimmunity by 8-12 months of age, these mice did contain increased T cell activation markers and autoantibody production relative to the HY, HY/YF5,6 and HY/YF1-6 male mice. Slight variations in the activation state of the T cells were noted, particularly the aged HY/YF1,2 male mice, which exhibited a significant increase in the percentage of $CD4^{+}$ T cells that express the early activation marker, CD69 (Figure 32 A). In addition, the expression of $CD25^{+}CD4^{+}$ T cells was significantly reduced in the HY/YF1,2 line when compared to the HY males (Figure 32 A). Surprisingly, there were no significant differences in the activation state of the $CD8^{+}$ T cells in the spleens of the aged HY/YF1,2 mice relative to the wild-type HY male mice (Figure 32 B). When the HY-specific $CD8^{+}$ T cells ($T3.70^{+}CD8^{+}$) were compared directly, there were no distinct differences in the expression patterns of CD44 and CD62L in the HY or HY/YF male

mice (data not shown). This data implies that despite no significant differences in the activation state of the $T3.70^{+}CD8^{+}$ T cells in the HY/YF1,2 male mice, other lymphocyte populations in the spleen are altered when compared to HY, HY/YF5,6 and HY/YF1-6 male mice. When the $T3.70^{-}CD8^{+}$ T cells were analyzed, the HY and HY/YF5,6 had diminished expression of both CD62L and CD44, while the HY/YF1,2 and HY/YF1-6 T cells maintained $CD62L^{hi}$ $CD44^{int}$ at levels comparable to that observed in the $T3.70^{+}CD8^{+}$ T cell population (data not shown). The increase in activated $CD4^{+}$ T cells and $CD11b^{+}B220^{+}$ B cells in the HY/YF1,2 male mice may be indicative of a higher potential for autoimmune disease that may require a triggering event such as infection.

Discussion

We have examined the contributions of different ITAMs (TCR ζ and CD3 ε) in the process of negative selection in the thymus. In particular, we characterized the role of the constitutively tyrosine-phosphorylated TCR ζ subunit (p21), as its expression has been linked to autoimmune progression. We demonstrate herein that the expression of p21, in the absence of p23 (HY/YF1,2 line), promotes the development of potentially autoreactive T cells in the HY TCR transgenic male mice. Two unique features of the HY/YF1,2 line include the emergence of a population of peripheral T cells expressing the autoreactive TCR and intermediate levels of CD8 on the cell surface, increased percentages of activated $CD4^+$ T cells and the expansion of B1 B cells in the spleen. In contrast, the HY/YF5,6 and HY/CD3 εM, both of which contain the same number of TCR ITAMs as the HY/YF1,2 line, had mature $T3.70^+CD8^{lo}$ T cells that resembled those from the HY male.

Most studies to date have revealed an additive effect for the ITAMs during thymopoeisis, wherein a reduction in the number of TCR ITAMs reduced the efficiency of positive and negative selection (114, 116). This was consistently observed in transgenic lines bearing low avidity TCRs. However, no experiments were undertaken to address the functions of p21. Our previous studies, in which we examined the contributions of phospho-ζ to T cell positive selection, are consistent with the notion that

the TCR ζ and CD3 ITAMs function additively in that sets of mice lacking 2 out of 10 ITAMs in the TCR complex (YF1,2; YF5,6) had similar reductions in the efficiency of positive selection. Somewhat surprisingly, the constitutive expression of p21 (YF1,2) offered no obvious selection advantage to T cells (175). Our current studies indicate that during negative selection, the ITAMs can also function in an additive manner, as the HY/YF1,2, HY/YF5,6 and HY/CD3 εM lines (all containing 8/10 TCR ITAMs) all contain increased percentages of $CD4^+CD8^+$ thymocytes. In fact, the inefficient negative selection in the HY/YF1-6 male mice seems to facilitate gene rearrangements of endogenous TCR α chains, as evidenced with the emergence of "normal" peripheral $CD4^+CD8^-$ T cells. However, the results described herein indicate a unique function for p21 in negative selection, resulting in the emergence of a $CD8^{int}T3.70^+$ population of T cells not previously identified in HY male mice or in the HY/YF5,6 or HY/YF1-6 male mice. This is the first demonstration that the different phosphorylated ITAMs can contribute differentially during negative selection.

How does the expression of p21, in the absence of p23, alter negative selection? One possibility is that the selective expression of p21 alters the functional capacity or spatial organization of signaling molecules required for negative selection. It has been established that p21 associates with an inactive pool of ZAP-70 (82). During negative selection events, the localization of signaling molecules, including Lck and TCR ζ, are distinct from that seen during positive selection or even mature T cell activation (182). Specifically, Lck is localized to the center of the immunological synapse while TCR ζ appears in a peripheral ring. Given the association of ZAP-70 with p21, ZAP-70 would

also be localized at the periphery. If p21 sequesters ZAP-70 in the peripheral ring of the synapse, the activation of ZAP-70 by Lck might be ineffective in p21 expressing thymocytes. In the wild type HY or the HY/CD3 εM male mice, TCR mediated induction of p23, the fully phosphorylated form of TCR ζ, could override this block. P23 might efficiently recruit new molecules of ZAP-70, allowing for their proper localization and activation. Alternatively, the p21/ZAP-70 complex may include attenuators of signal transduction such as cbl-b and/or Sts proteins, both of which can bind ZAP-70 (183, 184). In the wild-type HY and HY/CD3εM males, the induced expression of p23 could displace these signal inhibitors. In the absence of p23 in the HY/YF1,2 line, the failure to recruit sufficient forms of activated forms of ZAP-70 might prevent efficient removal of these signal attenuators. When examined during positive selection events in the female HY/YF mice, the expression of p21, in the absence of p23, had no discernable effects on ZAP-70 activation or positive selection (175). This could result from a different spatial organization of the signaling molecules that occurs during positive relative to negative selection events (182). In the absence of both p21 and p23 in the HY/YF5,6 and HY/YF1-6 lines, negative selection could be restored by activation signals mediated via the CD3 γε/δε signaling module. Notably, however, these lines exhibit a delay in negative selection, facilitating the development of $CD4^+CD8^+$ cells.

It has also been reported that mutations in key signaling molecules, including ZAP-70, can diminish the intensity of proximal TCR signaling events, leading to impaired thymic selection and the emergence of spontaneous autoimmunity (49). Thus, the sequestration of ZAP-70 by p21 in the HY/YF1,2 line could function in a similar

manner, diminishing the intensity of signals *in situ* that are required for negative selection. Interestingly, in our *in vitro* assays, the signaling capacity of the thymocytes and peripheral T cells from the HY and HY/YF lines are equivalent. Further experiments are being undertaken to explore these possibilities, but suggest that the CD3 γε/δε subunits form the predominant signaling module in *in vitro* assays (175).

Previous reports have identified a distinct phenotype for the cells that escape negative selection in the HY system, resulting in a change from CD8 αβ to CD8 αα cells, which more closely resemble the IELs found in the gut (180). These cells also have acquired characteristics of innate immune cells through the expression of certain activating NK receptors (NK1.1, CD94 and NKG2D) and NK cell specific signaling molecules (DAP12) (179, 180). In contrast to these studies, we did not detect innate cell markers, enhanced production of IFN γ or proliferation to IL-2 and IL-15 in the various HY/YF lines, even when one or more phosphorylated TCR ζ intermediates were eliminated (data not shown). Furthermore, when the activation state of the $T3.70^{+}CD8^{+}$ cells was analyzed in the HY/YF1,2 male mice, the expression patterns of CD25, CD69 and CD44 were similar to that observed in the HY male mice (data not shown). An analysis of the CD8αα IELs in the gut revealed similar percentages of these cells in the HY/YF1,2 line as observed in the wild-type HY males, indicating that the impaired negative selection in the HY/YF1,2 males does not result in an increase in CD8 αα IELs (data not shown). Although there were no gross autoimmune phenotypes in these mice, these HY/YF1,2 cells may still be capable of generating autoimmunity following some initial triggering event, such as infection.

When the HY/YF male mice were aged, we observed slightly elevated levels of autoantibodies and increased numbers of $CD11b^{+}B220^{+}$ B cells only in the HY/YF1,2 line. These markers are present on B1 B cells, which are generally involved in natural antibody production and function in an innate capacity (185). This increase in this subset of B cells may be indicative of a potentially autoimmune phenotype, as an increase in B1 B cells has been observed in murine models of SLE (186) . The increased presence of $CD11b^{+}B220^{+}$ B cells may be an indirect result of the select expression of p21, in the absence of p23, which may change the cytokine milieu to support the expansion of these cell types. In addition, during the aging process required for these studies, the death of two HY/YF1,2 male mice was noted. Without pathological evidence, it can only be speculated that the specific increase in mortality in the HY/YF1,2 was a result of autoimmunity. It is also unclear whether the HY-specific T cells are contributing to the pathogenesis, or whether a combination of HY-specific and endogenous TCR α-expressing T cells are necessary for disease progression.

In summary, the TCR ζ ITAMs contribute both additive and distinct functions during thymocyte negative selection, with the select expression of p21 attenuating negative selection. This is the first demonstration that the different phosphorylated ITAMs have distinct functions, and are simply not involved in an additive capacity. Current efforts are addressing the unique function of p21 during negative selection events.

Table V. *T Cell Development in the Thymus of HY/ TCR ζ Transgenic Male Mice*

Thymus	**Total Cell Number ($x10^{-6}$)**	**% $CD4^-CD8^-$ (Number x 10^{-6})**	**% $CD4^+CD8^+$ (Number x 10^{-6})**	**% $CD8^+CD4^-$ (Number x 10^{-6})**	**% $CD4^+CD8^-$ (Number x 10^{-6})**
HY Female	96 ± 28	21.5 ± 6.3 (14.2 ± 8.7)	57.0 ± 5.4 (54.6 ± 18.0)	15.4 ± 6.3 (16.8 ± 7.7)	7.7 ± 2.5 (7.3 ± 3.0)
HY[a] Male	18.9 ± 7.7	79.89 ± 6.1 (15.1 ± 6.4)	3.92 ± 2.2 (0.78 ± 0.7)	5.98 ± 2.1 (1.2 ± 1.1)	10.19 ± 5.9 (1.8 ± 1.2)
HY/YF1,2	18.5 ± 6.7	43.17 ± 13.7 (8.2 ± 1.5)	36.21 ± 16.0 (6.2 ± 3.7)	10.5 ± 4.0 (2.1 ± 0.98)	10.13 ± 2.9 (2.0 ± 1.0)
HY/YF5,6	12.5 ± 6.7	57.5 ± 10.9 (7.1 ± 2.8)	15.2 ± 7.7 (1.9 ± 1.6)	10.03 ± 2.8 (1.3 ± 0.8)	17.22 ± 2.9 (2.2 ± 1.8)
HY/YF1-6	15.8 ± 3.4	29.9 ± 7.1 (4.7 ± 2.7)	50.0 ± 10.9 (7.8 ± 1.8)	10.37 ± 3.5 (1.7 ± 0.85)	9.74 ± 3.3 (1.6 ± 0.88)
HY/CD3εM[b]	14.0	58.5 (8.19)	21.9 (3.07)	8.2 (1.15)	11.5 (1.61)

[a]Statistical values for the percentage and number of thymocytes in the HY and HY/YF male mice were determined from n=6.

[b]Two HY/CD3εM male mice were analyzed and exhibited similar numbers and percentages of cells.

Table VI. *T Cell Development in the Lymph Nodes of HY/TCR ζ Transgenic Male Mice*

Lymph Node	**Total Cell Number ($x10^{-6}$)**	**% $CD8^+CD4^-$**	**% $CD4^+CD8^-$**	**% $CD8^+T3.70^+$**
HY Female	16 ± 7.1	17.7 ± 3.0	27.9 ± 3.6	5.6 ± 1.8
HY[a] Male	24.0 ± 9.7	18.7 ± 3.9	5.6 ± 1.8	22.67 ± 7.9
HY/YF1,2	16.3 ± 4.9	19.6 ± 6.3	12.55 ± 4.2	20.71 ± 4.7
HY/YF5,6	19.1 ± 8.0	22.93 ± 4.0	8.19 ± 3.0	25.86 ± 4.0
HY/YF1-6	9.36 ± 3.3	11.59 ± 4.0	15.62 ± 0.9	10.24 ± 6.5
HY/CD3 εM[b]	13	33.0	8.5	30.6

[a]Statistical values for the percentage and number of cells in the HY and HY/YF male mice were determined from n=9.

[b]Two HY/CD3εM male mice were analyzed and exhibited similar numbers and percentages of cells.

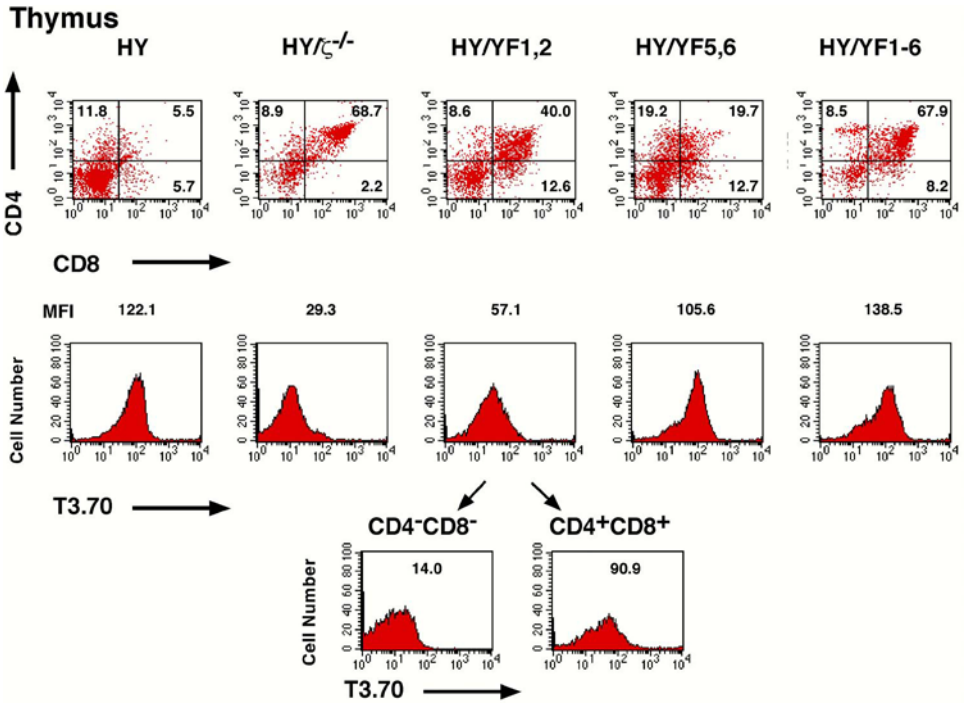


Figure 25. The Deletion of HY TCR Transgenic Thymocytes in Male Mice is Less Efficient with the Select Expression of the 21-kDa Tyrosine-Phosphorylated Form of TCR ζ. Thymocytes from male HY TCR transgenic mice were compared by flow cytometry with age-matched mice expressing the YF1,2; YF5,6; or YF1-6 TCR ζ constructs on the HY/TCR ζ-null background. Single cell suspensions were prepared and stained with mAbs to CD4 and CD8 and/or T3.70 and analyzed by flow cytometry. Results are representative of at least six independent experiments. MFI = mean fluorescence intensity.

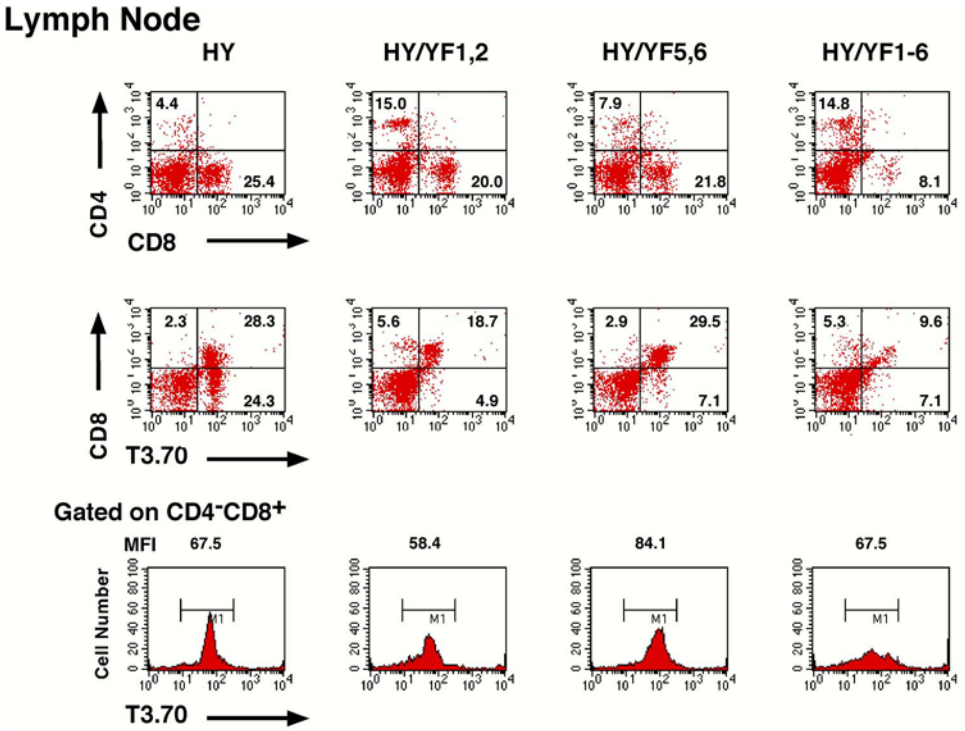


Figure 26. The Deletion of HY TCR Transgenic Thymocytes in Male Mice is Less Efficient with the Select Expression of the 21-kDa Tyrosine Phosphorylated Form of TCR ζ. Lymph node cells from male HY TCR transgenic mice were compared by flow cytometry with age-matched mice expressing the YF1,2; YF5,6; or YF1-6 TCR ζ transgenic constructs on the HY/TCR ζ-null background. Single cell suspensions were prepared and stained with mAbs to CD4 and CD8 and/or T3.70 and analyzed by flow cytometry. Results are representative of at least six independent experiments.

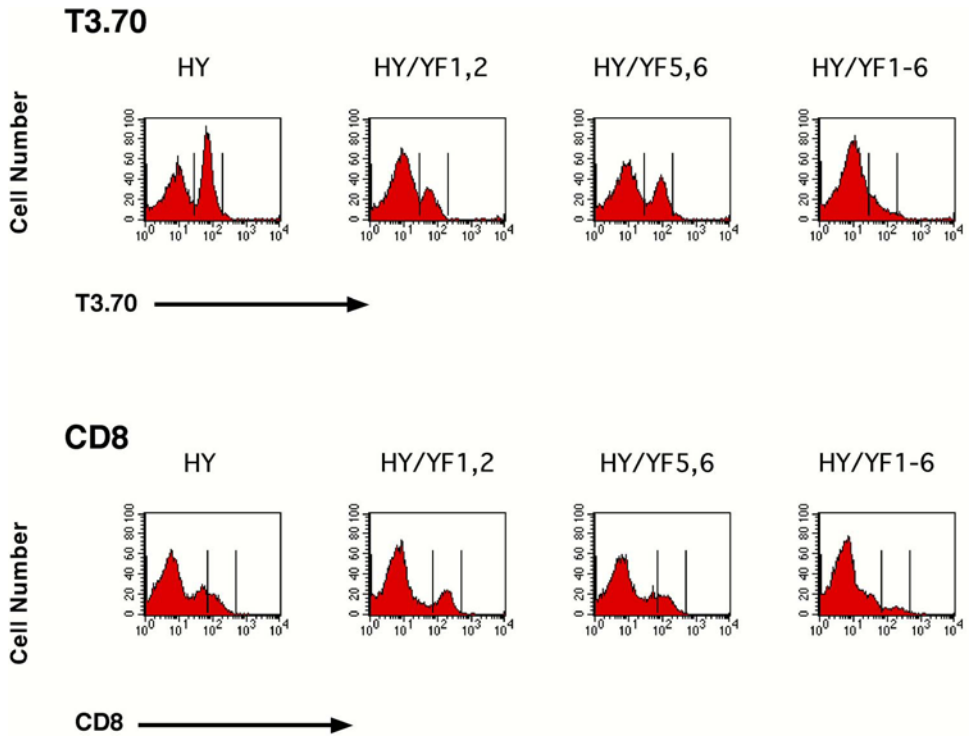


Figure 27. Altered Levels of TCR and CD8 Surface Expression when the 21-kDa Tyrosine-Phosphorylated Form of TCR ζ is Expressed. Lymph node cells from male HY TCR transgenic mice were compared by flow cytometry with age-matched mice expressing the YF1,2; YF5,6 or YF1-6 TCR ζ constructs on the HY/TCR ζ-null background. Single cell suspensions were prepared and stained with mAbs to CD4 and CD8 and/or T3.70 and analyzed by flow cytometry. Results are representative of at least six independent experiments.

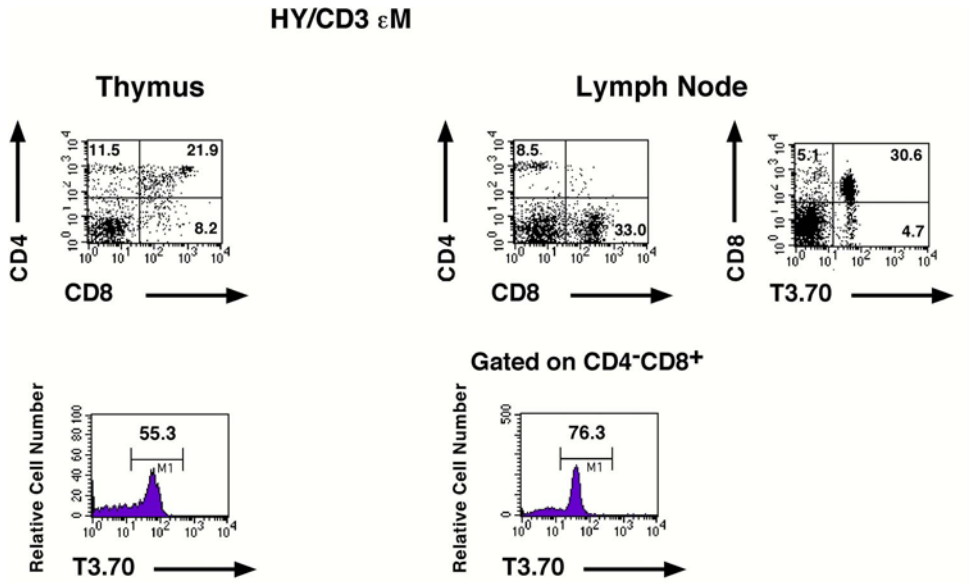


Figure 28. Negative Selection of Thymocytes is Normal in the Absence of CD3 ε ITAMs. Thymocytes and lymph node cells were isolated from HY/CD3 εM male mice. Single cell suspensions were prepared and stained with mAbs to CD4 and CD8 and/or T3.70 and analyzed by flow cytometry.

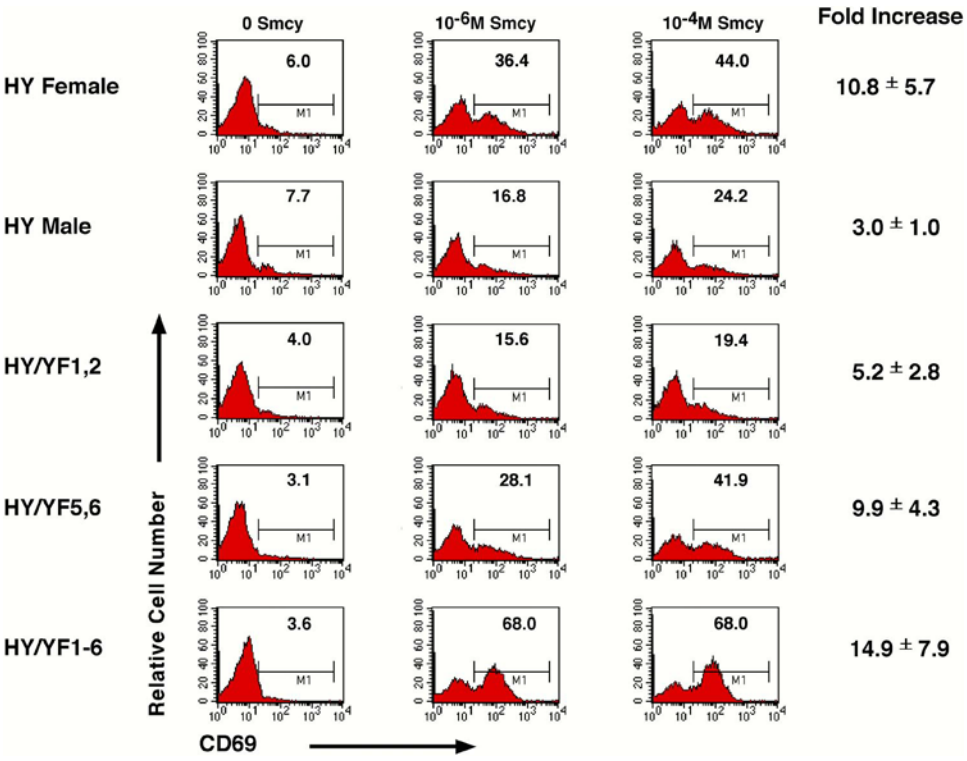


Figure 29. The Upregulation of CD69 is Impaired in HY and HY/YF1,2 Male Thymocytes. Thymocytes were isolated from the HY or HY/TCR ζ transgenic male mice and cultured for 19 hours at 37ºC with APC pulsed with agonist peptide (Smcy) or no peptide. The induction of CD69 on the cell surface was assessed by staining cells with mAbs against CD4, CD8 and CD69 followed by flow cytometric analyses. CD69 induction is represented as fold increase in the percent of cells expressing CD69 in response to high dose of agonist peptide (10^{-4} M) relative to no peptide control.

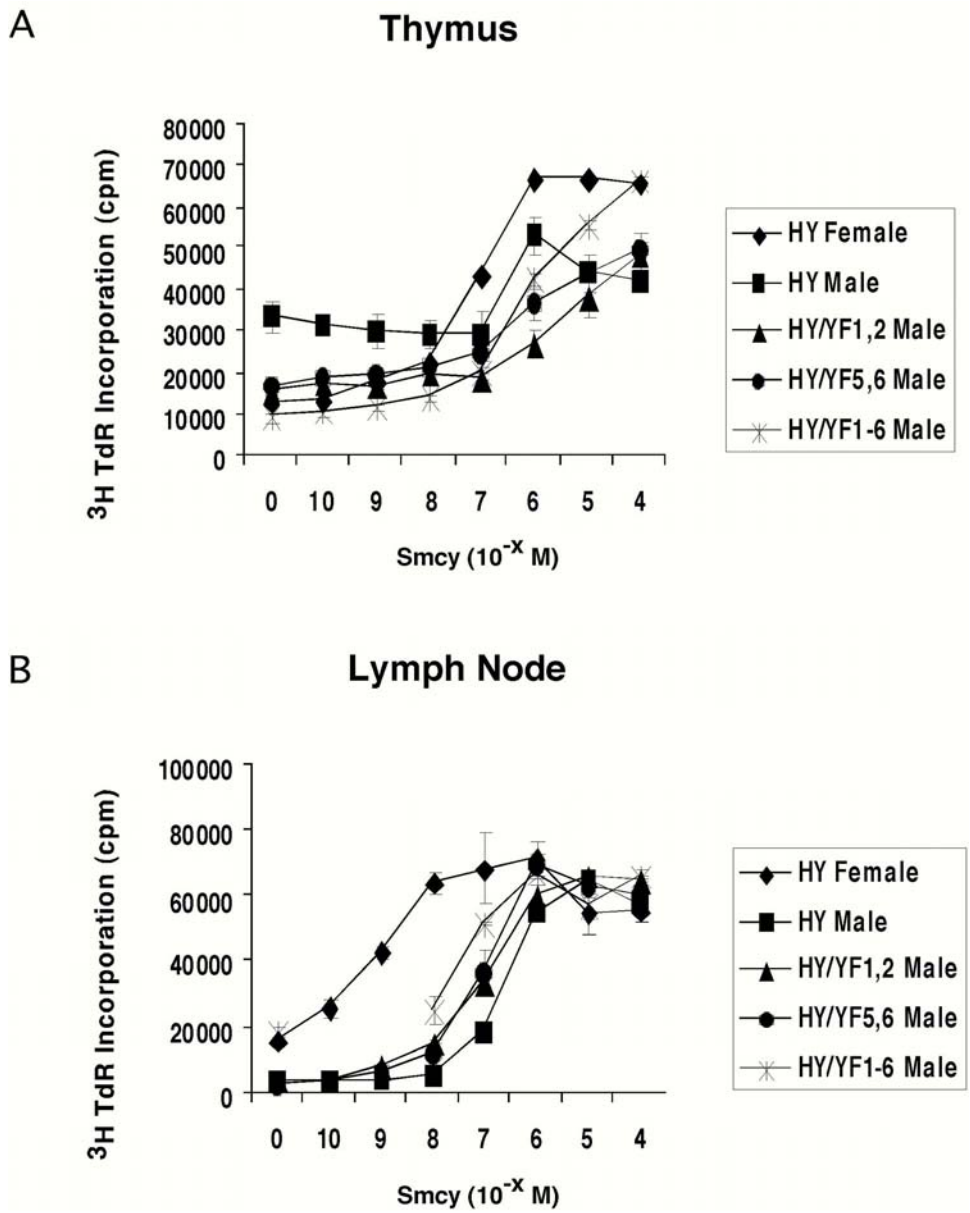


Figure 30. *in vitro* Proliferation of HY-Specific T Cells is Equivalent in HY versus HY/YF Male T Cells. Thymocytes (10^5) (A) or peripheral T cells (10^4 T3.70$^+$CD8$^+$) (B) were cultured for 72 hours with APCs that had been pulsed with increasing concentrations of the agonist peptide, Smcy, in the presence of 20 U/ml IL-2. Proliferation was measured by the incorporation of ^{3}H-thymidine, which had been added for the last 16 hours of culture.

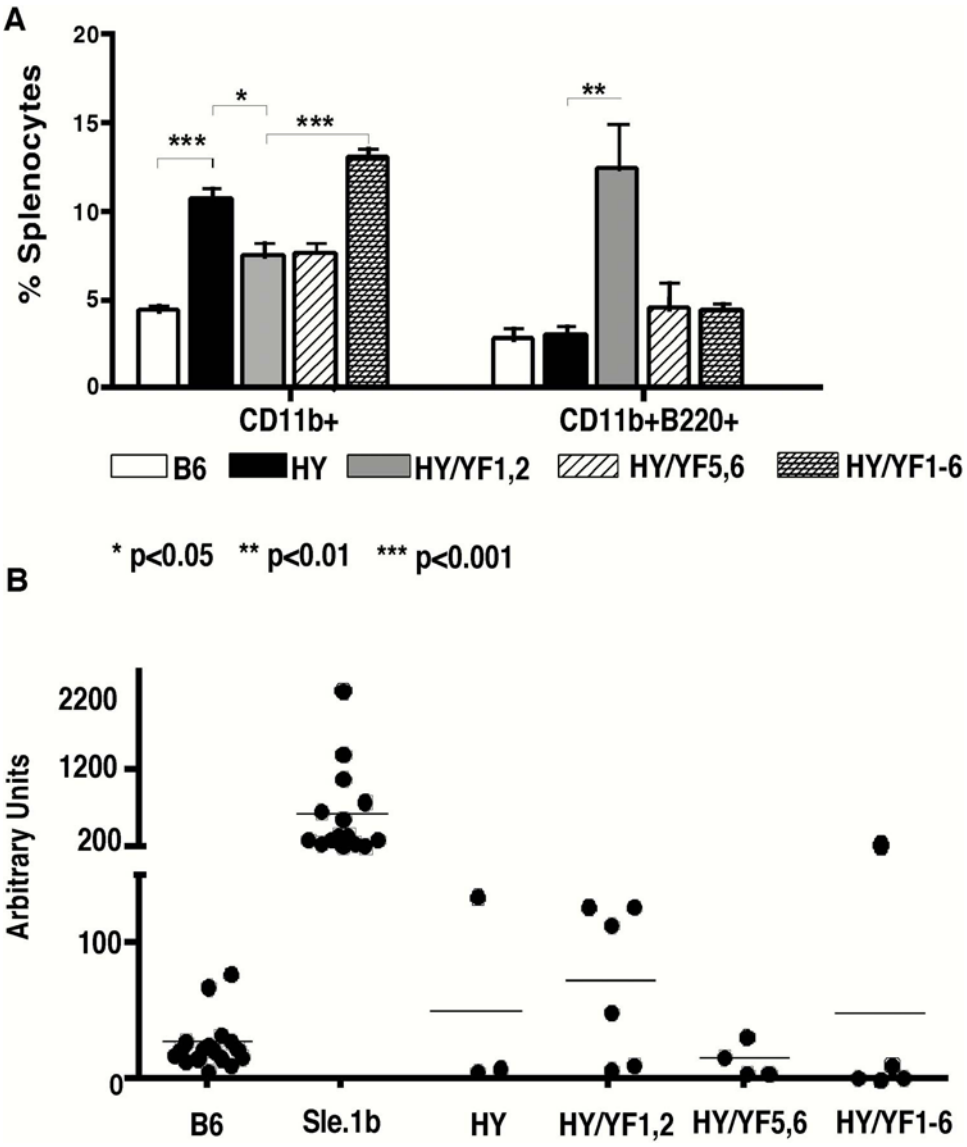


Figure 31. Aged HY/YF1,2 Male Mice Exhibit Altered Lymphocyte Populations without Autoimmunity. HY versus HY/YF1,2; HY/YF5,6; and HY/YF1-6 male mice that had been aged for eight to twelve months were compared for changes in lymphocyte populations in the spleen (A). The presence of autoreactive antibodies, including anti-histone/dsDNA and anti-dsDNA, in the serum of aged HY versus HY/YF male mice was assessed by ELISA (B).

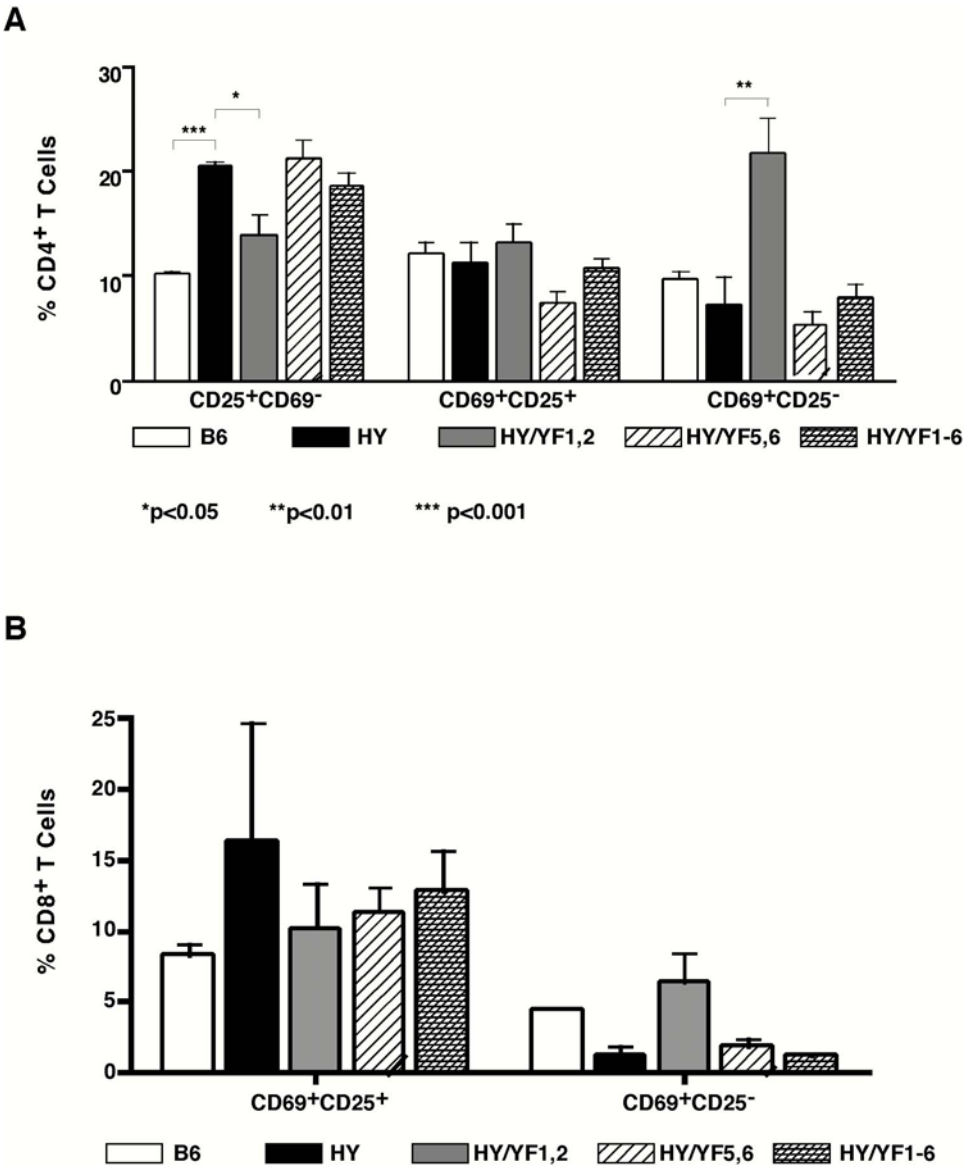


Figure 32. Aged HY/YF1,2 Male Mice Exhibit Increased Activation Markers in CD4+ T Cells. HY versus HY/TCR ζ transgenic male mice that had been aged either to twelve months were analyzed for changes in activation markers in $CD4^+$ and $CD8^+$ T cells in the spleen. Single cell suspensions of spleens were stained with mAb against CD25, CD69 and CD4 or CD8 and analyzed by flow cytometry.

Chapter VI - Discussion

The TCR is a unique receptor complex because it contains ten copies of the ITAM embedded in the cytoplasmic domains of the noncovalently associated signaling subunits. This is in contrast to other ITAM containing antigen receptors, which usually contain two to four copies of the ITAM motif (4). The TCR ITAMs are distributed within two independent signaling modules, TCR ζζ and CD3 γε/δε (Figure 33) (61). Based on initial findings, the two signaling modules were individually capable of generating the signaling events for full T cell activation (57-61). Historically, the TCR ζζ module was presumed to be the predominant signaling module in the TCR complex for several reasons. First, the TCR ζζ module possesses the majority of the ITAMs in the TCR complex (6/10) (Figure 33). Second, following TCR ligation, TCR ζζ is one of the more heavily tyrosine-phosphorylated proteins detected in thymocytes and peripheral T cells. In contrast to the TCR ζζ module, the CD3 γε/δε module is difficult to detect as phospho-proteins, even after TCR stimulation (34). Third, TCR ζζ can exist as two distinct phosphorylated intermediates, p21 and p23 (31, 32). Fourth, a portion of TCR ζ exists as the constitutively phosphorylated 21-kDa TCR ζ intermediate in thymocytes and peripheral T cells (43).

To study the role of the TCR ζζ module to T cell functions, in particular the 21- and 23-kDa phosphorylated TCR ζ intermediates, we generated a unique series of TCR ζ

transgenic mice expressing select phosphorylated TCR ζ intermediates (37, 91). In the YF1,2 TCR ζ transgenic line, p21 was expressed constitutively, in the complete absence of p23. Two other TCR ζ transgenic lines expressed only weakly inducible p19/p20 intermediates (YF5,6) or expressed no phospho-ζ intermediates (YF1-6) (37, 91). These TCR ζ transgenic mice, bearing tyrosine to phenylalanine mutations in the TCR ζ ITAMs, were used to definitively determine the functions of the TCR ζζ module in T cells. Importantly, this is the only system ever reported to examine the functions of p21 and p23, where p21 is maintained *in vivo*. The TCR ζ transgenic mice provided several advantages over previous studies using either truncated TCR ζ molecules or full-length TCR ζ molecules bearing ITAM mutations. First, our studies used modified full-length TCR molecules, containing amino acid substitutions in the ITAMs. This is in contrast to previous studies using truncated TCR ζ molecules. In some cases, the truncation of TCR ζ resulted in the abnormal, augmented tyrosine phosphorylation of the CD3 subunits (36). Second, the TCR ζ transgenic mice utilized in our studies represent the only system created to date that maintained the constitutive expression of p21 in the absence of p23. This enabled the direct examination of the functions of p21 in T cells. Third, the generation of the YF5,6 TCR ζ transgenic line, enabled a comparison of mice containing similar numbers of functional TCR ζ ITAMs (YF1,2 and YF5,6; 8/10 ITAMs) that express distinctly different phospho-ζ intermediates. These TCR ζ transgenic mice were used to examine the functions of p21 and p23 during antagonism of T cell responses, T cell activation and T cell development.

Based on the experimental results reported herein, we propose a revised model of TCR signal transmission (Figure 33). Within this model, we propose that the TCR ζζ and CD3 γε/δε signaling modules contribute both redundant and unique functions to T cells. Specifically, the TCR ζζ and CD3 γε/δε modules contribute redundant functions to thymocyte positive selection. This redundancy is likely mediated by the ten ITAMs that are distributed within the two TCR modules. However, positive selection also involves the TCR ζζ module, likely through alternative signaling pathways. Our model also proposes that the CD3 γε/δε module is mainly responsible for TCR mediated signal transmission, while the TCR ζζ module may play a secondary role in T cell activation. The findings leading to the development of this model are discussed below.

The CD3 γε/δε Signaling Module is Primarily Responsible for TCR-mediated Signal Transmission

Our data indicate that the CD3 γε/δε module is the principle regulator of TCR-mediate signal transmission. This conclusion is based on results where the elimination of p23, both p21 and p23 or all phospho-ζ intermediates did not alter the kinetics or amplitude of TCR-mediated signaling events compared to T cells with a fully intact TCR ζζ module. These findings were observed in three distinct systems, including wild-type T cells and TCR transgenic lines with containing high affinity and low affinity TCRs (P14 and HY, respectively). This interpretation supports previous studies using chimeric receptors containing the cytoplasmic domains of the TCR ζ and CD3 ε subunits that were

expressed in cells lines and in mice (57-63). Similar observations were made using TCR ζ transgenic mice containing mutant TCR ζζ modules bearing ITAM substitutions or truncations, where signaling was normal in the absence of one or all TCR ζ ITAMs (36, 58, 61, 114-116). However, our studies have significantly advanced these initial observations. We have analyzed TCR signal transmission in a TCR ζ transgenic line that maintained the constitutive p21 form of TCR ζ (YF1,2). In addition, we analyzed TCR signal transmission in a system where T cell development is contingent upon a full complement of ITAMs (HY). In all of these systems and in all of the TCR ζ transgenic lines analyzed, membrane proximal early T cell activation events, including PTK activation and intracellular calcium mobilization, were equivalent. Furthermore, later T cell activation events, including CD69 upregulation and T cell proliferative responses, were comparable to wild type T cells over a range of agonist and APL doses. Thus, we propose that the CD3 γε/δε signaling module is primarily responsible for the activation of the classical TCR signaling pathways.

The initial studies using chimeric receptors had indicated that TCR ζ and CD3 ε ITAMs could individually transmit the intracellular signals leading to PTK activation and T cell activation. One particular study using TCR ζ and CD3 ε chimeric receptors had also indicated that distinct signals were generated through the CD3 ε and TCR ζ ITAMs (60). Our model supports the notion of distinct signals through the CD3 γε/δε module, which is primarily responsible for T cell activation events (Figure 33). The identification of distinct protein interactions with CD3 ε subunit suggest a unique role for CD3 ε and further support the notion of a primary role for the CD3 γε/δε module in signal

transmission (Table 1). In fact, the interactions between CD3 ε and Nck or CAST may also contribute to the predominant signaling role for the CD3 γε/δε module (78, 79). Recent studies oppose the requirement for Nck association with CD3 ε for the initiation of TCR-mediated signals. In an analysis of T cells harboring mutations in the proline-rich region of CD3 ε, which interacts with Nck, no defects in T cell activation events were noted (D. Vignali, personal communication). Based on this, the predominant role of the CD3 γε/δε signaling module is not dependent on an association of Nck. However, other yet unidentified proteins may associate with the CD3 γε/δε module to bestow unique functions to this signaling module in T cell activation. We have preliminary evidence that the CD3 ε subunit specifically associates with a serine/threonine kinase in thymocytes and T cells (L. DeFord and N. S. C. van Oers, unpublished data). The identification and function of this serine/threonine kinase is unknown, but it may potentially contribute to the predominant function of the CD3 γε/δε module in TCR-driven signal transmission.

In contrast to previous studies, our analyses refuted a potential inhibitory role for 21-kDa phosphorylated TCR ζ intermediate (99, 101, 102, 156). The selective expression of p21, in the absence of p23, in the YF1,2 TCR ζ transgenic line promoted equivalent early and late TCR signaling events compared to wild-type T cells. Furthermore, the expression of p21 in the absence of p23 did not enhance the antagonism of T cell responses to APLs. While the 21- and 23-kDa phospho-ζ intermediates were not required for TCR-mediated signaling transmission or T cell antagonism, some phospho-ζ was required for T cell antagonism by antagonist peptides in our system, since

no inhibition of T cell proliferative responses was observed in the absence of phospho-ζ. This suggests that phospho-ζ may contribute to ligand discrimination, possibly through weakly phosphorylated intermediates that are difficult to detect. Although the mechanisms involved in the detection and responses to different ligands are unclear, distinct protein interactions may also be involved in the signals for T cell antagonism. The differential recruitment of various signal activators or attenuators may follow agonist or antagonist ligands. Alternatively, the phosphorylation of the CD3 ITAMs may contribute to the signals involved in ligand discrimination.

Redundant Roles for the TCR ζζ and CD3 γε/δε Signaling Modules During Thymocyte Positive Selection

Given the fact that the TCR ζζ and CD3 γε/δε modules contain multiple ITAMs, which can all interact with ZAP-70 PTK, one would expect some functional redundancy to exist within the two modules. In the revised model for TCR signal transmission, we propose that the functional redundancy for the TCR ζζ and CD3 γε/δε signaling modules occurs during T cell development. Previous findings showed a reduction in positive and negative selection were observed in the absence of functional TCR ITAMs, but only in T cells with low affinity TCRs (114, 116, 119, 121). This additive function for the ITAMs during positive selection events is partially supported by our experimental results. In the low affinity HY TCR transgenic system, the efficiency of positive selection was directly dependent on the total number of functional ITAMs present in the TCR complex. Our

data indicated similar reductions in positive selection when only 8/10 TCR ITAMs were functional, despite expressing distinct phospho-ζ intermediates. In fact, the select expression of p21 did not either inhibit or enhance positive selection, indicating that the distinct phospho-ζ intermediates did not contribute unique functions during positive selection events. Thus, we propose that an equivalent number of ITAMs, contributed by the TCR ζζ or CD3 γε/δε ITAMs, or a combination of ITAMs from the TCR ζζ and CD3 γε/δε modules, could induce similar signals involved in positive selection. However, some of the signals involved in thymocyte positive selection require phospho-ζ, since the complete absence of phospho-ζ almost completely ablated positive selection in the HY/YF1-6 mice. Therefore, phospho-ζ may be involved in alternative signaling pathways required for positive selection that are not yet clearly defined. Alternative signaling pathways mediated by phospho-ζ may involve the adaptor protein Shc. Since Shc associates with phospho-ζ and Shc is required for T cell development, it is possible that this interaction between phospho-ζ and Shc is important in the activation of non-classical TCR-mediated signaling pathways during positive selection events (70, 71).

We also found that T cell development was normal in all of the TCR ζ transgenic lines when analyzed on a wild type or high affinity TCR transgenic system (P14). Seeing as a dependence on the TCR ITAMs was not observed in either a wild-type or the high affinity P14 TCR transgenic systems, it may be that only a minimal number of functional ITAMs (4/10) are required for development of most T cells, while T cells with low avidity TCRs may require all ten functional ITAMs for efficient development.

The TCR ζζ Module Provides a Unique Role to Thymocyte Negative Selection

Surprisingly, when we examined the roles of the phospho-ζ intermediates during negative selection events, a unique role for p21 was revealed. Specifically, the select expression of p21 attenuated negative selection of thymocytes in the low affinity HY TCR transgenic system. The expression of p21 in the absence of p23 promoted the emergence of T cells with a distinct phenotype that suggested potential autoreactivity. We propose that p21 exerts its unique function through sequestration of critical signaling proteins (ZAP-70) or by associations with signal attenuators. When normal TCR ζ molecules are present, the formation of p23 may override any potential negative effects of p21 on negative selection. In the absence of both p21 and p23 in the YF5,6 line, the available TCR ζζ and CD3 γε/δε ITAMs may provide sufficient signals required for efficient negative selection. This suggests that signaling strength, mediated partially by the TCR ITAMs, is important for efficient negative selection. Thus, an impaired signaling strength can result in the escape of autoreactive T cells from the thymus into the periphery, leading to autoimmunity. A reduction in TCR signal strength during selection events leading to autoimmunity has been shown *in vivo*. Point mutations in key signaling molecules, including ZAP-70 and LAT, which reduce TCR-mediated signals during development, have been identified that reduce the efficiency of negative selection events and lead to autoimmune T cells in the periphery (49, 188).

Altogether, within our revised model of TCR signaling, the TCR ζζ and CD3 γε/δε modules are both functionally redundant and distinct. Both the TCR ζζ and CD3 γε/δε

modules contribute to positive selection events. The functional redundancy that exists within the TCR ζζ and CD3 γε/δε modules is mediated through the ten ITAMs within the TCR complex. However, the CD3 module is primarily responsible for TCR signal transmission, including PTK activation, T cell proliferation and CD69 upregulation. The TCR ζζ module, particularly the constitutive p21 phospho-ζ intermediate, provides a unique function in the attenuation of negative selection events. In addition, the phosphorylated TCR ζζ intermediates contribute in an unknown manner to positive selection of thymocytes.

The Functional Role of the TCR ζζ Module

If the CD3 γε/δε module is primarily responsible for T cell activation, what then, are the functions of the TCR ζζ module in T cells? Can the TCR ζ ITAMs and thus the distinct phospho-ζ intermediates act as sensors for the pMHC ligands engaged by the TCR? To some extent, TCR ζ does appear to function as a molecular sensor. The constitutive expression of p21 is clearly the result of TCR interactions with self pMHC complexes (82, 84, 139, 141). Interestingly, the signals generated by TCR/self-pMHC interactions that induce the formation of p21 are distinct from the signals required for positive selection. In fact, nonselecting self-pMHC ligands in the thymus induce levels of p21 equivalent to those induced by positively selecting ligands (A. White and N. S. C. van Oers, unpublished data). Thus, TCR ζ can act as a molecular sensor to detect the presence of self-pMHC in the thymocytes and peripheral T cells, resulting in the formation of p21. However, since p21 expression does not translate into positive

selection, the function of the constitutively expressed p21 intermediate in thymocytes and peripheral T cells is unclear.

In the periphery, the interactions with self-MHC that induce the constitutive expression of p21 have been proposed to enhance T cell responsiveness (141). The elimination of TCR interactions with self-MHC *in vivo* or *in vitro* has been shown to cause a severe reduction in the constitutive expression of p21 (141). In these studies, the reduction in p21 translated into diminished T cell responsiveness following TCR stimulation (141). Thus, the TCR interactions with self-pMHC in the periphery that generate p21 may serve to augment T cell responses to antigen, both during positive selection and mature T cell activation in the periphery (141, 171). However, our experimental data might refute this notion. The select expression of p21 neither enhanced nor inhibited T cell positive selection events. Nonetheless, the proliferative responses of the YF1,2 T cells were slightly augmented when compared to control T cells. This slight augmentation in T cell proliferation in the YF1,2 T cells was not as impressive as the increase in T cell proliferative responses in the presence of p21 observed by Stefanova *et al* (141). In addition, our data indicated that at high peptide doses, the proliferative responses of the YF1,2 and wild-type T cells were similar and any augmented proliferative responses with the select expression of p21 only occurred with low peptide doses. How might this enhanced responsiveness to antigen be accomplished by the recognition of self-pMHC? One possibility is that self-pMHC interactions promote the formation of p21, which can increase the local concentration of signaling molecules, such as ZAP-70, to the TCR complex (141). This contrasts the recruitment of

negative regulators of signal transmission, such as SHP-1, that have been observed following stimulation with antagonist ligands, despite similar patterns of phospho-ζ (101-103, 141, 155). An increase in the local concentration of TCR proximal signaling molecules is a plausible explanation for enhanced signaling since TCR ζ, in its constitutively partially phosphorylated form (p21), complexes with an inactive pool of ZAP-70. However, the majority of the data presented here indicated that the elimination of p21 does not impair TCR-mediated signaling events. Thus, p21 may augment responses to weak stimulatory conditions, while the CD3 γε/δε module is primarily responsible for initiating TCR-mediated signaling events.

The Functions of the TCR ζζ Module in Peripheral T Cell Survival

A substantial amount of evidence has indicated that tonic signaling through the TCR is required for T cell survival in the periphery (84, 134, 137). It has been proposed that the constitutive expression of p21 is directly related to peripheral T cell survival (84). To address the role of phospho-ζ, particularly the constitutively expressed p21 intermediate, in the maintenance of peripheral T cells, we compared the survival of T cells from the various TCR ζ transgenic mice expressing distinct phospho-ζ intermediates. Based on previous data that suggested that p21 was required for T cell survival, it would be expected that the YF5,6 and YF1-6 T cells, which don't express p21 in a constitutive or inducible manner, would have a decreased lifespan compared to wild-type and YF1,2 T cells (84). Enriched T cell populations from either wild-type or the various TCR ζ transgenic mice were labeled with CFSE and adoptively transferred into

unirradiated littermate mice. At various timepoints, from one to five weeks after the adoptive transfer of T cells, the spleens and the lymph nodes of the recipient mice were removed and the presence of $CFSE^+$ donor T cells was analyzed. Wild-type T cells, which constitutively express p21, were detected in the lymph nodes and spleens of recipient mice at least three weeks after adoptive transfer into recipient mice (Figure 34). The wild-type CFSE labeled T cells could be detected even five weeks after transfer (data not shown). Unexpectedly and in contrast to wild type T cells, T cells from the YF1,2 mice, which constitutively expressed p21 in the absence of p23, were undetectable in the spleens or lymph nodes of recipient mice by three weeks (Figure 34). It is important to note that the YF1,2 T cells in these experiments expressed high levels of p21 compared to wild-type mice (founder 0563; Table II). Furthermore, in the absence of constitutive or inducible p21, the YF5,6 and YF1-6 T cells were maintained in the spleens and lymph nodes of recipient mice up to five weeks after adoptive transfer (Figure 34). These data suggest that the constitutive expression of p21 is not required for T cell survival in the periphery, as T cells lacking both p21 and p23 (YF5,6) or all phospho-ζ (YF1-6) survive as well or better than wild-type T cells. These data also imply that p21 attenuates T cell survival. In fact, we had observed a dose-responsive effect on the survival of T cells from various YF1,2 founders that expressed different levels of p21 (data not shown). Thus the relative levels of p21 may control T cell survival. In particular, high levels of p21 may reduce T cell survival in the periphery. It should be noted that these experiments were preliminary and after a period of two years, the specific loss of the YF1,2 cells from the peripheral lymphoid compartments by three weeks after adoptive

transfer was not reproducible. This is likely due to the reduction in expression of the TCR ζ transgene in the YF1,2 line used (as noted in Table II).

How might high levels of p21 be obtained in a cell? In addition, how might p21 attenuate T cell survival? Since p21 is the result of TCR interactions with self-pMHC in the periphery, it is conceivable that the levels of p21 are representative of past TCR engagements. This hypothesis is based on the assumption that the formation of p21 in a cell is stable following TCR ligations. Along these lines, the overall levels of p21 would increase in a cell over its lifespan. When high levels of p21 are obtained within a cell, a certain threshold set during development may be exceeded such that signals may be generated to program that cell for death. To assess whether high levels of p21 in a cell can diminish T cell survival, the lifespan of T cells can be compared *in vivo.* Thymectomizing the various TCR ζ transgenic mice followed by a kinetic analysis of the survival of peripheral T cells may reveal distinct differences between the YF1,2 TCR ζ transgenic and wild-type mice. If the number of T cells is depleted from the periphery in the YF1,2 line more rapidly that in the wild-type, YF5,6 and YF1-6 lines, it would suggest a unique role for p21 in the attenuation of T cell survival. In addition, the generation of a second YF1,2 TCR ζ transgenic mouse, containing high levels of the transgene could be used to confirm our preliminary findings. If the proposed experiments are consistent with our preliminary results, where high levels of p21 diminish T cell survival, the mechanisms involved in the specific attenuation of T cell survival by high levels of p21 will need to be addressed. It is possible that the select expression of p21 may negatively regulate survival factors, such as the transcription factor LKLF, which

maintains T cells in a quiescent state (189, 190). Analyses could be used to compare the expression patterns of apoptotic or survival factors, including LKLF, in the various TCR ζ transgenic mice.

An alternative interpretation of these results is that the CFSE-labeled transferred YF1,2 cells from the spleens and lymph nodes of the recipient mice after three weeks may be undetectable as a result of the YF1,2 T cells trafficking to other organs. The distinct phospho-ζ intermediates may regulate the expression of chemokines and chemokine receptors on T cells. One such chemokine receptor to examine would be CCR7, which is involved in lymphocyte homing to lymphoid compartments. A comparison of relative expression levels of chemokine receptors of the different TCR ζ transgenic T cells would verify if the loss of YF1,2 T cells from recipient lymphoid organs is a result of distinct patterns of T lymphocyte trafficking. These proposed studies will verify whether T cell survival or trafficking is dependent on the expression of p21.

The Role of the TCR ζζ Module in the Adaptive Immune Response to Pathogens

Surprisingly, with the number of studies that have focused on the functions of TCR ζζ module during T cell development and activation, very few studies have examined the functions of TCR ζζ during responses to pathogens *in vivo*. To address the functions of the TCR ζζ module during adaptive immune responses, the TCR ζ transgenic mice were subjected to *in vivo* infections with *Listeria monocytogenes*. *Listeria monocytogenes* (LM) is a gram positive intracellular bacterium. *In vivo* infections with

Listeria monocytogenes have been used frequently to study both innate immune responses as well as T cell-mediated adaptive immune responses to intracellular pathogens. It has been shown that the majority of LM traffic to the liver immediately after infection (191). Both $CD4^+$ and $CD8^+$ T cells are involved in the clearance of LM and the generation of protective immunity against LM (192). In addition, cytokines, particularly TNFα and IFN-γ, are crucial mediators of immunity to LM. To evaluate the contribution of the TCR ζζ module to the clearance of pathogens in vivo, TCR ζ transgenic mice, including the YF5,6 and YF1-6 mice, were challenged with *Listeria monocytogenes*. Wild-type YF5,6 and YF1-6 TCR ζ transgenic mice were initially infected intravenously with a low dose (0.1 LD_{50}) of LM. Four to six weeks after the initial infection, the mice were rechallenged with a high dose of LM (10 LD_{50}). Five days later, the number of CFU in the spleens and livers of control and infection mice were analyzed. After five days, wild-type mice had completely eliminated the LM from the spleen (Figure 35). In addition, the majority of wild-type C57Bl/6 mice had completely cleared LM from the liver. In contrast, the YF5,6 mice, which express only weakly phosphorylated p19/p20 intermediates in T cells, were overwhelmed with LM in the liver five days post infection (Figure 35). The YF5,6 mice were variable in their ability to eliminate LM from the spleens after five days. The YF1-6 mice, which contain no phospho-ζ intermediates, completely eliminated the LM from both the spleen and liver within five days. These data indicate a specific defect in the YF5,6 mice, which are impaired in their ability to respond to a secondary challenge with LM. In addition, in the absence of any phosphorylated TCR ζ intermediates, the YF1-6 mice were able to respond to and eliminate LM from the liver and spleen as well or slightly better than

wild-type mice. To determine whether there was a specific defect in the expansion of the $CD4^+$ and $CD8^+$ T cells in these mice following LM infection, the percentages of $CD4^+$ and $CD8^+$ T cells in the spleens of the YF5,6 TCR ζ transgenic were compared to wild-type LM-infected mice. In wild-type mice that had been infected with LM, an expansion of $CD8^+$ T cells was detected in splenocytes after five days, resulting in a ratio of $CD4^+$ to $CD8^+$ T cells of approximately one (Figure 36 A). A similar expansion in the $CD8^+$ T cell population was observed in the YF5,6 mice. Thus, it does not appear that a specific defect in the expansion of the $CD8^+$ in response to LM infection is causing the high titers of LM in the livers and spleens of the YF5,6 mice.

Cytokines, including IFN-γ and TNFα, are critical in the clearance of LM (191). To determine whether the T cells in the YF5,6 TCR ζ transgenic mice were capable of eliciting $CD8^+$ T cell effector functions, the wild-type and TCR ζ transgenic mice were stimulated *in vitro* with LM-infected macrophages overnight. The cultured $CD8^+$ T cells were then analyzed for their ability to secrete IFN-γ by intracellular cytokine flow cytometry. Following stimulation with LM-infected macrophages, splenocytes in the wild-type mice contained CD8+ T cells that secreted IFN-γ (11.2%) (Figure 36 B). $CD8^+$ T cells from the YF5,6 mice were also capable of secreting IFN-γ in response to LM-infected macrophages, however, the number of $CD8^+IFN\text{-}\gamma^+$ cells were reduced nearly two-fold compared to wild-type cells (6.7% vs. 11.2%). The $CD8^+$ T cells from the YF1-6 mice were also able to secrete IFN-γ in response to APCs presenting LM peptides (Figure 36 B). In addition, the percentage of $CD8^+$ $IFN\text{-}\gamma^+$ T cells (9.6%) in the spleen of the YF1-6 mice was nearly equivalent to that observed in wild-type mice. This data

suggests that the YF5,6 mice are impaired in their ability to effectively eliminate a secondary infection with *Listeria monocytogenes*. However, preliminary evidence indicates that the defect in the YF5,6 mice is not a result of deficiencies in $CD8^+$ T cell expansion or secretion of IFN-γ. A potential mechanism for the specific defect in the YF5,6 mice may be that the YF5,6 T cell are differentially localized during infection. This may be a result of abherrent patterns of expression of various chemokine receptors. As described above, CCR7 is a chemokine receptor that is involved in lymphocyte homing to the lymphoid organs. If the YF5,6 mice expressed elevated levels of CCR7, these T cells may be retained in the spleens and lymph nodes rather than trafficking to the liver. In addition, other chemokines and chemokine receptors may also be involved in the attenuated response of the YF5,6 mice to clear LM from the liver.

An alternative possibility is that the YF5,6 T cells are specifically antagonized during LM infection. We have previously noted that the YF5,6 T cells express some weakly phosphorylated TCR ζ intermediates of p19/p20 after stimulation, which may contribute to the antagonism of T cell responses. Initial studies had shown that monophosphorylated ITAMs can reduce T cell responsiveness to agonist ligands (156). Since the YF5,6 T cells do not express any stable phospho-ζ intermediates, the presence of monophosphorylated ITAMs may contribute to the diminished T cell responses. It is also plausible that the TCR ζ and CD3 ITAMs can differentially associate with adaptor/effector proteins. Specific protein interactions with the phospho-ζ intermediates in the YF5,6 transgenic T cells may negatively regulate T cell responses.

Additional experiments are required to determine how the YF5,6 T cells are specifically impaired in their response to *Listeria monocytogenes*. These will include a more extensive analysis of the various TCR ζ transgenic mice, including the YF1,2 line. Based on the preliminary findings, it is unclear whether the defective adaptive immune response to LM is specific to the YF5,6 TCR ζ transgenic line, since these experiments did not include an analysis of the YF1,2 TCR ζ transgenic mice. Additional analyses of the activation state of the T cells and the ability to elicit effector functions of these cells will be required. This will include an analysis of T cell activation markers (CD69, CD25, CD44), cytokine secretion, including TNFα, and the ability to induce cytolysis of LM-infected targets. In addition, the analysis of a second YF5,6 TCR ζ transgenic mouse will determine if the specific defects in T cell responses observed in the YF5,6 line are a result of transgene integration site.

Conclusions

The aforementioned studies have assisted in resolving some of the previous contentions as to the functions of p21 and p23 in thymocyte development and T cell activation. Based on these results, we proposed a revised model of TCR signaling (Figure 32). Within this model, the CD3 γε/δε module is primarily responsible for TCR-mediated signaling events. A predominant role for the CD3 γε/δε module is likely mediated through unique protein interactions. The TCR ζζ and CD3 γε/δε modules both contribute to the efficiency of positive selection of thymocytes in certain systems. This

redundancy is a function of the multiple ITAMs within the two modules. In our model, we also propose that the TCR ζζ module, particularly the constitutively expressed 21-kDa TCR ζ intermediate, contributes a unique function in the attenuation of thymocyte negative selection. In addition, the TCR ζζ module may have important roles in maintaining T cell survival and in the adaptive immune response to pathogens.

As an extension of the findings presented here, additional studies are required to further understand the functions of the two TCR signaling modules. To definitively address the functions of the TCR ζζ versus CD3 γε/δε ITAMs to thymocyte selection and activation, a complete analysis of mutations in the ITAMs of the various TCR subunits is required. This will require many years of animal matings to obtain various combinations of TCR ζ and/or CD3 mutants. An analysis such as this may reveal distinct functions between the ITAMs of the various TCR subunits. However, a complete analysis of the TCR ITAMs will likely not be sufficient to fully determine the functional complexity of the TCR complex. Recently, the non-ITAM regions in the TCR ζ and CD3 subunits have been shown to contribute to the functions of the TCR ζζ and CD3 γε/δε signaling modules. In fact, both the ITAMs and the distinct non-ITAMs regions in the TCR ζ and CD3 subunits have been shown to associate differentially with effector/adaptor proteins. These associations likely confer distinct functions to the TCR complex. Thus, to completely delineate the functions of the TCR ζζ and CD3 γε/δε modules future experiments would require the generation and analysis of combinations of mutants in the ITAM, as well as the non-ITAM regions of the TCR ζ and CD3 subunits. Despite the wealth of knowledge that we have already gained on the functions of the two TCR

signaling modules to T cell biology, we are just beginning to understand the complexities of the TCR complex.

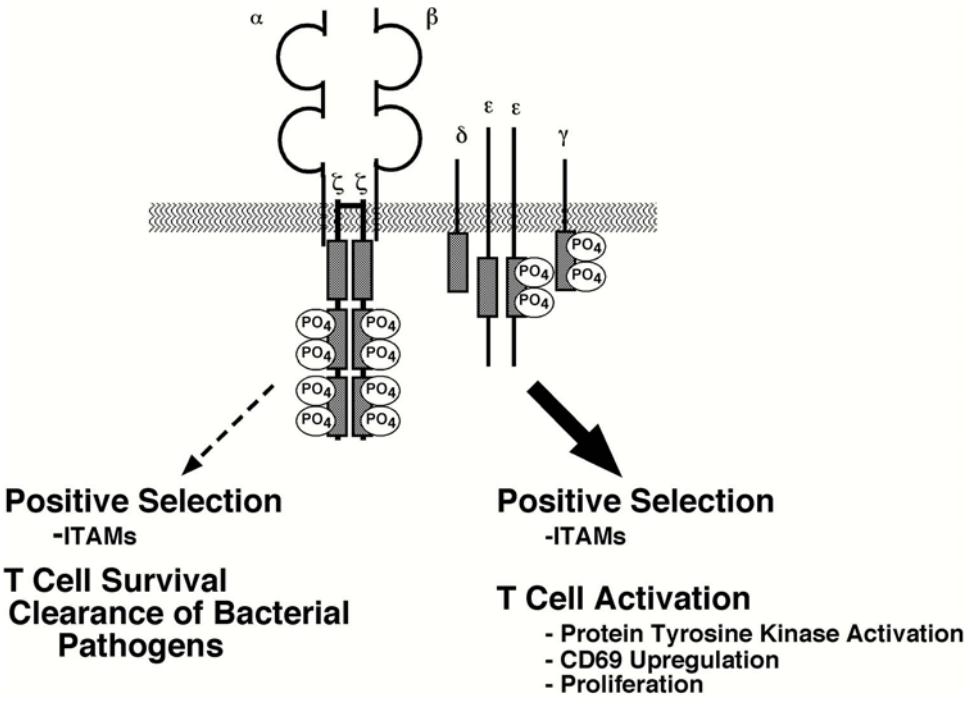


Figure 33. Revised Model of TCR Signal Transmission. The TCR is comprised of two independent signaling modules with redundant and distinct functions. The CD3 γε/δε-module is primarily responsible for T cell activation events. The TCR ζζ-module is involved in thymocyte positive selection, T cell survival and the clearance of bacterial pathogens.

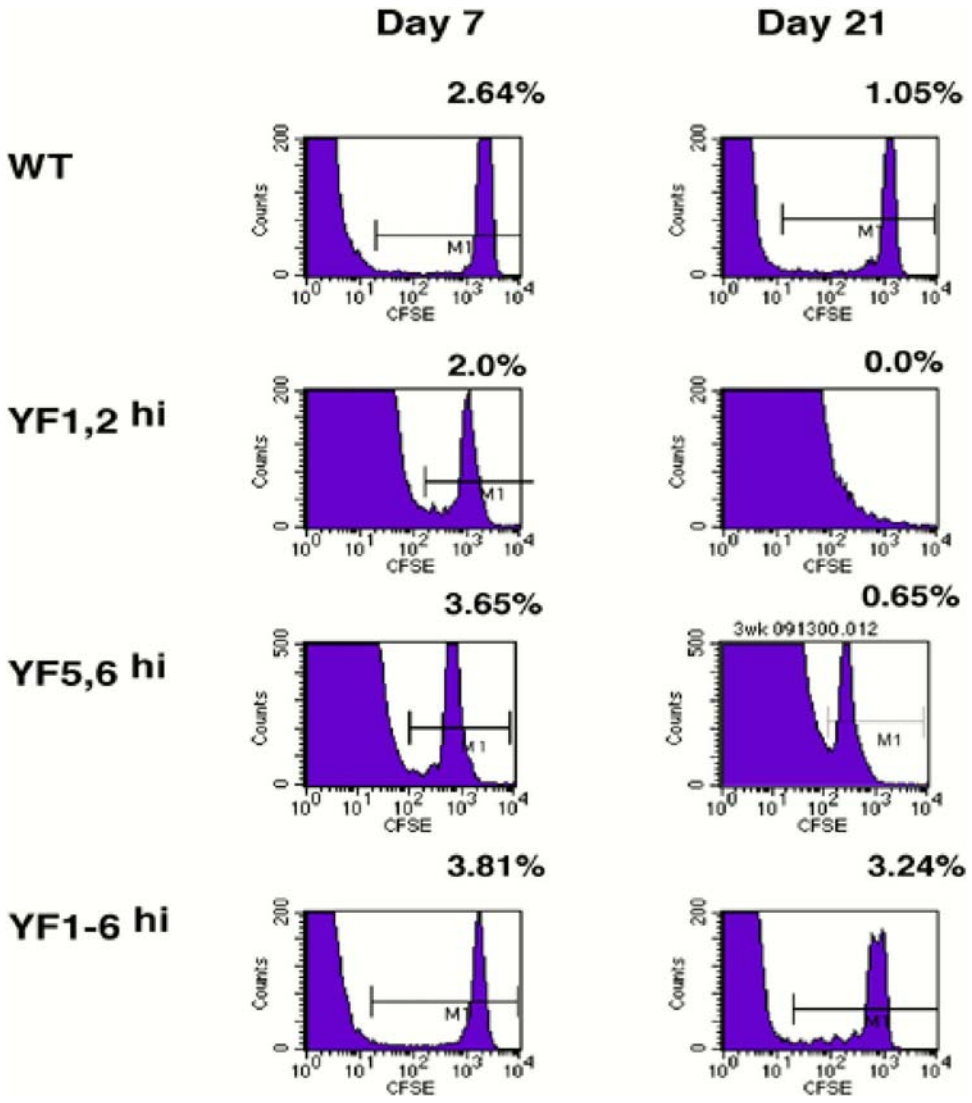


Figure 34. The Constitutive p21 Phosphorylated Intermediate of TCR ζ Attenuates T Cell Survival. T cells from wild-type, YF1,2; YF5,6 and YF1-6 TCR ζ transgenic mice were isolated, labeled with CFSE and transferred into unmanipulated littermate mice. The lymph nodes of recipient mice were analyzed at day 7 or day 21 for the presence of CFSE labeled T cells by flow cytometry.

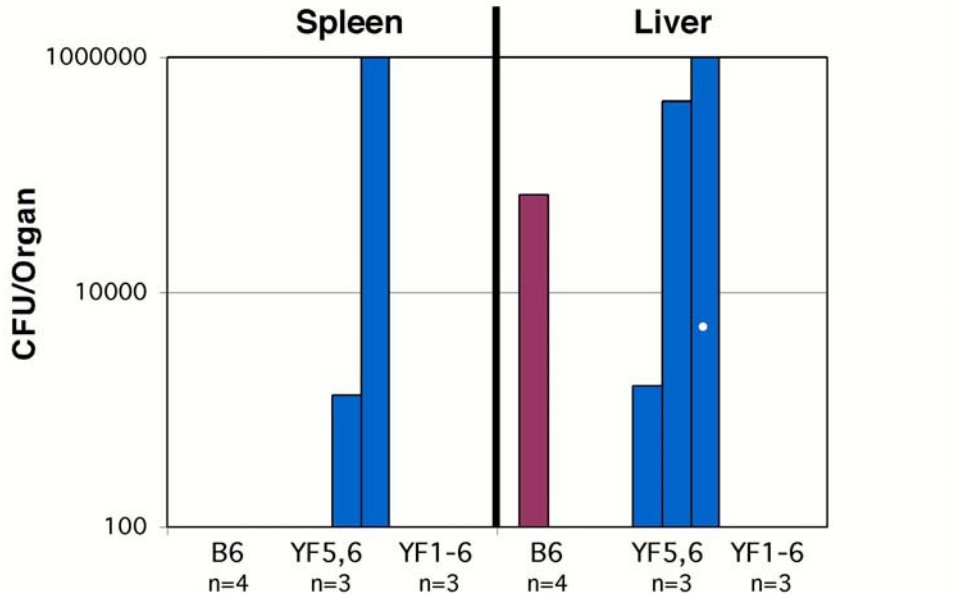


Figure 35. The YF5,6 TCR ζ Transgenic Mice are Unable to Clear a Secondary *Listeria monocytogenes* Infection. Wild-type, YF5,6 and YF1-6 TCR ζ transgenic mice were administered a sublethal dose of *Listeria monocytogenes* intravenously. Four to six weeks after initial infection, the mice were re-infected with a high dose (10 LD_{50}) of Listeria monocytogenes. Five days later, the number of *Listeria monocytogenes* colony forming units (CFU) was determined in the spleen and liver of infected mice.

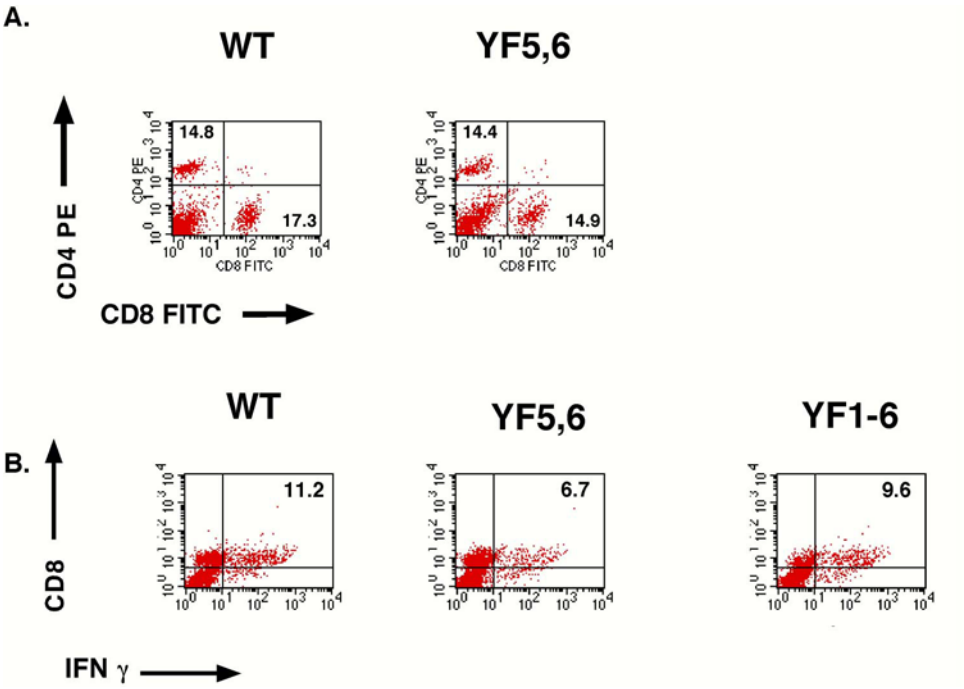


Figure 36. T Cells from YF5,6 TCR ζ Transgenic Mice can Expand and Produce IFN γ in Response to Stimulation with Listeria-infected Macrophages. Splenocytes from Listeria-infected wild-type and YF5,6 TCR ζ transgenic mice were stained with mAb against CD4 and CD8 and analyzed by flow cytometry (A). Splenocytes from Listeria-infected wild-type and YF5,6 TCR ζ transgenic mice were culture overnight with Listeria-infected macrophages. The production of IFN γ by CD8 T cells was assessed by intracellular cytokine flow cytometry.

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VITAE

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