Title
On the signaling of the pre-TCR

Author
Palacios, Emil H.

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On the signaling of the pre-TCR

by

Emil H. Palacios

DISSERTATION

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Preface

A lot goes into getting to the point of writing this preface. And so a lot of folks have helped me get to this point. The biggest thanks goes to my mentor Art Weiss, who after me, probably has the most vested in watching me succeed professionally. It says a lot that Art actually worries about his graduate students and how their projects are progressing. So I am grateful for his ongoing support and interest in my projects and my career. I've always respected and tried to learn as much as I can from his passion for science, his desire to ask important questions (not just 'archival' science) and his perspective that scientific questions should always consider whether the human condition is reflected.

Personally, my wife Marta has been more supportive than I have had the right to expect. We met right after I joined this lab and so she has seen all of the ups and downs of my projects. Moreover, when things haven't gone so well, she's usually been the one to get the worst of it. Or the worst of me, as it were. For her heroic patience and understanding I am very grateful. I am also grateful for our three boyz, the munchkins, Pablo, Diego and Joaquin. The not only keep me grounded in what's important, but they are just a pure joy to have in my life. I am also grateful to my parents for their constant support throughout the years. They've always been great advocates of higher education and that has clearly had a positive impact on me.

In the lab, several people have been important in helping me get through graduate school. Larry Kane and Michelle Hermiston helped me in the early years. Larry was a great guy to have in the lab, despite his Bostonian leanings and he got me excited about joining the lab after rotating with him, though he probably didn't even realize it. Michelle taught me the basics of mouse research when my projects were first starting, plus a nice

bonus of working near Michelle was that her experiments always made mine appear modest and not wasteful. Marianne, the "glue" of the lab always offered a smile and served as my paragon of middle-America, except for maybe the grey duck thing, that was just odd. Marianne, Michelle and Jeroen always offered sound parenting advice for issues like "what if they fight for the orange cup?" or "when do you let them pick out their own outfits" and such. Card carrying members of the "coffee club" and the "lunch club" including Larry, Mike, and "Vick" Vikas will truly be missed. I'd also like to thank my baseball trash-talking friend Lyn who faithfully supported her beloved A's who served as a wonderful foil to my Angels. "Better luck next year!" as I got used to saying to her, never got old. Everyone in the lab has helped make it a great environment in which to work and I will always remember it as such.

Lastly, I'd like to thank my former boss Bob Grant and also Warner Greene for encouraging me and helping me get into grad school. They gave me a lot of autonomy early on as a research technician and gave me my first taste of "biomedical" research for which I will always be grateful.

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Palacios, E. H., and Weiss, A. (2007). Distinct roles for Syk and ZAP-70 during early thymocyte development. J Exp Med *204*, 1703-1715.

Abstract

On the signaling of the pre-TCR

by

Emil H. Palacios

The function of tyrosine kinase activity during canonical T cell antigen receptor (TCR) signaling in T cells has been intensively studied over the past 18 years. This signaling pathway is critical at multiple stages of T cell development. Which kinase family members are used at these stages has been largely described for the Src-family kinases (SFK), but less is known of the Syk-family kinases. Previous studies have suggested that neither of the Syk-family kinases, Syk or ZAP-70, serves a unique function during the earliest stage that requires signaling through the antigen receptor, the β-selection stage that uses the pre-TCR. Moreover, ZAP-70 has previously only been shown to have a unique role in propagating thymic selection signals. This thesis establishes the relative levels of both Syk-family kinases in all major thymocytes subsets. It will demonstrate that Syk and ZAP-70 serve unique, temporally separable functions during early thymocyte development and that pre-TCR signaling continues well after the originally established DN3 stage and on through multiple developmental stages until immediately before positive selection. Further, it will be shown that the phosphatase CD45, a positive regulator of antigen receptor signaling, is also critically required for early developmental progression, including the newly described sustained pre-TCR stages.

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Chapter 1

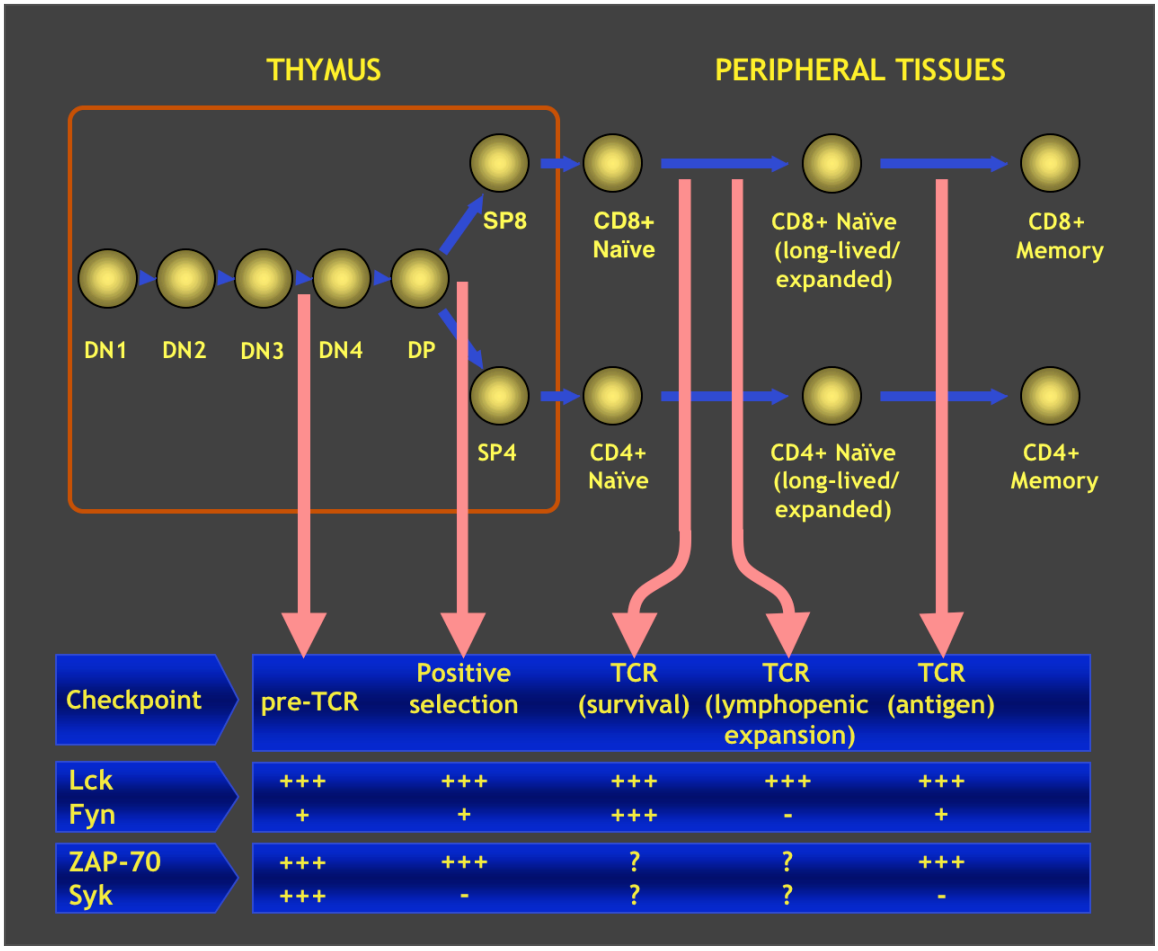
Introduction to TCR-based signaling through development

A primer on T cell development

It is becoming clear that TCR signaling affects the selection and survival of T cells at every stage of development. Since the TCR signaling pathway critically depends on the function of SFKs and Syk-family kinases, a broad understanding of T cell development is necessary in order to appreciate the varying functions of these kinases in the T lineage (Figure 1.1). Thymocyte development proceeds through well-defined stages and has been reviewed in detail elsewhere (Sebzda et al., 1999; Shortman and Wu, 1996). Briefly, lymphocyte progenitor cells (that retain B, T, natural killer, and dendritic cell potential) migrate from the bone marrow to the thymus via the bloodstream. In the thymus, these "DN1" cells ("double-negative", as they do not yet express the T cell co-receptors CD4 or CD8) receive survival and instructive signals to initiate the αβ T cell developmental pathway. DN thymocytes can be divided into at least four major subsets, based on differential expression of the surface proteins CD44 and CD25. The earliest DN1 are $CD44^{+}CD25^{-}$ (and also $CD117^{+}$) and are the cells that initially seed the thymus from the bone marrow. Other DN subsets are defined as $CD44^{+}CD25^{+}$ (DN2), $CD44^{-}CD25^{+}$ (DN3), and $CD44^{-}CD25^{-}$ (DN4). Initially, at least, Notch and IL-7 signals are required to proceed from the DN1 to the DN2 and DN3 stages.

After instructive signals from Notch ligands expressed on thymic stromal cells, the DN3 stage is where the first critical checkpoint during thymocyte development occurs. Progression and expansion past the DN3 stage requires surface expression of the product of a productive chromosomally rearranged TCRβ chain. This newly formed TCRβ chain pairs with an invariant pre-Tα chain. This heterodimer then is transported to the plasma membrane in association with the CD3 and TCRζ chains in order to produce a

Figure 1.1 TCR-based signaling uses SFKs and Syk-family kinases and is necessary throughout T-cell development. The canonical TCR signaling mechanism is fundamental to all stages of T-cell development and peripheral function. Lck & Fyn and ZAP-70 & Syk are critically involved at each stage, but their overlapping function has made accurate assessments of their individual contributions difficult. Animal studies with kinases that are deleted, overexpressed, dominant negative, and dominant active suggest that Lck primarily mediates ITAM phosphorylation throughout the T-cell lineage. However, Lck and Fyn seem equally capable of low-level basal ITAM phosphorylation during nonspecific self-MHC recognition in lymphoid tissue, which is necessary for naïve T-cell survival. Thus, the relative contributions of each kinase presented here are inferred from the currently reported ex vivo studies. Similarly, analysis of Syk-family kinases using deletion or overexpression mutants suggests that either Syk or ZAP-70 function equally well in early thymocyte development, but direct evidence for this is lacking. No data currently is reported for Syk-family function during homeostatic maintenance or lymphopenic expansion although ZAP-70 is implicated due to its relatively high expression compared to Syk in peripheral T cells. A direct measurement of SFK or Syk-family kinase contribution for TCR activation awaits direct analysis in single cells at different stages of development. In addition to cytokines, it is unclear whether TCR signals contribute to memory T-cell survival. See the text for details.


THYMUS
PERIPHERAL TISSUES
DN1
DN2
DN3
DN4
DP
SP8
SP4
CD8+ Naïve
CD8+ Naïve (long-lived/ expanded)
CD8+ Memory
CD4+ Naïve
CD4+ Naïve (long-lived/ expanded)
CD4+ Memory
Checkpoint
pre-TCR
Positive selection
TCR (survival)
TCR (lymphopenic expansion)
TCR (antigen)
Lck
Fyn
+++
+
+++
+
+++
+++
+++
-
+++
+
ZAP-70
Syk
+++
+++
+++
-
?
?
?
?
+++
-

TCR-like signal. This "pre-TCR" signal requires the same adapter proteins, LAT and SLP-76 that are required for TCR signaling in model cell lines and in mature T cells (Figure 1.2). The pre-TCR signal fulfills at least four separable functions, 1) rescue from apoptosis, 2) differentiation, 3) entry into cell cycle, and 4) allelic exclusion (reviewed in (Kruisbeek et al., 2000)). Cells unable to generate a proper pre-TCR signal are arrested and die at the DN3 stage (technically, this is the TCRβ selection stage, but we will refer to all stages by their 'DN' or 'DP' nomenclature for convenience). Proper signaling allows progression through the DN4 stage and to the DP stage ("double-positive", as these thymocytes now express both CD4 and CD8). The next critical checkpoint occurs during the DP stage and is also dependent on a TCR-based signal. Thymocytes are "educated" which means they are selected for non-autoreactivity and proper MHC-restricted usefulness at the DP stage. This stage requires that a newly and properly rearranged TCRα chain pair with the previously expressed TCRβ chain. Since the repertoire of TCRs is stochastically generated via random rearrangements of gene segments and the random pairing of TCRα and β chains, selection for a useful repertoire of TCR bearing cells is critical. This newly formed TCRαβ interacts with complexes of self-MHC molecules that have bound self-peptides and are expressed on stromal or antigen presenting cells, resulting in one of three outcomes. Very strong interactions are likely representative of autoreactivity; these cells are "negatively" selected and die via apoptosis. Cells expressing TCRs unable to generate a positively selecting signal are arrested and die at the DP stage. Medium strength signals are likely to be useful and these cells are "positively" selected. These DP thymocytes down-regulate either CD4 or CD8, becoming "single-positive" (SP) thymocytes, and eventually migrate to the periphery.

Introduction to SFK and Syk-family kinases

Progress towards understanding the basic mechanisms of the mammalian immune system has rapidly advanced over the past 20 years. Whereas many immunologists focused on understanding which cell populations perform specific functions, others focused on elucidating the molecular aspects of cellular activation. Current immunological research is often expected to do both, explain a physiological phenomenon in the context of a proposed biochemical mechanism. As the technologies for studying cellular and molecular immunology continue to improve, so do the models of how molecules function within immune cells under different physiologic circumstances (i.e., development or disease). Our current understanding of the function of the SFKs (Lck and Fyn) and the Syk-family kinases (Syk and ZAP-70), which are key tyrosine kinase families expressed in T cells, exemplifies this trend.

In T cells the function of SFKs and Syk-family kinases is largely described at a biochemical level. Recent work correlates these biochemical characteristics with cellular functions relevant to the organism. The following is a summary of SFK and Syk-family kinase regulation and function in T cell development, homeostasis and activation. First, I present an overview of the distribution of these kinases within the hematopoietic system, including T cells. Then I discuss how the biochemical properties of SFKs and Syk-family kinases are implicated in the activation and function of the TCR signaling pathway. Finally, I introduce the functions of SFK and Syk-family kinases during thymocyte developmental and in peripheral T cells.

SFK hematopoietic distribution

The hematopoietic system contains several SFK members to perform functions that are highly specialized to serve the various lineages. T cells primarily express Lck and Fyn-T (a hematopoietic cell specific isoform which contains an alternatively spliced form of exon 7 that encodes a part of the kinase domain (Cooke and Perlmutter, 1989)). Lck protein is expressed at a fairly constant level throughout development. Fyn protein is transiently decreased at the double positive (DP; CD4+/CD8+) stage of thymocyte development (see below) but increases thereafter in more mature cells (Olszowy et al., 1995) . The expression of Src and Yes and most other SFKs has not been rigorously characterized in all stages of T cell development using contemporary sensitive serologic reagents.

Lck and Fyn domain structure and function

Lck and Fyn are 56 and 59 kDa proteins, respectively, with a domain organization similar to other SFK members. Both molecules have the following domains in common: N-terminal attachment sites for saturated fatty acid addition, a unique region enabling protein-protein interactions, a Src-homology 3 (SH3) domain, an SH2 domain, a tyrosine kinase domain (SH1), and a C-terminal negative regulatory domain.

The N-terminal attachment sites allow covalent addition of the saturated fatty acid chains from myristic and palmytic acid moieties (Koegl et al., 1994; Paige et al., 1993). Whereas Lck and Fyn are both myristoylated and palmitoylated, their localizations are non-overlapping within T cells, suggesting that other domains, including the unique domain of Lck, further regulate localization (Ley et al., 1994; Lin et al., 2000). Whereas

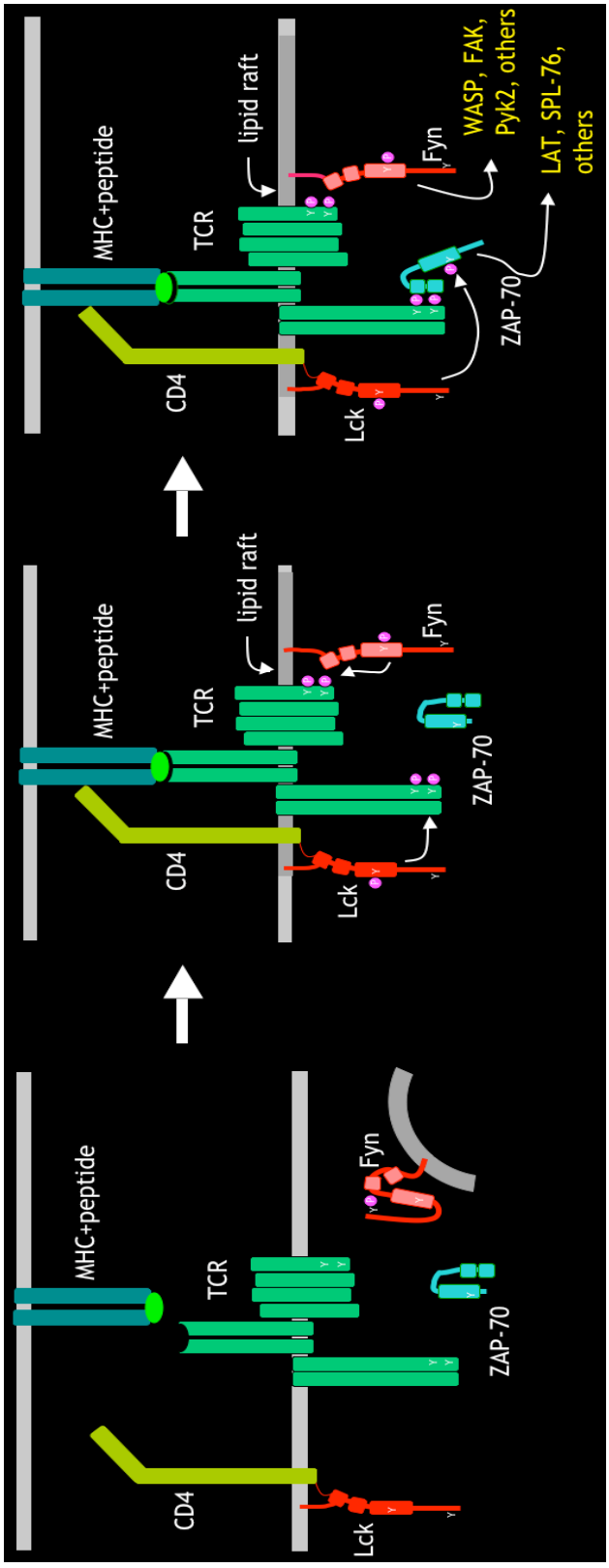
Lck is more abundantly found at the plasma membrane, Fyn colocalizes with centrosomal and mitotic structures.

Model of regulation and activation of proximal events during canonical TCR activation

It has been more than 18 years since Rudd, et al., and Veillette, et al., first demonstrated that Lck is non-covalently associated the CD4 and CD8 coreceptors in T cells (Rudd et al., 1989; Veillette et al., 1988). Soon thereafter, biochemical work in the somatically mutated leukemic T cell line Jurkat (J.CaM1), firmly established the role of the SFK Lck in proximal TCR signal transduction (Goldsmith and Weiss, 1987; Straus and Weiss, 1992). Here, we briefly summarize what has been learned since then (Figure 1.2).

Lck is regulated like other SFKs, via its C-terminal negative regulatory tyrosine. In normal thymocytes or T cells, this site is normally not phosphorylated, rendering Lck in a so-called "primed" state, ready to be activated. During TCR-MHC/peptide interactions, Lck is recruited to the stimulated TCR complex via its non-covalent association with either CD8 or CD4 coreceptors. It is likely that Lck molecules transphosphorylate their activation loop tyrosines (Y394) upon clustering of the coreceptors during antigen recognition. After co-ligation of the TCR and either of the coreceptors, active Lck is proximally positioned to phosphorylate specific tyrosine residues within ITAMs located within the CD3 and TCRζ signaling chains of the TCR, including the three tandem ITAMs within each of the covalently-linked TCRζ chains. ITAMs consist of evolutionarily conserved amino acid sequence motifs of

Figure 1.2 Current model of TCR-based signaling that uses SFKs and Syk-family kinases sequentially in canonical ITAM based signaling. Lck is primarily used for ITAM phosphorylation of TCRζ while Fyn may be phosphorylating the other CD3 chains. In mature T cells, the Syk-family kinase ZAP-70 binds to TCR and is activated by Lck. ZAP-70, in turn, is critical for phosphorylating downstream targets and for generation of secondary messengers. It is unknown which Syk-family kinase is used during pre-TCR signaling, as discussed in the text and shown in Figure 1.3.


MHC+peptide
TCR
CD4
Lck
Fyn
ZAP-70
lipid raft
WASP, FAK,
Pyk2, others
LAT, SPL-76,
others

D/ExYxxLx(7-8)YxxL (Reth, 1989). Sufficient TCR/coreceptor clustering leads to doubly phosphorylated ITAMs and recruitment of Syk-family kinases (ZAP-70 in T cells) to the ITAMs via their tandem SH2 domains. Lck then phosphorylates and activates ZAP-70, which likely trans-autophosphorylates to achieve its full activation. Subsequently, ZAP-70 phosphorylates T cell-specific adapters, such as LAT and SLP-76 leading to the recruitment and activation of other kinase families and enzymes, resulting in secondary messenger generation and culminating in T cell activation. T cell activation is characterized by entry into cell cycle and changes in gene expression consistent with T cells achieving full effector function.

As is true for c-Src, Lck can be sterically inactivated when phosphorylated at a C-terminal tyrosine, Y505 (human protein sequence numbering). When phosphorylated, Y505 promotes intracellular interactions involving the SH2 and SH3 domains of Lck, driving the protein towards an inactive state. The tyrosine kinase Csk serves to phosphorylate Lck Y505, which makes Csk a negative regulator of Src-kinase activity. Csk, via its SH2 domain, constitutively associates with tyrosine phosphorylated PAG/Cbp (PAG), a lipid raft targeted transmembrane adapter protein (Brdicka et al., 2000; Kawabuchi et al., 2000). PAG expression and localization should therefore also affect SFK function (Lindquist et al., 2003). TCR stimulation appears to induce the transient dephosphorylation of PAG, thereby releasing Csk from its plasma membrane anchor (Davidson et al., 2003; Torgersen et al., 2001). The release of Csk from its proximity with Lck may serve to facilitate and/or sustain the activation of Lck and Fyn.

Work in this laboratory and others established that after SFKs are recruited and activated by TCR ligation, they have two important functions for propagating and

amplifying TCR signaling. First, SFKs must phosphorylate ITAMS within the TCR signaling complex and then must phosphorylate and activate Syk family kinases, which are recruited via their tandem SH2 domains to the ITAMs. Studies of the Lck-deficient mouse revealed that levels of TCRζ phosphorylation in resting or stimulated thymocytes were severely reduced (primarily DP thymocytes, see below). Peripheral T cells also had a more moderate decrease in constitutively phosphorylated TCRζ chains (van Oers et al., 1996a). Both observations are consistent with lower Fyn expression in DP thymocytes and higher Fyn expression in mature T cells. These data suggest that major initial downstream targets of Lck are the TCRζ chain ITAMs, and that Fyn can substitute for Lck in peripheral T cells but to a lesser extent.

Finally, after SFKs phosphorylate tyrosine residues within ITAMs, Syk-family kinases are recruited via their tandem SH2 domains to the doubly phosphorylated ITAMs. Once recruited into the TCR complex SFKs then phosphorylate the Syk kinases on tyrosines within interdomain B and thereby activate the recruited Syk-family kinase member. ZAP-70 is the Syk-family kinase primarily expressed in T cells and is highly dependent on a Src kinases for its activation (Iwashima et al., 1994a). Syk may be less dependent on SFKs for its catalytic activation, as it is able to trans-autophosphorylate and activate itself, although its activation is clearly facilitated by SFK (Chu et al., 1998a; Turner et al., 2000). This 2-step model of recruitment of Src- and then Syk-family kinases is a fundamental feature of the TCR tyrosine kinase cascade and has proven generally applicable to all ITAM-based immunoreceptor signaling systems (Latour and Veillette, 2001).

These studies raise various other issues that have not been fully resolved. For example, in resting primary T cells and thymocytes, ZAP-70 is associated with phosphorylated TCRζ chains, yet it itself is not phosphorylated, nor activated, until cross-linking of the TCR. Additionally, despite constitutive TCRζ ITAM phosphorylation, ITAMs within other CD3 chains were not phosphorylated in *ex vivo* T cells (Pitcher et al., 2003). Current data suggest that constitutively phosphorylated TCRζ and bound ZAP-70 may transduce low level signals necessary for mature T cell survival, for lymphopenic expansion within the periphery, or for sensitizing the TCR for antigen stimulation (see below within *Mature T cell function*), but no function for Syk-family kinases has been shown in T cell survival or lymphopenic expansion to date.

SFK function in early T cell development

Lck and Fyn are critical during thymocyte development as evidenced by genetic studies. Equal numbers of DN3 cells are generated in the Lck deficient mouse but DP cells are reduced >10-fold and SP cells are almost undetectable (Molina et al., 1992b). A strict requirement for SFK activity was discovered after Lck and Fyn deficient mice were crossed together and an absolute block at the transition from the DN3 to DN4 stage of thymocyte development was reported (Groves et al., 1996; van Oers et al., 1996b).

Interestingly, when Lck/Fyn doubly-deficient mice were injected with anti-CD3 antibodies, thymocytes could be induced to differentiate to the DP stage, along with a modest expansion in cell number (Chu et al., 1999). When this treatment is given to Rag deficient mice (whose thymocytes are also blocked at this stage due to an inability to rearrange TCRβ chains), DP cells are generated to wild-type levels (Levelt and

Eichmann, 1995). The antibody is thought to cross-link very low levels of CD3 molecules that are able to reach the surface of Rag DN3 cells that lack a pre-TCR. This treatment has no effect in LAT (Zhang et al., 1999) or SLP-76 (Pivniouk et al., 1998) deficient mice, indicating these adaptor proteins are non-redundant and play critical signaling functions needed for pre-TCR signaling and β-selection. When Chu, *et al*, assayed DN3 cells from Lck/Fyn double knockouts or from Rag mice for expression of other SFKs, they noted specific expression of Fgr—normally expressed in myeloid cells – in Lck/Fyn deficient mice. Therefore, Fgr can be expressed at the DN3 stage (though this was not assayed in wild-type DN3s) but is not sufficient for initiating pre-TCR signaling under physiologic conditions.

The regulation of Lck and Fyn during pre-TCR signaling is poorly understood. Classically in mature T cells, Lck is purportedly recruited and cross-linked via its association with CD4 and/or CD8, with the TCR-peptide/MHC complex. DN3 cells have no CD4 or CD8 to recruit Lck. The pre-TCR requires plasma membrane expression for signaling (O'Shea et al., 1997). DN3 cell membranes are reportedly highly enriched for lipid rafts suggesting an equilibrium shift from elemental to coalescent rafts as a potential mechanism for spontaneous Lck clustering and activation once the ITAM-containing pre-TCR is expressed (Dykstra et al., 2003; Haks et al., 2003; Saint-Ruf et al., 2000). One study reported constitutive recruitment of Lck and phosphorylation of ZAP-70 in cells expressing a pre-TCR (Saint-Ruf et al., 2000). However, it should be noted the transformed cell lines used in that study may not reflect physiologic DN3 cells newly expressing a TCRβ chain. A definitive understanding of Src- and Syk-family kinase recruitment and activation during pre-TCR signaling will require analysis of endogenous

DN3 cells, either by biochemical or by single-cell analysis. Moreover, when pre-TCR signaling has been studied in developing cells, most investigators have either inactivated or over-expressed proteins early in the T cell lineage to understand their function. Thus, while we know what can affect pre-TCR signaling, it is not clear what actually happens in the normally developing cell. Whereas CD45, Csk, and PAG are all expected to affect SFK function, little is known of their activity, localization and recruitment dynamics in the DN subset of cells.

Syk-family kinase tissue distribution

Mammalian genomes possess only two family members of the Syk family kinases, ZAP-70 and Syk. The hematopoietic lineages express both kinases among the various subsets where Syk appears to be more broadly expressed than ZAP-70. Syk is also expressed in non-hematopoietic tissues, including some epithelial cells where loss of Syk expression has been linked to cancer progression (Coopman et al., 2000; Stewart and Pietenpol, 2001).

Within the hematopoietic system, Syk is expressed in nearly every lineage, including early thymocytes, B cells, natural killer (NK) cells, dendritic cells, and most myeloid cells (Law et al., 1994). By contrast, ZAP-70 is expressed by a much more restricted subset of lineages (Chan et al., 1992). T cells represent the cell type that uniquely expresses high amounts of ZAP-70 and do not express Syk. Conversely, mature B cells express high amounts of Syk and virtually no ZAP-70. NK cells express lower amounts of both kinases relative to T and B cells. Compared to B cells, myeloid cells express lower amounts of Syk. Recent work indicates that ZAP-70 can have aberrantly

high and disregulated expression within chronic lymphocytic leukemia B cells and its expression correlates with poor disease prognosis (Chen et al., 2005; Rai and Chiorazzi, 2003).

Syk-family kinase structure

Syk and ZAP-70 are 72 and 70 kDa proteins, respectively. Both kinases have the following conserved domain structure, a pair of N-terminal SH2 domains ("tandem domains") separated by a small linker region termed, interdomain A. The two SH2 domains are followed by a long interdomain B region, which is followed by the kinase domain cells (Law et al., 1994). Like all tyrosine kinases, both Syk and ZAP-70 have a conserved "activation loop" that gets phosphorylated on tyrosines (human ZAP-70 Y493 and human Syk Y525 and Y526) when the enzymes are activated (Chan et al., 1995; Kurosaki et al., 1995). Also, complete activation of Syk and ZAP-70 is concomitant with phosphorylation of several conserved tyrosines within interdomain B including human ZAP-70 Y292, Y315, and Y319 (human Syk Y317, Y348, Y352, respectively) (Di Bartolo et al., 1999; Watts et al., 1994). One unusual difference between Syk and ZAP-70 is that for ZAP-70 the N-terminal SH2 domain is structurally incomplete and requires regions of the C-terminal SH2 domain to become complete. Syk has two fully intact SH2 domains (Hatada et al., 1995).

Cellular localization patterns within a given cell of Syk and ZAP-70 are largely overlapping, suggesting that, unlike SFKs, localization is not thought to dictate specificity, if any (Huby et al., 1997). Both enzymes localize to the T cell cortex area near the plasma membrane and do not change much after TCR stimulation. Interestingly,

Ley and colleagues found that the SH2 domains were largely dispensable for proper localization, and that an active kinase domain was instead necessary (Huby et al., 1997). A later study found that ZAP-70 was found within sustained clusters within the cytosol immediately after activation by surface bound ligands (Bunnell et al., 2002). While these clusters lasted at least 20 min, ZAP-70 actively dissociates and reassociates from them. Similar analysis of Syk activity after TCR stimulation was not examined. Both of these reports did however use over-expression systems to analyze localization and therefore may be subject to artifact.

Syk family kinase function in early T cell development

Previous work has shown that both ZAP-70 and Syk are expressed in early thymocyte development, yet mature T cells only express ZAP-70 (Chan et al., 1994). This laboratory reported that the pre-TCR signal itself is upstream of Syk regulation and this signal leads to loss of Syk expression in the T-lineage (Chu et al., 1999). The exact pattern of ZAP-70 RNA and protein expression has not been quantitatively measured in all established thymocyte subsets. Moreover, it remains unclear what signals lead to initial ZAP-70 expression.

Functionally, the genes encoding both proteins have been inactivated in the genome and gross lymphocyte development has already been examined (Cheng et al., 1995; Kadlecek et al., 1998; Negishi et al., 1995b; Turner et al., 1995). Loss of Syk was originally reported to have no gross phenotype on T cell development. αβT cells populated the periphery in roughly normal numbers. Syk has a critical but poorly described function in vascular development and as such, newborn mice deficient for Syk

die immediately after birth making analysis of Syk-deficient T cells difficult. Thus, in these and many subsequent studies, analysis of Syk function in T cells required creating chimeric mice using *syk*$^{-/-}$ stem cells reconstituted into irradiated mice.

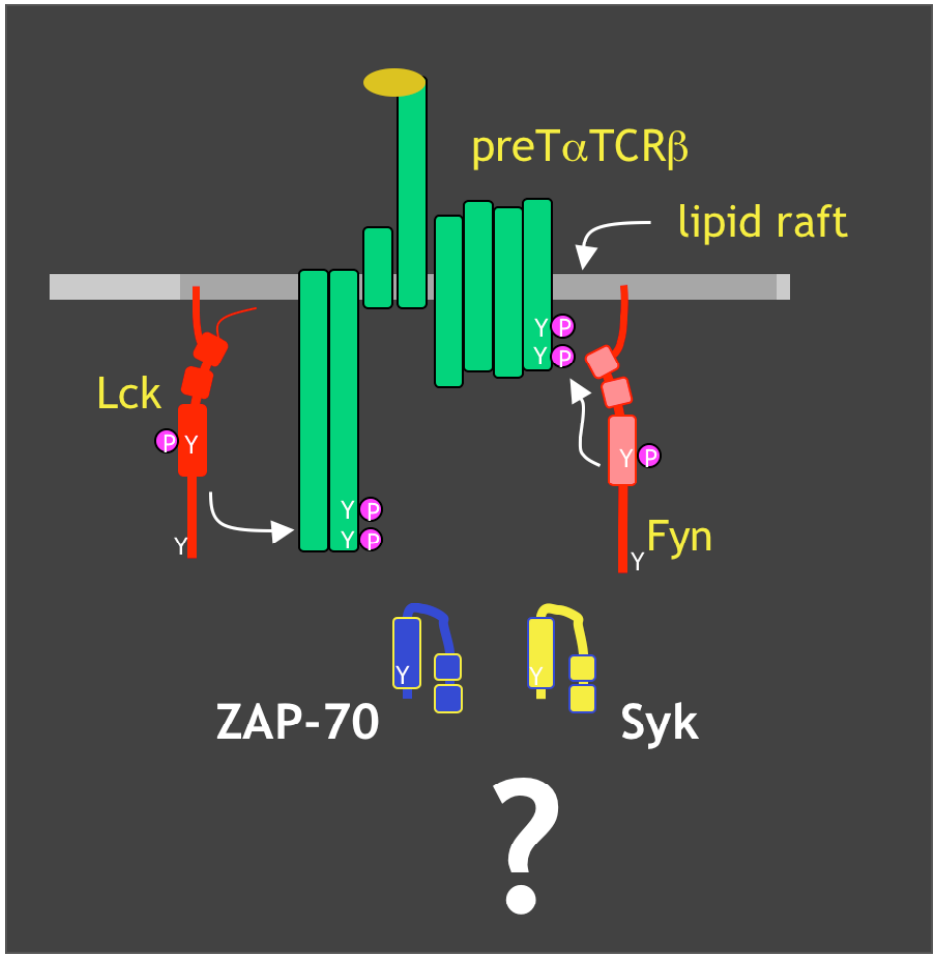
More recently, Colucci and colleagues reported that Syk might generally function to promote overall T cell production, including αβT and γδT cells (Colucci et al., 2000). Reconstitution of partially irradiated Rag2 and cytokine receptor common γ chain *γc*$^{-/-}$ *rag2*$^{-/-}$ double-deficient hosts with *syk*$^{-/-}$ fetal liver stem cells resulted in all major T cell subsets being represented, including CD4^{+}, CD8^{+}, NKT, and γδT cells. However, absolute counts of all subsets were reduced by approximately 1/3 to ½ when compared to mice reconstituted with WT stem cells in parallel assays. Moreover, these researchers also showed that DN3 cells appeared to have accumulated within DN subsets, suggesting that Syk promotes survival and/or signaling at this stage and generally augments αβT cell development. γδT cells also initially develop beginning around this stage (Hayday et al., 1999). Some studies argued γδT development was uniquely impaired in Syk-deficiency, but the results of Colluci, et. al. suggested this was an artifact of making reconstituted mice.

ZAP-70 has a much more established role in T cell development. Knockout studies of ZAP-70 clearly established that this kinase is critical for transducing both positive and negative selection signals during thymocyte education. In these studies, total cellularity appeared largely unchanged, yet no mature SP thymocytes were present. Even after crossing to TCR transgenic receptors that normally result in either positive or negative selection, no SP mature cells developed and no cells were deleted, respectively (Negishi et al., 1995b). This was recapitulated in *in vitro* assays of TCR induced cell

death as a model for negative selection. A recent study identified the *skg* mouse that carried a unique mutation in the C-SH2 domain of ZAP-70 that reduced its association with the TCR and resulted in markedly reduced strength of signaling through the TCR during thymic selection (Sakaguchi et al., 2003). The resulting "shift" in repertoire selection yielded T cells that were specific toward self antigens and could cause autoimmune disease in a genetically susceptible mouse strain. This study provided strong experimental evidence that impairments in TCR signaling during thymic selection could produce self-reactive T cells and that these signals required proper ZAP-70 function.

No specific role for ZAP-70 during the early TCRβ selection stage was observed in these original studies. However, a crucial role for Syk-family kinases in TCRβ selection was revealed when Syk and ZAP-70 mice were crossed together to produce double knockout *syk*$^{-/-}$*zap70*$^{-/-}$ mice (Cheng et al., 1997). Thymocyte development was completely blocked at the DN3 stage, with phenotypic profiles and cellular counts recapitulating that of *rag1*$^{-/-}$mice. *TCR* rearrangements themselves seemed normal. Thus, the initial pre-TCR signal that is supposed to occur as soon as the TCRβ locus rearranges and the newly created pre-TCR achieves surface expression was completely impaired and development completely halted, and this signal depended entirely on either Syk or ZAP-70 (Syk-family kinases) being present. This result has lead to the conclusion that Syk and ZAP-70 are largely redundant through this stage of development (Borowski et al., 2002; Cantrell, 2002; Michie and Zuniga-Pflucker, 2002), and that thymocyte development makes no preferential use of either kinase at any stage until thymic selection occurs in DP cells (Figure 1.3).

Figure 1.3 Current model of pre-TCR-based signaling that uses SFKs and Syk-family kinases and is necessary primarily at the DN3 thymocyte stage. ZAP-70 and Syk are assumed to redundantly function downstream of SFK. Before this thesis, it was unknown whether the pre-TCR preferentially uses one Syk-family kinase or the other. Moreover, expression data of ZAP-70 has never been clearly established in the various early thymocyte subsets. It is believed the pre-TCR does not require any ligand and instead is proposed to initiate a signal when the receptor spontaneously assembles on the cell surface after an "in-frame" TCRβ chain has rearranged. This signaling is characterized by a coalescence of lipid rafts that may serve to cluster the newly assembled pre-TCRs. See text for details.


preTαTCRβ
lipid raft
Lck
Fyn
ZAP-70
Syk
?

As T cells are the only lineage that have evolved to completely replace Syk with ZAP-70, it raises the question of whether there is a unique functionality that T cells require that ZAP-70 performs that is not provided by Syk. This question was provisionally addressed by Chan and colleagues (Gong et al., 1997). Those researchers elegantly expressed Syk within the T lineage and asked whether Syk could, at least when over-expressed, replace ZAP-70 through development. When Syk was over-expressed using the *lck*-proximal promoter, thymocyte development through to the SP stage was grossly rescued and T cells were exported to the periphery. Positive selection appeared to function normally, as loss of the SFK positive regulator CD45 still blocked SP generation. This study did not address several questions, however. Since the *lck*-proximal promoter was used to drive Syk expression, peripheral T cell function could not be evaluated since Syk protein was lost in the resulting T cells. With regard to thymic development, the level of expression of Syk protein was never addressed and this may have been much greater than that expressed in early thymocytes or even in the B cell lineage. Further still, it remains unknown whether these developing thymocytes are indeed as "developmentally fit" as their normal counterparts. Since differentiation and expansion from the early DN3 stage to the DP stage involves several cell divisions (Penit and Vasseur, 1988; Penit et al., 1988), a relative impairment, may look "normal" when first analyzed.

Thymocytes at the SP stage are thought to be either positively selected cells that wait to be seeded to the periphery or are cells that may yet receive a death-inducing negative selection signal, also delivered through the TCR (and perhaps other co-receptors) (Palmer, 2003). One recent study suggested that ZAP-70 may have a unique

role in positively regulating progression of SP cells and the positively selecting signal depends on continued expression of ZAP-70 even after CD69 and TCR up-regulation (Liu et al., 2003).

Other functions for Syk have not been explored in great detail. Of note, one study used DNA microarrays to investigate the transcriptional profile of wild-type thymocytes at most major stages of development and assayed for *syk* mRNA among thousands of other transcripts (Hoffmann et al., 2003). Interestingly, this study found that while *syk* transcripts were down-regulated immediately after the DN3 initial pre-TCR signaling stage in accordance with protein analysis work performed in this laboratory (Chu et al., 1999), *syk* mRNA was found unexpectedly high in DP thymocytes. This result was not explained or elaborated on, but might suggested some role for Syk in DP cells not yet discovered.

Kinase function during the DP to SP transition

After early thymocytes pass the pre-TCR signaling checkpoint, they clonally proliferate, upregulate the coreceptors CD4 and CD8 and wait to be selected or deleted at the DP selection stage. The DP thymocyte is the stage where autoreactive T cells are deleted, useful self-MHC restricted T cells are selected to progress to the most mature SP stage of development, and useless cells die by neglect. It is also a stage where each individual cell is truly bipotential, able to become either a CD4+ or CD8+ T cell, under proper stimulation. While very distinct, these two theoretically intriguing concepts have fueled intense study and have been extensively reviewed (Germain, 2002; Sebzda et al., 1999; Singer, 2002). Knockouts of the coreceptors, MHC molecules, ZAP-70 and CD45

(among others) have clearly established that TCR signaling, after proper self-peptide/MHC molecule interactions, is necessary for positive and negative selection. However, a major problem with studying TCR signaling during DP selection is that when a mouse is made deficient in a critical adapter protein or an entire kinase family, it usually exhibits a complete block in thymocyte development at the earlier, pre-TCR signaling stage. Thus, the compound knockouts of Lck/Fyn or Syk/ZAP-70, or the unique adapters LAT, or SLP-76 do not yield any DP thymocytes, making loss-of-function studies of these classes of enzymes or adaptor proteins at the DP checkpoint impossible.

Efforts to explain the nature of the signals involved in thymocyte selection and distinct lineage commitment (CD4 vs CD8 lineages) has reached the status of immunology lore, where paradigms have shifted from "classic" models of instructive to stochastic and back to instructive. The complex nature of CD4 and CD8 regulation during thymocyte selection obscured early models of lineage commitment (Germain, 2002). Nowadays, the large amount of empirical data that have emerged suggest models that are not so different and are all based on the strength of the TCR signal, in some form. It should be pointed out, however, that differences in duration of the TCR signal as opposed to the intensity of the signal may underlie lineage commitment of CD4 versus CD8 cells (Singer, 2002).

Since CD4 and CD8 associate with and recruit Lck, and CD4 and CD8 coreceptors are required to direct cells into their respective lineages, it was quickly inferred that Lck transduces signals of positive selection, and evidence supports this (Hernandez-Hoyos et al., 2000; Legname et al., 2000). Moreover, until recently, it was

not formally known if other upstream molecules are involved. When the SFK negative regulator Csk was deleted from the T cell lineage, all TCRs were directed to the CD4 lineage, regardless of MHC restriction or even in the absence of MHC molecules (Schmedt et al., 1998; Schmedt and Tarakhovsky, 2001). Taken together, these studies strongly indicate that Lck is responsible for transmitting to downstream pathways the positive selection signal and that this signal also determines CD4 versus CD8 lineage fate.

Effects of CD45 on TCR signaling and thymocyte development

The receptor-like tyrosine phosphatase CD45 is highly expressed on all nucleated hematopoietic cells. In fact, CD45 is so highly expressed in lymphoid cells, it comprises approximately 10% of the entire cell surface protein mass, suggesting a prominent conserved function (Thomas, 1989). Evidence of CD45 function in T lineage cells is found in loss of function studies performed in cell lines, mouse models, and in human studies (Byth et al., 1996; Kishihara et al., 1993; Kung et al., 2000; Mee et al., 1999). Various T-lymphoid cell lines, including the Jurkat human T lymphoid cell line that lack CD45 are highly defective in TCR signaling (Peyron et al., 1991; Volarevic et al., 1992). As discussed previously (in the SFK section), CD45 appears to be the major phosphatase responsible for dephosphorylating the negative regulatory tyrosine within the C-terminus of Lck and Fyn. In all these CD45-deficient cell lines and in *in vivo* models reported, Lck and to a lesser extent Fyn, are hyperphosphorylated at the LckY505 equivalent position and display decreased *in vivo* kinase activity when tested (Koretzky et al., 1991; Ostergaard et al., 1989; Stone et al., 1997). As mentioned previously, phosphorylation of

the C-terminal tyrosine by Csk promotes intramolecular interactions that include the SH2 and SH3 domains, keeping the SFK in a "closed" conformation. The major function of CD45 is expected to be dephosphorylation of the inhibitory C-terminal tyrosine, rendering Lck and Fyn open and "primed" and ready for activation. Thus, CD45 and Csk function to positively and negatively regulate SFK function in T cells, respectively, with directly antagonistic actions.

Thymocyte development is nearly completely impaired in CD45-deficient mice at the positive selection stage (DP stage) of development when signaling through the mature TCR is first required. This leads to a failure to develop peripheral T cells. These blocked DP cells also exhibit hyperphosphorylation of Lck at Y505. Similarly, humans with inactivating mutations in CD45 also have a paucity of peripheral T cells and are impaired in TCR signaling (Byth et al., 1996; Kishihara et al., 1993).

Interesting, development through the TCRβ-selection stage is only mildly impaired in CD45-deficient mice, about 2-fold when measured as a ratio of DP to DN cells (Byth et al., 1996). This stage critically requires signaling through the pre-TCR, which also uses the canonical signaling machinery (see earlier section, *A primer on T cell development*). Thus, either another phosphatase can substitute, or CD45-independent pre-TCR signaling can facilitate progression past this critical checkpoint. Since SFK signaling appears critical for this pre-TCR signaling stage (Groves et al., 1996; van Oers et al., 1996b) while CD45 is not, either SFK or Syk-family kinase activation is occurring without CD45 at this developmental stage, *in vivo*. Whether CD45 is necessary at stages between the DN3 pre-TCR and the DP positive selection checkpoint has never been addressed.

CD45 regulation of Syk-family kinase function

Previous work in this laboratory has provided strong evidence that the Syk-family tyrosine kinases, Syk and ZAP-70, are differentially regulated by SFK and by CD45. Chu and colleagues found that in Jurkat T-lymphoid cell lines CD45 and Lck were dispensable for TCR signaling provided Syk was highly expressed instead of only ZAP-700 (Chu et al., 1996).

Two CD45-deficient Jurkat derivatives were compared, JS-7 and J45.01. The J45.01 clone was completely impaired in TCR signaling, while the JS-7 clone had a relatively normal overall tyrosine phosphorylation response after TCR stimulation. Immunoblotting of various TCR signaling proteins, showed at least one marked difference between J45.01 and JS-7 cells: differential expression of Syk. Both cells expressed roughly equal amounts of other TCR signaling components, such as TCRζ, Lck, and even ZAP-70. However, only the JS-7 expressed robust amounts of Syk. They further showed Syk co-immunoprecipitated with TCRζ after TCR stimulation, and that only Syk could propagate TCR signals in the absence of Lck when transfected into the Lck-deficient JCam1.6 cell line. Thus, in Jurkat T-lymphoid cells, Syk can mediate TCR signaling independently of CD45 or Lck upstream activation. It is unclear if Syk still required some other residual SFK function, as expression of other SFK members was never addressed in these cells, but it is clearly not as dependent on Lck or CD45 function as ZAP-70.

Whether the loss of CD45 differentially affects Syk or ZAP-70 function has not been addressed in primary cells since peripheral lymphocytes express either Syk (B cells)

or ZAP-70 (T cells) and not both. NK cells do express both kinases and ITAM signaling is blocked when Syk and ZAP-70 are both deleted (Colucci et al., 2002), but the role of CD45 has not been addressed within the context of Syk and ZAP-70 function in NK Fc-receptor signaling.

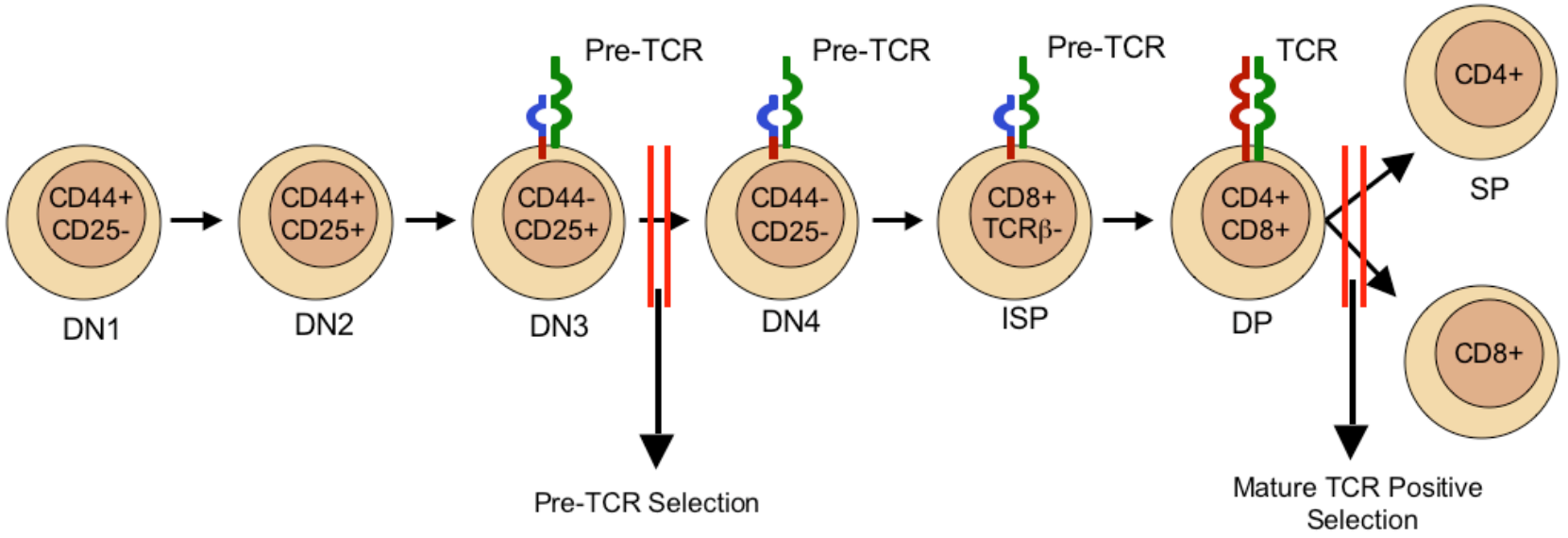
One interesting feature of T-cell development is that both Syk and ZAP-70 are coexpressed early in development as thymocyte progenitors differentiate down the αβTCR developmental pathway (Figure 1.4). Previous work in this laboratory indicated that early DN thymocytes, when considered in aggregate and addressed by immunoblotting, express both ZAP-70 and Syk (Chan et al., 1994). This work, however, did not address the various discrete subsets that comprise the DN cells. These early stages of thymocyte development may therefore provide a unique model to investigate the *in vivo* effects of CD45 on ZAP-70 and/or Syk function in a primary T lineage cell.

Conclusions

SFK and Syk-family kinases appear to be involved in nearly all stages of T lymphocyte development and function (Figure 1.1). Whereas much is known of SFK and Syk-family kinases mechanism and activity, a complete understanding of SFK and Syk-family kinases activity in individual cells that are developing, surviving and responding in the animal is still lacking. Moreover, we are only beginning to understand the real-time effects of modifiers of kinase function, whose activity may also change during development and upon activation. This thesis will focus on the *in vivo* function of the Syk family kinases through early thymocytes development. Currently, the role for each

Figure 1.4 Current model of pre-TCR/TCR signaling stages and Syk-family kinase function throughout development. “Expression” depicts schematic of relative expression patterns now established for Syk-family kinases for all thymocyte stages. “Function” indicates proposed requirement for pre-TCR signaling and which Syk-family kinase is preferentially used. The initial pre-TCR signal is propagated as soon as a TCRβ chain is properly expressed is uses either Syk or ZAP-70 equally well. This signal leads to many rounds of cell division and gene expression changes that occur during normal DP cell development. Syk expression is down-regulated and only ZAP-70 can signal for positive or negative selection. No pre-TCR or TCR signal is postulated to be necessary for progression through the DN4, ISP or DP stage until positive selection.

Function: **ZAP-70/Syk** **ZAP-70** **ZAP-70**



kinase, Syk and ZAP-70 is schematically shown in Figure 1.4 for thymocyte development.

This thesis will describe evidence that addresses whether Syk and ZAP-70 truly are equally expressed and functionally redundant during the earliest stages of thymocyte development. The following will be presented. Chapter 2 will describe the protein expression patterns of both Syk and ZAP-70 for all distinguishable thymocyte subsets using optimally labeled-antibodies. Then, an *in vivo* "loss of function" line of experiments will describe whether thymocytes lacking Syk or ZAP-70 have impaired developmental fitness when forced to compete against each other or to WT cells. Chapter 3 will describe the effects of genetically deleting both ZAP-70 and CD45 in the same mouse and how early thymocyte development is perturbed after loss of two purportedly positive regulators. Chapter 4 will discuss implications of these findings and suggest future avenues of research. A revised version of the schematic shown in Figure 1.4 will be proposed as a model of Syk-family kinase function in early thymocyte development.

Chapter 2

Distinct roles for Syk and ZAP-70 during early thymocyte development

Abstract

The spleen tyrosine kinase (Syk) and ζ-associated protein of 70 kDa (ZAP-70) tyrosine kinases are both expressed during early thymocyte development, but their unique thymic functions have remained obscure. No specific role for Syk during ß-selection has been established, and no role has been described for ZAP-70 before positive selection. We show that Syk and ZAP-70 provide thymocytes with unique and separable fitness advantages during early development. Syk-deficient, but not ZAP-70–deficient, thymocytes are specifically impaired in initial pre-TCR signaling at the double-negative (DN) 3 ß selection stage and show reduced cell-cycle entry. Surprisingly, and despite overlapping expression of both kinases, only ZAP-70 appears to promote sustained pre-TCR/TCR signaling during the DN4, immature single-positive, and double-positive stages of development before thymic selection occurs. ZAP-70 promotes survival and cell-cycle progression of developing thymocytes before positive selection, as also shown by in vivo anti-CD3 treatment of recombinase-activating gene 1–deficient mice. Our results establish a temporal separation of Syk family kinase function during early thymocyte development and a novel role for ZAP-70. We propose that pre-TCR signaling continues during DN4 and later stages, with ZAP-70 dynamically replacing Syk for continued pre-TCR signaling.

Introduction

ZAP-70 and Syk are cytoplasmic protein tyrosine kinases involved in immunoreceptor signaling. Both have been implicated in TCR signaling during thymocyte development. The ZAP-70-deficient mouse displays an absolute block in thymocyte development at the positive selection stage (transition from $CD4^+CD8^+$ double positive "DP" to $CD4^-CD8^+$ or $CD4^+CD8^-$ single positive "SP" stages) whereas the Syk-deficient thymus shows no obvious block (Cheng et al., 1995; Turner et al., 1995). Strikingly, the combined loss of both kinases results in an absolute block at the earlier TCRβ selection stage, a stage where thymocytes are selected for rearrangements of in-frame TCRβ chains (Cheng et al., 1997). This finding, combined with an absolute requirement for Src-family kinase signaling (Groves et al., 1996; van Oers et al., 1996b) is consistent with the canonical two-step immunoreceptor pathway originally described in T cell lines also applying to this early step of thymocyte development (Iwashima et al., 1994b; Palacios and Weiss, 2004; Weiss and Littman, 1994). That is, sequential Src-family kinase dependent ITAM (immunoreceptor tyrosine-based activation motif) phosphorylation, followed by Syk-family kinase signaling, is required for normal progression through β-selection.

Successful rearrangement and expression of an in-frame TCRβ chain creates a protein that pairs with the invariant pre-Tα (pTα) chain to form a pre-TCR. The pre-TCR engages the already expressed signaling machinery in the immature thymocyte in an apparent ligand-independent manner and generates a signal (Irving et al., 1998). It is widely assumed that simple expression of a functional pre-TCR at the β-selection stage generates a signal that, combined with other signals such as TNF and Notch signaling, are sufficient to generate normal DP cells that are ready to undergo thymic selection

(Borowski et al., 2002; Cantrell, 2002; Ciofani et al., 2004; Michie and Zuniga-Pflucker, 2002). The pre-TCR signal has many purposes including rescue from apoptosis, proliferation and differentiation among others (Borowski et al., 2002; Michie and Zuniga-Pflucker, 2002). Mice unable to rearrange TCR genes or signal through the pre-TCR illustrate the critical importance of β-selection. For example, *rag*$^{-/-}$ (Rag KO) thymocytes cannot rearrange TCRβ or TCRγδ loci and are completely arrested at this stage with thymic cellularity reaching only 2% that of wild-type (WT).

The β-selection stage is readily identified in the mouse. Thymocytes from mice deficient in components of pre-TCR signaling are blocked at the CD3$^-$4$^-$8$^-$11b$^-$19$^-$DX5$^-$ Gr1$^-$TCRγδ$^-$CD44$^-$CD25$^+$ (Lin$^-$) double-negative 3 (DN3) stage. Thus, *rag*$^{-/-}$ thymocytes or those lacking non-redundant adapters Slp-76 or LAT or enzymes such as Src- or Syk-family kinase members are completely blocked at DN3 (Cheng et al., 1997; Clements et al., 1998; Groves et al., 1996; Pivniouk et al., 1998; van Oers et al., 1996b; Zhang et al., 1999). Moreover, since no signal is generated, the DN3 cells remain small and quiescent, unable to enter cell cycle and achieve a blast phase morphology as occurs in normal development. Although the double knockout of ZAP-70 and Syk suggests a degree of redundancy of these two kinases, it is not known whether ZAP-70 or Syk is preferentially used during β -selection.

After TCRβ selection and pre-TCR signaling, thymocytes proceed through a sequence of proliferation and differentiation before reaching a second critical selection checkpoint at the CD4$^+$CD8$^+$ double-positive (DP) stage. Similar to the DN3 pre-TCR signaling stage, thymic selection also critically depends on the TCR signaling machinery. Positive (and negative) selection depends on ZAP-70, TCRα and MHC molecules as

shown by the respective knock-outs (Berg and Kang, 2001). However, absolute blocks at the DN3 stage may preclude assessing a role at later stages. For example, it had been assumed that Slp-76 is required for positive/negative selection and peripheral T cell activation yet direct evidence was lacking until recently for thymic selection (Maltzman et al., 2005). Similarly, the pre-TCR may be necessary and functioning at later stages, such as between DN3 and DP, and this may be masked by absolute blocks at DN3 (Slp-76 and LAT knockouts) or progression to the DP stage because of kinase family redundancy (Fyn and Lck, as well as ZAP-70 and Syk knockouts). The transcription factors bHLH, TCF/LEF and Egr-families and the nuclear hormone receptor RORγ are implicated (reviewed in (Kruisbeek et al., 2000)) during these stages. These factors are thought to act at the immature single-positive $CD8^{+}CD4^{-/lo}TCR\beta^{lo}$ (ISP) stage immediately preceding the DP stage or at the DP stage, before selection. Aside from these factors there are few reports of mutations that interfere with development between the DN3 and DP stages. It is possible that proper pre-TCR signaling using either Syk or ZAP-70 protein is necessary for normal transcription factor induction and progression during the stages between DN3 and DP.

To investigate the individual roles of Syk and ZAP-70 during early thymocyte development, we competed thymocyte progenitors from *syk*$^{-/-}$ and *zap70*$^{-/-}$ donors against each other as they repopulated a normal thymus after depleting the endogenous thymocytes. We found that only *syk*$^{-/-}$ thymocytes are specifically impaired in progressing past the DN3 β-selection stage of development despite upregulation of ZAP-70 within TCRβ-selected DN3. Unexpectedly, only *zap70*$^{-/-}$ thymocytes are uniquely impaired after the DN3 stage and before DP, despite continued Syk expression within DN4 and ISP

stages. Our experiments indicate unexpected and distinct functions for Syk and ZAP-70 during early development and suggest that pre-TCR/TCR signaling continues during DN4, ISP, and DP stages to generate normal numbers of DP cells before they undergo thymic selection.

Results

Syk and ZAP-70 Proteins provide thymocytes with differential fitness during development

We used competitive repopulation of thymus in irradiated hosts as a sensitive and discriminating assay for *in vivo* thymocyte development. Such a competitive repopulation strategy might reveal subtle differences in proliferative capacities, or usage of limiting growth factors, for example. We used stem cell progenitors from *zap70*$^{-/-}$ and *syk*$^{-/-}$ embryos or animals and distinguished the two donor populations based on the congenic markers, CD45.1 and CD45.2 (Shen et al., 1985). This strategy is outlined in the Figure 2.1. Lethal irradiation of WT hosts and reconstitution with donor stem cells resulted in about 98-99% replacement of all thymocytes within 4 weeks by all genotypes tested (unpublished data). The 1-2% residual host cells detected were always within the mature SP subsets. All other subsets were routinely replaced to >99% by donor-derived cells.

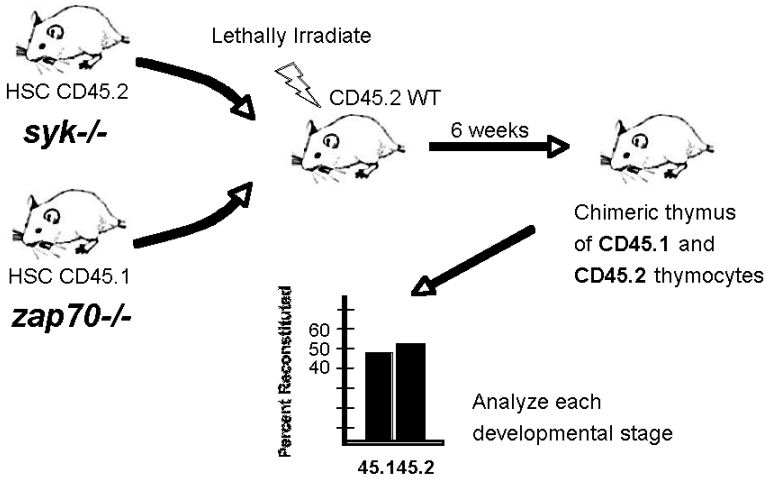
Since thymocyte development occurs as a well-characterized series of stages, the percent represented by each genotype at a given stage is indicative of the developmental fitness of the thymocyte up until that stage. Any impairment or enhancement in fitness would result in a decrease or increase, respectively, at the next stage of development. The competitive repopulation of the thymus using *syk*$^{-/-}$ versus *zap70*$^{-/-}$ progenitors is shown in Figure 2.2. Injection of equal amounts of bone marrow (BM) or fetal liver (FL) resulted in roughly equal representation at the earliest DN2 and DN3 stages identified. Equal representation of *syk*$^{-/-}$ and *zap70*$^{-/-}$ thymocytes within these earliest subsets suggests neither kinase has a significant function in development up until at least the

Figure 2.1. General strategy used for creating and analyzing chimeric mice for competitive repopulation of thymus using fetal liver (FL) stem cells.

(A) FL are harvested from E15.5 fetuses from timed matings.

(B) Assessment of contribution of each donor to various subsets determined by flow cytometry. To insure an unbiased assessment, all subpopulations were identified first, followed afterward by interrogation of the CD45 allele marker.

A


Lethally Irradiate
HSC CD45.2
syk-/-
CD45.2 WT
6 weeks
HSC CD45.1
zap70-/-
Chimeric thymus
of CD45.1 and
CD45.2 thymocytes
Percent Reconstituted
60
50
40
45.145.2
Analyze each
developmental stage

B

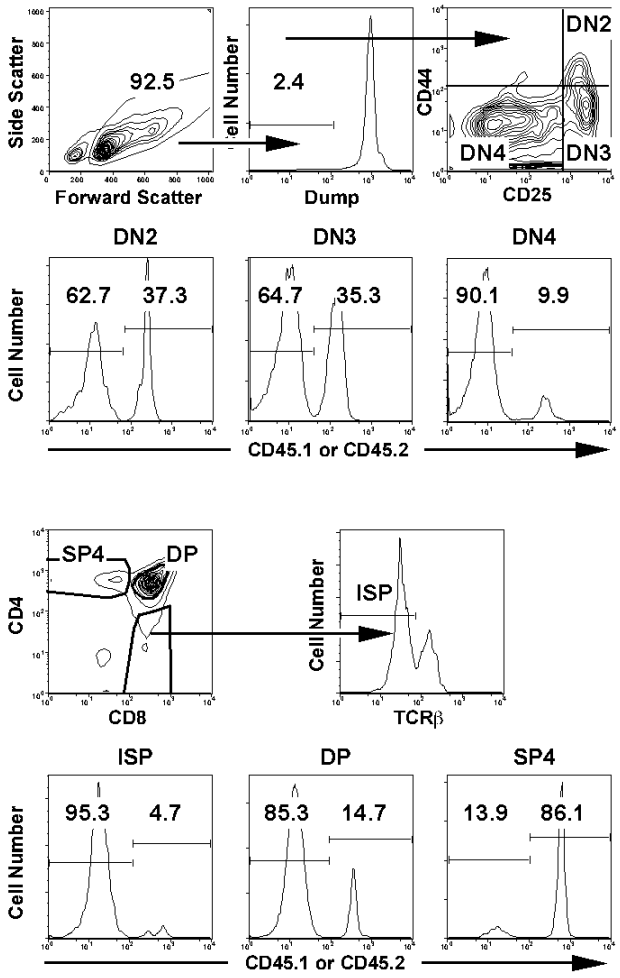
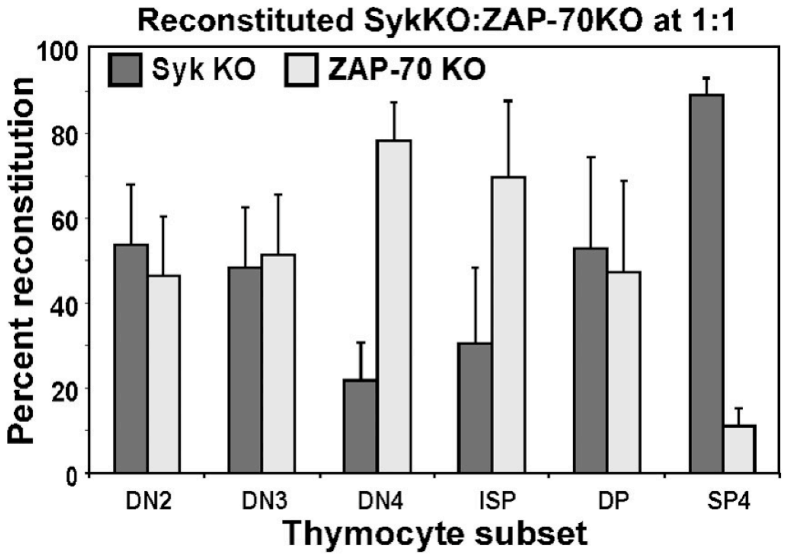

Side Scatter
92.5
Forward Scatter
Cell Number
2.4
Dump
CD44
DN2
DN4
DN3
CD25
DN2
DN3
DN4
Cell Number
62.7
37.3
64.7
35.3
90.1
9.9
CD45.1 or CD45.2
CD4
SP4
DP
CD8
Cell Number
ISP
TCRβ
ISP
DP
SP4
Cell Number
95.3
4.7
85.3
14.7
13.9
86.1
CD45.1 or CD45.2

Figure 2.2. Syk-deficient and ZAP-70-deficient thymocytes show differential fitness during development

Competitive repopulation assay where *syk*$^{-/-}$ and *zap70*$^{-/-}$ bone marrow (BM) cells were injected at a 1:1 ratio and the reconstituted chimeric thymus was analyzed 6 weeks later. Syk-deficient BM was created by reconstituting lethally irradiated WT mice with *syk*$^{-/-}$ fetal liver (FL) and harvested after 8 weeks. Data are representative of four mice. Bars depict mean +/-SD. Experiment was repeated using FL from both genotypes with similar results.


Reconstituted SykKO:ZAP-70KO at 1:1
Syk KO
ZAP-70 KO
Percent reconstitution
100
80
60
40
20
0
DN2
DN3
DN4
ISP
DP
SP4
Thymocyte subset

DN3 stage. In striking contrast, the two populations are differentially represented at multiple stages immediately after DN3. At least two changes seemed to occur. *Syk*$^{-/-}$ thymocytes became underrepresented at the DN4 stage when compared to DN3. After that, *zap70*$^{-/-}$ thymocytes became underrepresented during progressing from DN4 to DP stages. These could be explained in multiple ways. One possibility is Syk provides a positive regulatory role at the DN3 stage of development (thus *syk*$^{-/-}$ accumulate at DN3) while ZAP-70 provides a different positive regulatory role at the DN4 and ISP stages. Another possibility is ZAP-70 provides a negative regulatory role at DN3 allowing *zap70*$^{-/-}$ thymocytes a greater fitness advantage in transitioning from DN3 to DN4. Similarly, *syk*$^{-/-}$ thymocytes may have an advantage in progression from DN4 to DP. Our results differ from previous work that had reported that ZAP-70's only unique function in thymocyte development was during thymic selection, at the DP stage (Negishi et al., 1995a). We also saw this effect in our assays as ZAP-70 deficiency resulted in a complete block in positive selection as shown by the decrease in representation of *zap70*$^{-/-}$ thymocytes between the DP and SP4 stages. These data reveal that Syk and ZAP-70 uniquely contribute to the developmental fitness of early thymocytes at distinct stages well before positive selection occurs.

Syk And ZAP-70 Proteins are Inversely Expressed During Early Thymocyte Development

To understand the basis of differential fitness between *syk*$^{-/-}$ and *zap70*$^{-/-}$ developing thymocytes, we sought to determine the expression patterns of both kinases during all stages of thymocyte development. To investigate the expression patterns of Syk and

ZAP-70 kinases, we quantitated the relative levels of mRNA in all thymocyte subsets. We sorted cells and used real-time fluorogenic RT-PCR to quantitate mRNA expression. The DN subsets were isolated based on the standard phenotyping: $CD25^{+}CD44^{+}$ (DN2), $CD25^{+}CD44^{-}$ (DN3), $CD25^{-}CD44^{-}$ (DN4). We found that *Syk* mRNA was robustly expressed at DN2, whereas *Zap70* was hardly detectable, almost 100-fold less than in peripheral T cells (Figure 2.3A). *Zap70* is first upregulated within the DN3 population and increases thereafter. Interestingly, *Syk* mRNA expression decreases from DN3 to DN4, and spikes at the DP stage, before decreasing again at the SP stage. This pattern of Syk-family kinase mRNA expression is consistent with another recent study (Hoffmann et al., 2003).

After characterizing mRNA expression, we investigated ZAP-70 and Syk protein expression. We previously characterized Syk (Chu et al., 1999), but not ZAP-70 protein expression during thymocyte development. To do so, we conjugated a monoclonal (mAb) antibody previously made in this laboratory against ZAP-70 (Qian et al., 1997) to AlexaFluor dyes. This gave a 25-fold increased signal in T versus B cells. This was specific as NK cells and the 1-2% remaining T cells from $zap70^{-/-}$ mice provided a negative control (unpublished data).

Expression of ZAP-70 and Syk in all stages of thymocyte development is shown in Figure 2.3B. The overall pattern of expression of Syk agrees with our previous findings (Chu et al., 1999). We found ZAP-70 expressed at low levels during the earliest stages of development. In fact, robust ZAP-70 expression is not seen until DN4, which exhibits a 4-5-fold increase in ZAP-70 levels compared to DN3. ZAP-70 protein continues to increase thereafter (Figure 2.3B-2.3C). $CD8^{+}TCR\beta^{hi}$ SP thymocytes had

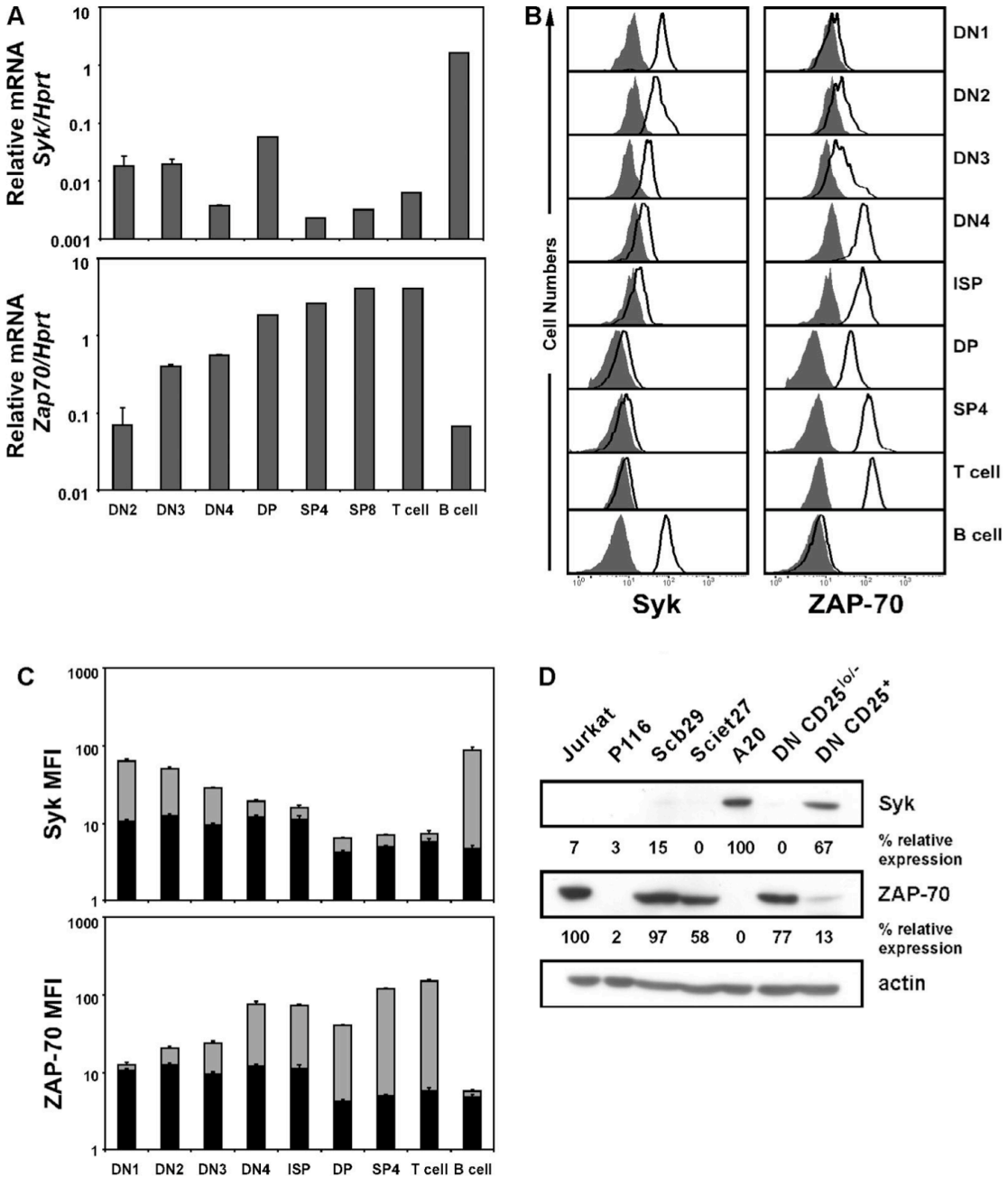
Figure 2.3. ZAP-70 and Syk protein are differentially expressed during thymocyte development

(A) The bar graphs (below) indicate mean +/-SD of real-time quantitation of *Zap70* and *Syk* mRNA from the sorted cells (>98% purity) relative to a control gene (*Hprt*). The entire sort and RNA quantitation was performed twice using two groups of 20 mice with similar results.

(B) Flow cytometric histograms showing ZAP-70 and Syk protein expression in all major thymocytes subsets. Shaded histograms show an isotype-matched mouse IgG1 conjugated to the same fluorescent dye.

(C) Bar graphs quantitatively present the means +/-SD of ZAP-70 and Syk MFI protein expression as shown in (B) from 3 adult animals. This was repeated at least 16 times with similar results. Black bars indicate background IgG1 MFI.

(D) Immunoblot of ZAP-70 and Syk protein expression in sorted $CD25^{+}$ (DN2/3) and $CD25^{lo/-}$ (DN1/4) DN thymocytes at >98% purity (unpublished data) and various lymphoid cell lines. Densitometry was used to quantitate the relative expression levels of the kinases in each lane.


A
Relative mRNA Syk/Hprt
Relative mRNA Zap70/Hprt
10
1
0.1
0.01
0.001
DN2 DN3 DN4 DP SP4 SP8 T cell B cell
B
Cell Numbers
DN1
DN2
DN3
DN4
ISP
DP
SP4
T cell
B cell
Syk
ZAP-70
C
Syk MFI
ZAP-70 MFI
1000
100
10
1
DN1 DN2 DN3 DN4 ISP DP SP4 T cell B cell
D
Jurkat
P116
Scb29
Sciet27
A20
DN CD25lo/-
DN CD25+
Syk
7 3 15 0 100 0 67
% relative expression
ZAP-70
100 2 97 58 0 77 13
% relative expression
actin

slightly more ZAP-70 than $CD4^+$ SP thymocytes, owing to their slightly larger size (unpublished data).

We sorted $CD25^+$ DN cells (pooled DN2/3) and performed immunoblot analysis on lysed cells (Figure 2.3D) to confirm low ZAP-70 protein expression in DN3. We also sorted $CD25^-$ DN cells and compared these cells to various lymphoid cell lines. This analysis confirmed that Syk is easily detectable within DN2/3 cells while ZAP-70 is expressed at low levels. Overall, Syk and ZAP-70 are reciprocally expressed across thymocyte development, where Syk is highly expressed in early development and ZAP-70 is highly expressed in later development. However, there is significant overlap where both kinases are detectable, including the DN4 and ISP stages.

Syk is Necessary for Normal Progression Past DN3 Only

Intracellular flow cytometry combined with our initial competitive repopulation assays suggested that Syk may be serving a positive regulatory role at DN3 and serves a reduced role from DN4 through DP stages as its protein expression disappears. To test this possibility, we competed Syk-deficient progenitors against WT in a repopulating thymus. This ruled out confounding effects brought on by competing against *zap70*$^{-/-}$ thymocytes. Because Syk is highly expressed in DN1 and possibly earlier, we assayed representation of DN1 after varying the ratios of FL injected into the host as a measure of fitness of colonizing the thymus. Analysis of DN1 representation revealed no significant changes in donor ratios (Figure 2.4A) compared to what was injected. Therefore, colonization is not significantly affected in *syk*$^{-/-}$ thymocyte progenitors after lethal irradiation of the host consistent with our results in Figure 2.2.

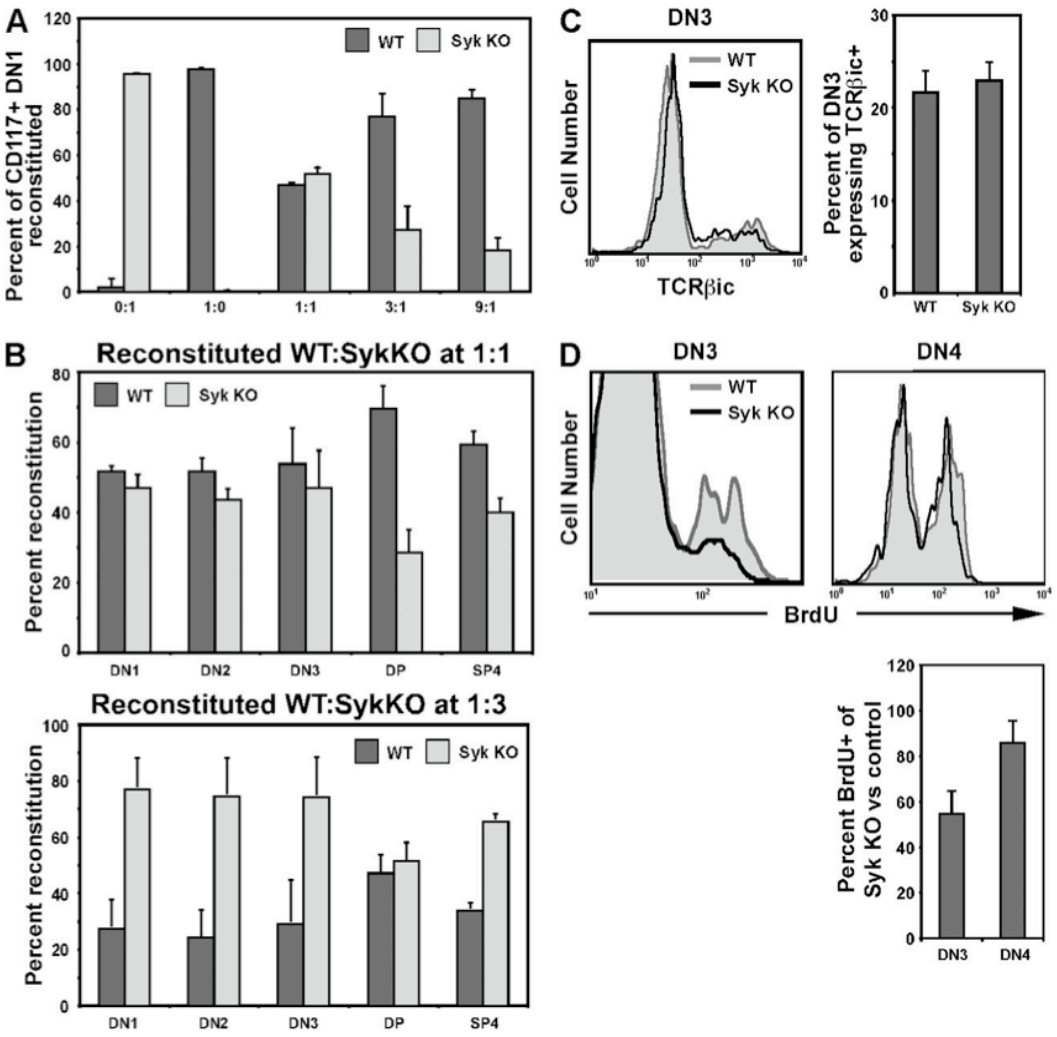
Figure 2.4. Syk is uniquely required for optimal progression past DN3

(A) WT and *syk*$^{-/-}$ deficient FL cells from E15.5 embryos were injected at various ratios into lethally irradiated B6 hosts and the reconstituted chimeric thymus was analyzed 5-7 weeks later. DN1 was characterized by further excluding CD25^{+} events while gating on CD44^{+}CD117^{+}. Data indicate 3-5 mice per group in all experiments. Bars depict mean +/-SD.

(B) Contribution of each genotype to major subsets after competing at 1:3 and 1:1 ratios.

(C) Histograms showing representative intracellular TCRβ within DN3 for WT and *syk*$^{-/-}$ within the same mouse. Bar graph depicts mean +/-SD of percent TCRβ^{+}.

(D) Histograms of representative BrdU incorporation within DN3 and DN4 of WT and *syk*$^{-/-}$ within the same mouse 1.5 hrs after injecting 2 mg BrdU. Bar graph indicates mean +/-SD of BrdU^{+} *syk*$^{-/-}$ thymocytes as a percentage of the mean of BrdU^{+} WT thymocytes within the same mice.


A
Percent of CD117+ DN1 reconstituted
WT
Syk KO
0:1
1:0
1:1
3:1
9:1
B
Reconstituted WT:SykKO at 1:1
Percent reconstitution
DN1
DN2
DN3
DP
SP4
Reconstituted WT:SykKO at 1:3
C
DN3
Cell Number
TCRβic
Percent of DN3 expressing TCRβic+
D
DN4
BrdU
Percent BrdU+ of Syk KO vs control

We then assessed donor representation within subsequent stages of thymocyte development. Figure 2.4B shows *syk*$^{-/-}$ thymocytes were consistently underrepresented only after DN3. This was true at all starting ratios. Interestingly, *syk*$^{-/-}$ thymocytes were overrepresented within the CD4^{+} SP and CD8^{+} SP TCR^{hi} (unpublished data) populations. This was true whether the irradiated host was CD45.1^{+} or 45.2^{+}, suggesting *syk*$^{-/-}$ thymocytes may be more fit than WT at progressing from the DP to the SP stage.

We focused our studies on whether Syk-deficiency impairs TCRβ rearrangement and protein expression. DN3 cells from WT and *syk*$^{-/-}$ thymocytes had equal percentages of intracellular TCRβ (TCRβic^{+}) cells (Figure 2.4C). However, cell cycle progression was impaired 40-50% in *syk*$^{-/-}$ DN3 (Figure 2.4D) as shown by decreased BrdU incorporation. In contrast, DN4 cell cycling was comparable in *syk*$^{-/-}$ and WT cells, indicating less of a role for Syk at this stage. Because apoptosis is difficult to detect within a steady-state thymus, we could not detect reproducible differences in apoptosis between genotypes within the mixed chimeras. Therefore, our competitive repopulation experiments indicated Syk-deficient thymocytes have a fitness defect compared to WT thymocytes at the DN3 stage and loss of Syk is only partially compensated for by ZAP-70. Furthermore, whereas TCRβ rearrangement seems to occur normally, Syk-deficiency impairs cell cycle progression at the DN3 stage.

Syk is Preferentially Required for the Initial DN3-E Pre-TCR Signal that Leads to DN3-L Blast Phase

DN3 cells are a mixture of at least two populations, small cells actively rearranging TCRβ loci (DN3-E or DN3a) and large cells that have successfully expressed a TCRβ

chain, initiated pre-TCR signaling and have entered cell cycle (DN3-L or DN3b) (Hoffman et al., 1996; Taghon et al., 2006). Our repopulation assays suggested Syk is required for normal progression through one or both of these stages. To investigate which stage is affected, we analyzed TCRβic^{+} expression and compared it to cell size to discriminate DN3-E and –L cells within WT and *syk*$^{-/-}$ thymocytes. For a given mouse, TCRβic^{+} correlated well with increased cell size in WT thymocytes. However, in *syk*$^{-/-}$ thymocytes within the same mouse, TCRβic^{+} did not correlate with increased cell size, where the DN3-L population was greatly reduced despite normal levels of TCRβic^{+} expression (Figure 2.4C, 2.5A and 2.5B). We performed similar analysis of DN3 from WT and *zap70*$^{-/-}$ mixed chimeric mice (further discussed below). *Zap70*$^{-/-}$ thymocytes had normal numbers of DN3-L cell formation after TCRβic^{+} expression. Similar results were seen in mice reconstituted with varying ratios, or non-competitively reconstituted and in non-reconstituted WT and *zap70*$^{-/-}$ mice. Taken together, these results indicate that Syk has a non-redundant function in early pre-TCR signaling and is needed for normal blast formation and cell cycle progression after TCRβ selection and protein expression. Additionally, our mixed chimeric studies indicate that this defect is thymocyte intrinsic.

We next wished to directly characterize the biochemical nature of the initial pre-TCR signal in WT and *syk*$^{-/-}$ thymocytes. However, traditional biochemical methods were prohibitive due to the paucity of DN subsets and the limitations of making sufficient chimeric mice. As an alternative to traditional biochemistry, we used flow cytometry to detect phosphorylation-site specific antibody staining in TCRβic^{-} DN3-E cells before and after CD3 cross-linking as a correlate of kinase activation. The phospho-ZAP-70 (Tyr319)/Syk (Tyr352) antibody used detects activated forms of both ZAP-70 and Syk.

Small amounts of CD3 reach the surface of Rag1-deficient DN3 cells (Shinkai and Alt, 1994) and we speculated this would be true of normal TCRβic$^-$ DN3-E cells. Anti-CD3 stimulation of WT DN3-E thymocytes resulted in a 2-fold increase in phosphorylated Syk/ZAP proteins (Figure 2.5C, top-left panel). Pre-incubating with PP2, a Src-kinase specific inhibitor, decreased the phospho-ZAP/Syk signal below background (unpublished data), confirming the signal emanates from a CD3 Src-kinase dependent pathway. Similar stimulations of *syk*$^{-/-}$ DN3-E thymocytes revealed that any phosphorylation of ZAP-70 is undetectable (Figure 2.5C, top-right panel). As a control, CD4$^+$ SP thymocytes from WT and *syk*$^{-/-}$ thymocytes (which express equal levels of ZAP-70 and no Syk) were equally phosphorylated on Syk-family kinases after CD3 cross-linking. These data suggest Syk, not ZAP-70, is preferentially activated downstream of the CD3 complex within WT DN3-E cells and this may account for the blast defect seen in *syk*$^{-/-}$ TCRβic$^+$ DN3 thymocytes.

Reduced levels of surface CD27 and intracellular TCRβ and ZAP-70 on Syk-deficient DN3b cells indicate defects in initial pre-TCR signal strength

We further tested Syk-deficient DN3 cells for impaired strength of signal by examining markers that increase with pre-TCR signaling. CD27 has recently been reportedly upregulated immediately after initial pre-TCR signaling and is the first reported surface marker that distinguishes newly selected pre-T cells (Taghon et al., 2006). Consistent with this, we found that cells that are completely blocked in pre-TCR signaling, such as LckFyn-double deficient (LckFyn) and LAT-deficient (LAT) thymocytes, fail to upregulate CD27 as shown in Figure 2.6. These pre-TCR signaling

Figure 2.5. Syk-deficiency impairs initial DN3 pre-TCR signaling and blast formation in a cell autonomous manner

(A) Left histogram depicts cell size of DN3 cells before TCRβic expression. Middle histogram compares the cell size of *syk*$^{-/-}$ and WT TCRβic^{+} DN3 within one mixed chimeric mouse. Right histogram compares the cell size of *zap70-/-* and WT TCRβic^{+} DN3 within one mixed chimeric mouse.

(B) Quantitation of data in (A) where each genotype is represented by at least nine chimeric mice. Bars depict percent mean +/-SD of TCRβic^{+} that are DN3-L using an arbitrary gate applied to all samples equally.

(C) Activation state phosphorylation of Syk-family kinases is measured in small CD25^{+}CD4^{-}CD8^{-} DN cells and CD4^{+}CD8^{-} SP after *in vitro* stimulation of unfractionated thymocytes with anti-CD3. Shaded histograms represent basal levels while open histograms are after 2 min stimulation. Experiment was repeated three times with similar results.

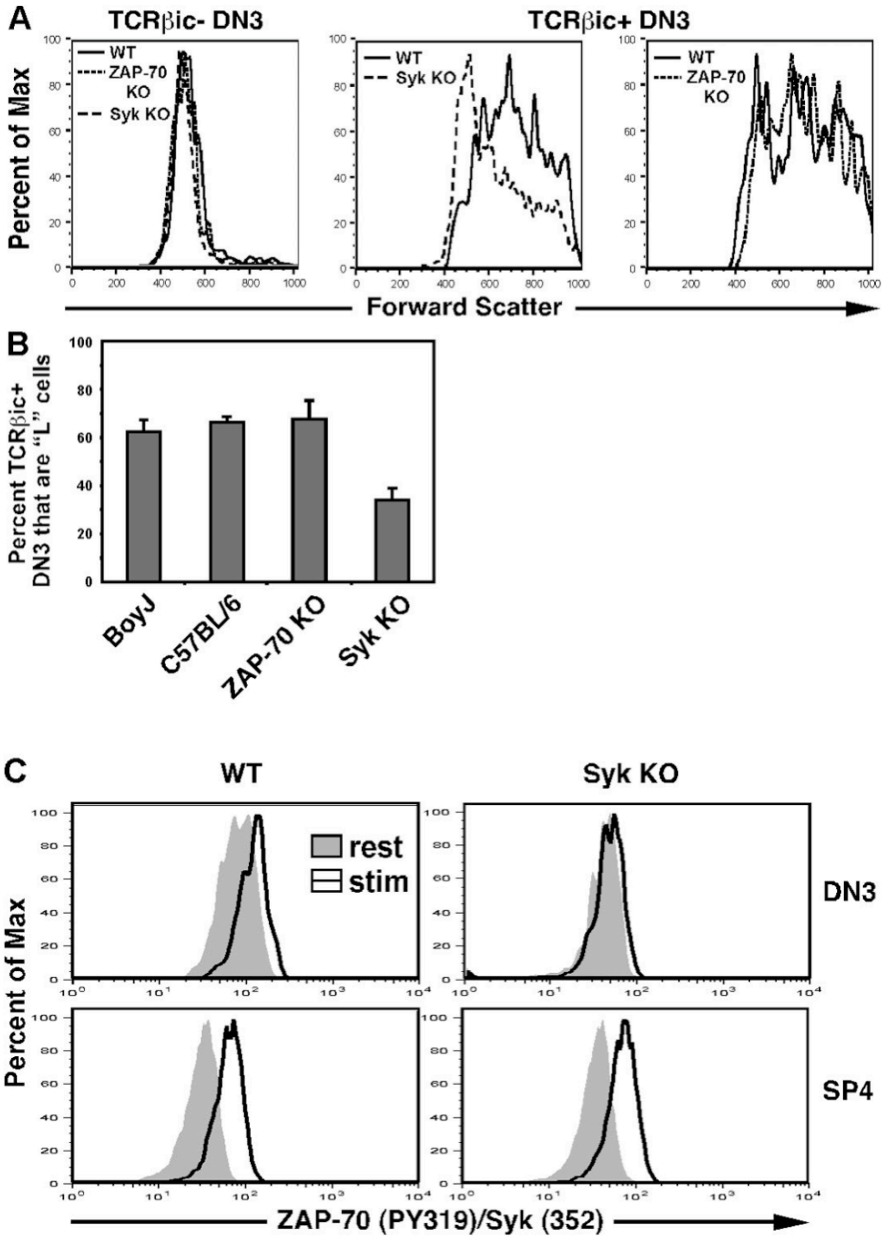
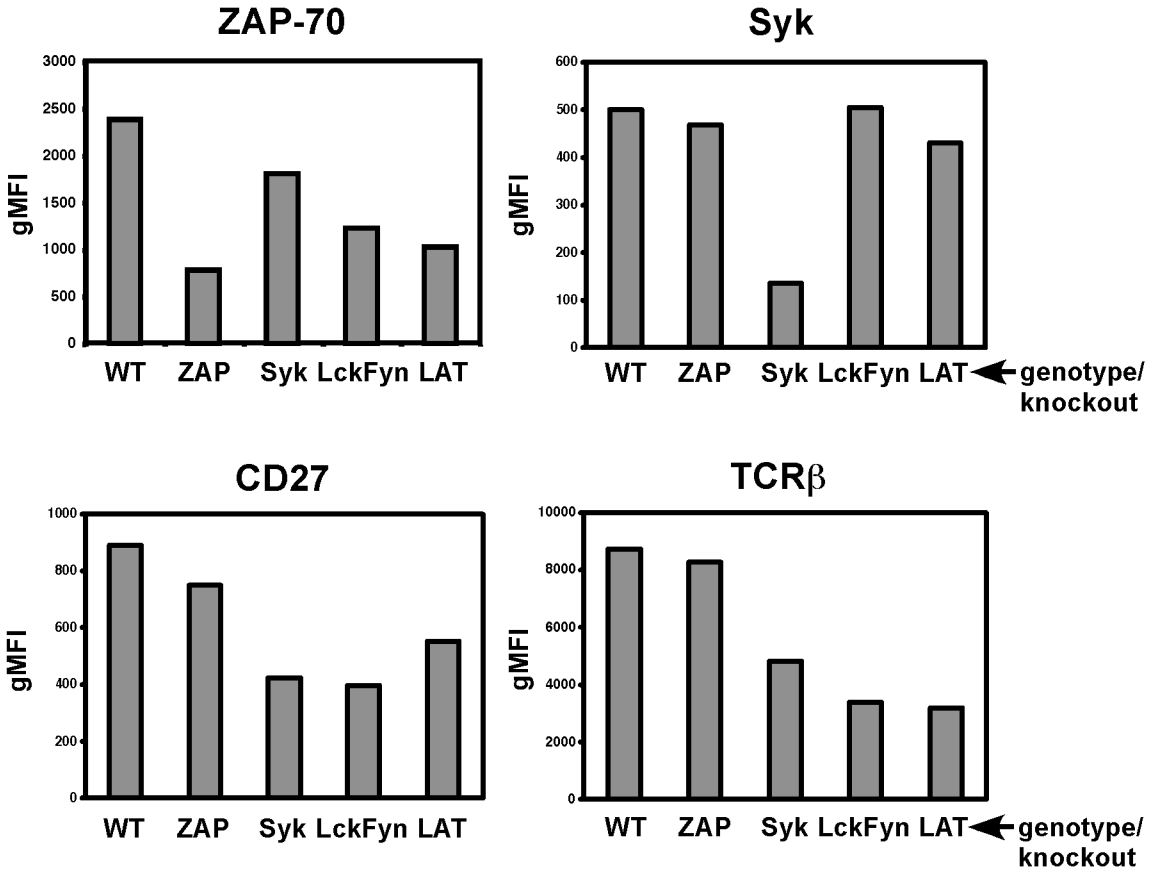

A
TCRβic- DN3
TCRβic+ DN3
WT
ZAP-70 KO
Syk KO
Percent of Max
Forward Scatter
B
Percent TCRβic+ DN3 that are "L" cells
BoyJ
C57BL/6
ZAP-70 KO
Syk KO
C
WT
Syk KO
rest
stim
DN3
SP4
Percent of Max
ZAP-70 (PY319)/Syk (352)

Figure 2.6. Phenotypic profile of DN3b (intracellular TCRβ^{+} DN3) cells

Quantitation of flow cytometric data from DN3b cells of WT, *zap70*$^{-/-}$, *syk*$^{-/-}$, *lck*$^{-/-}$*fyn*$^{-/-}$, and *lat*$^{-/-}$ mice. DN cells were defined as being negative for surface CD3, 4, 8, 11b, 19, Gr1, DX5, TCRγδ. Cells were simultaneously stained intracellularly for ZAP-70, Syk, and TCRβ. Cells were also stained for CD27. Bar graphs indicate MFIs of ZAP-70 & Syk (top graphs) and CD27 & intracellular TCRβ (bottom graphs) on TCRβ^{+}-gated DN3 cells (DN3a). Results are representative of two experiments.


ZAP-70
gMFI
3000
2500
2000
1500
1000
500
0
WT ZAP Syk LckFyn LAT
Syk
gMFI
600
500
400
300
200
100
0
WT ZAP Syk LckFyn LAT
genotype/
knockout
CD27
gMFI
1000
800
600
400
200
0
WT ZAP Syk LckFyn LAT
TCRβ
gMFI
10000
8000
6000
4000
2000
0
WT ZAP Syk LckFyn LAT
genotype/
knockout

blocked thymocytes exhibit a complete block at the DN3 stage of development, similar to *rag*$^{-/-}$ thymocytes. Unlike *rag*$^{-/-}$ thymocytes, LckFyn and LAT thymocytes express normal percentages of intracellular TCRβ^{+} cells within DN3 (DN3b) (data not shown). Thus, TCR rearrangements appear to occur normally. We find that the magnitude of intracellular TCRβ expression is, however, drastically reduced when compared to WT (Figure 2.6). Thus, lack of initial pre-TCR signaling also results in lack of TCRβ upregulation. Additionally, we find that ZAP-70 is not substantially upregulated in pre-TCR signaling blocked DN3 cells from LckFyn- and LAT-deficient mice (Figure 2.6).

When we examined similarly gated intracellular TCRβ^{+} DN3 (DN3b) cells from Syk-deficient thymus, we find that Syk-deficient DN3b cells have greatly reduced levels of CD27, ZAP-70 and TCRβ (Figure 2.6). ZAP-70-deficient thymocytes have comparatively normal levels of CD27 and TCRβ (Figure 2.6). These data further underscore the notion that Syk, but not ZAP-70 primarily functions in initial pre-TCR signaling and aides in proper transitioning from DN3a to DN3b stages. These data also further argue that Syk is primarily functioning downstream of pre-TCR signaling, as opposed to having some other important function that merely correlates with this stage.

ZAP-70 has an Unexpected and Unique Function in Generating DP Thymocytes, before Positive Selection Occurs

We next wished to further test whether ZAP-70 serves a positive regulatory role before positive selection. Our initial competitive repopulation experiments suggested ZAP-70 might provide two novel functions: a negative regulatory function at DN3 and also a positive function immediately after DN3 and before positive selection. Unlike *syk*$^{-/-}$

thymocytes, *zap70*$^{-/-}$ thymocytes had no discernable fitness impairment or enhancement at the DN3 stage as measured by competitive repopulation versus WT (Figure 2.7A). Blast size, TCRβ rearrangement and protein expression were similar to the WT as was cell-cycle status in DN3 and DN4 cells (Figure 2.7B and 2.7C). These data suggest that ZAP-70 has no notable role (positive or negative) at the DN3 stage that is not compensated for by Syk in the *zap70*$^{-/-}$ thymocyte during competitive repopulation. Competitive repopulation revealed that SP thymocytes are lacking in *zap70*$^{-/-}$ thymocyte subsets, consistent with the reported phenotype (Negishi et al., 1995a).

Surprisingly, *zap70*$^{-/-}$ thymocytes were also significantly impaired before the DP stage. *Zap70*$^{-/-}$ thymocytes were largely impaired even before the $CD8^{+}CD4^{-/lo}TCR\beta^{lo}$ ISP stage (Figure 2.7A). Thus, competition of *zap70*$^{-/-}$ versus WT and *syk*$^{-/-}$ versus WT are entirely complementary with our results from directly competing *syk*$^{-/-}$ against *zap70*$^{-/-}$ (Figure 2.2, 2.4 and 2.7). Taken together, these data suggest a novel positive regulatory function for ZAP-70 during DN4, ISP and DP stages and before positive selection as well as a distinct positive role for Syk at DN3-E. The individual competitions against WT argue against any "enhanced" fitness upon deleting the kinases and any obvious negative regulatory functions.

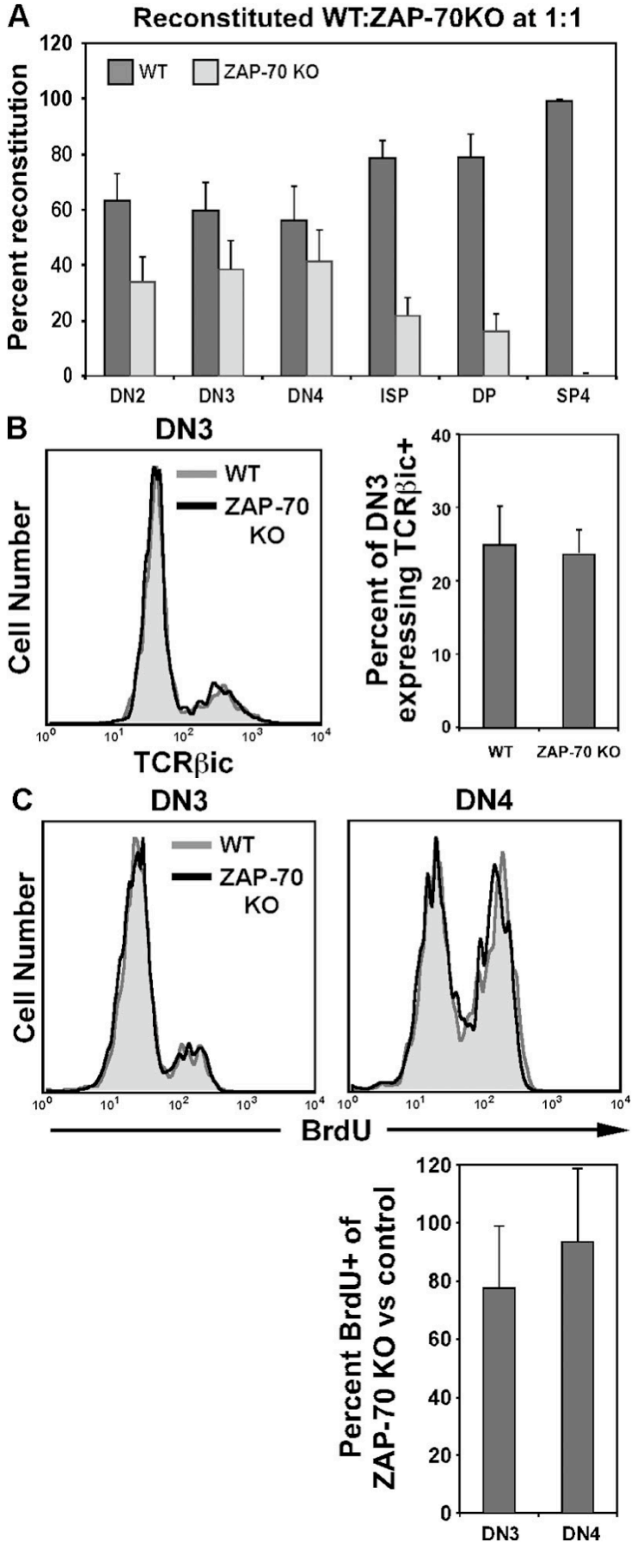
ZAP-70 is Necessary for Normal Generation of DP Thymocytes after Cross-Linking CD3 on Rag DN3 Thymocytes

It is possible that ZAP-70 is not involved in pre-TCR signaling but has some other function that is important and merely correlates with the pre-TCR^{+} cellular stage. For example, the pre-TCR may generate a single Syk-dependent signal, at the DN3-E stage

Figure 2.7. ZAP-70 is unexpectedly and uniquely required before positive selection for normal generation of DP thymocytes in a cell autonomous manner

(A) Competitive repopulation assay where 5 X 10^6 WT and *zap70-/-* BM cells were injected at a 1:1 ratio and the reconstituted chimeric thymus was analyzed 7 weeks later. Experiment represents seven mice and was repeated three times with similar results. Bars depict mean +/-SD.

(B-C) Intracellular TCRβ and BrdU incorporation were analyzed as in Figure 2.4C and 2.4D.


A
Reconstituted WT:ZAP-70KO at 1:1
Percent reconstitution
WT
ZAP-70 KO
DN2
DN3
DN4
ISP
DP
SP4
B
DN3
Cell Number
WT
ZAP-70 KO
TCRβic
Percent of DN3 expressing TCRβic+
WT
ZAP-70 KO
C
DN3
DN4
Cell Number
WT
ZAP-70 KO
BrdU
Percent BrdU+ of ZAP-70 KO vs control
DN3
DN4

that leads to the expected 6-9 cell divisions and characteristic gene expression changes, while ZAP-70 functions in a parallel but different pathway. Alternatively, initial Syk-dependent pre-TCR signaling may give way to continuous ZAP-70-dependent pre-TCR and/or TCR signaling that is MHC-independent between DN3 and DP stages.

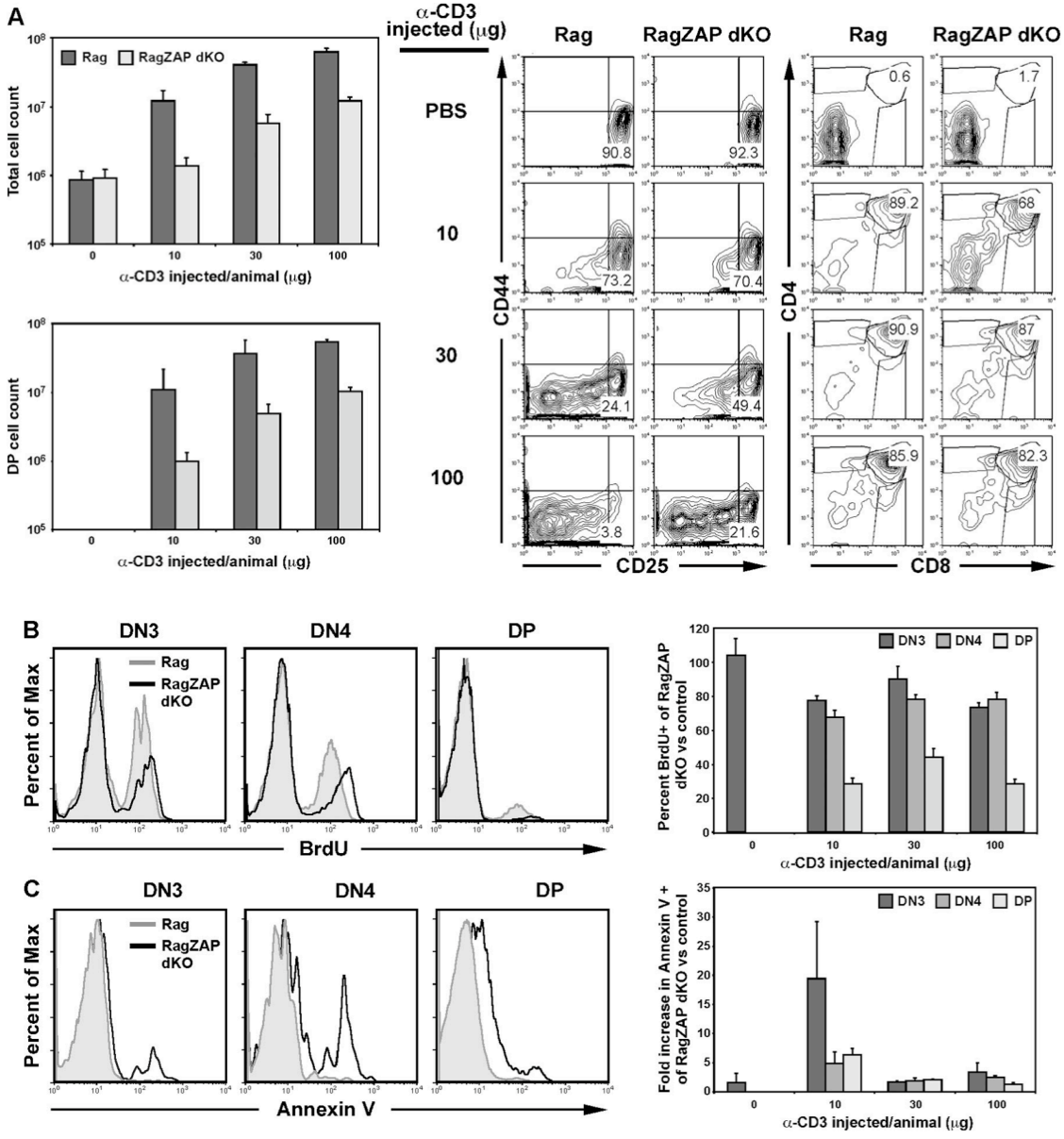
To test this, we used an *in vivo* model of pre-TCR signaling. *Rag1*$^{-/-}$ (Rag) thymocytes can be induced to differentiate into DP thymocytes by *in vivo* stimulation with anti-CD3 mAb (Jacobs et al., 1994; Shinkai and Alt, 1994). This process yields normal numbers of DP thymocytes and is dependent on kinases and the non-redundant T-cell adapters Slp-76 and LAT (Pivniouk et al., 1998; Zhang et al., 1999). We injected increasing amounts of anti-CD3 into *rag1*$^{-/-}$ mice to determine whether DP development was titratable. Increasing amounts of anti-CD3 resulted in a dose-dependent increase in total thymocyte production after 5 days and was primarily representative of DP production (Figure 2.8A, bar graphs). Absolute DN4 production also increased with increasing antibody, while DN3 cells decreased (unpublished data), probably owing to the slow rate of replacement from BM stem cells and increased differentiation to DN4. This assay allowed us to ask whether ZAP-70 is necessary for DP generation when we control the strength and specificity of a "pre-TCR-like" signal. Anti-CD3 stimulation resulted in 7-10-fold less DP generation at all antibody concentrations in *rag1*$^{-/-}$*zap70*$^{-/-}$ (RagZAP dKO) compared to Rag controls. The difference slightly decreased with increasing antibody concentrations, suggesting increased signaling can lead to normalization of cell numbers. Nevertheless, within the time frame studied, RagZAP dKO DN3 required 10-fold more antibody injected to achieve the same overall cellularity (compare RagZAP dKO at 100 μg and Rag at 10 μg). We believe this result represents a

Figure 2.8. ZAP-70 is required for efficient generation and survival of DP cells in Rag-deficient mice after *in vivo* anti-CD3 stimulation

(A) Top graph shows total cellularity from *rag1*$^{-/-}$ and *rag1*$^{-/-}$*zap70*$^{-/-}$ thymocytes after 5 days of *in vivo* treatment with increasing anti-CD3 mAb. Lower graph shows DP cellularity. Each group represents three mice. Representative plots illustrate CD25/CD44 profile of DN subsets and CD4/CD8 for DP subset. Numbers on DN plots indicate percent DN3. Numbers on DP plots indicate percent DP.

(B) Representative histograms of incorporated BrdU after 100 μg anti-CD3 treatment. Right bar graph shows the average percentage of BrdU^{+} cells in *rag1*$^{-/-}$*zap70*$^{-/-}$ subsets divided by the average of *rag1*$^{-/-}$ control for 0, 10, 30 and 100 μg mAb injected.

(C) Histograms of Annexin V^{+} staining in *rag1*$^{-/-}$*zap70*$^{-/-}$ dKO and *rag1*$^{-/-}$ thymocytes after anti-CD3 treatment as depicted in (B). Representative example shown is after 10 μg anti-CD3. Right bar graph is similarly quantitated as in (B).


A
Rag
RagZAP dKO
Total cell count
α-CD3 injected/animal (μg)
DP cell count
α-CD3 injected (μg)
PBS
10
30
100
CD44
CD25
CD4
CD8
90.8
92.3
0.6
1.7
73.2
70.4
89.2
68
24.1
49.4
90.9
87
3.8
21.6
85.9
82.3
B
DN3
DN4
DP
Percent of Max
BrdU
Percent BrdU+ of RagZAP dKO vs control
C
Annexin V
Fold increase in Annexin V + of RagZAP dKO vs control

ZAP-70-dependent cell intrinsic defect downstream of the CD3 pathway before the positive selection stage.

Representative phenotypic profiles of Rag and RagZAP dKO thymocytes are shown in Figure 2.8A, right panels. No overall increase in cell numbers was seen in RagZAP dKO stimulated with 10 μg anti-CD3, yet 68% of cells differentiated to the DP stage, reflecting decreased absolute DN3s. At 30 μg the frequency of DP is similar with or without ZAP-70, yet the overall and DP cellularity is >7-fold less without ZAP-70. The absolute counts of DN3 and DN4 are also decreased 2-5-fold in RagZAP dKO compared to Rag mice (unpublished data).

To determine how ZAP-70 affects generation of DP cells in this assay, we measured levels of BrdU incorporation and Annexin V binding as markers of cell cycle status and apoptosis, respectively. For both genotypes, the fraction of cells in cell cycle in all subsets increased with increasing antibody. Examples of BrdU incorporation at 100 μg are shown in Figure 2.8B. RagZAP dKO DN3 and DN4 cells incorporated about 80% BrdU compared to respective Rag KO cells and RagZAP dKO DP cells incorporated only ~35% (Figure 2.8B). Interestingly, the relative decrease in cell cycle in RagZAP dKO compared to Rag subsets was constant at varying antibody concentrations. These data suggest that the fraction of cells in cell cycle in all subsets is proportional to the CD3-mediated signal (i.e., pre-TCR) and that this signal depends on ZAP-70.

Since the initiating CD3-based signal was largely synchronized in these experiments, assessment of apoptosis was possible. For both genotypes, apoptosis was highest after minimal stimulation with 10 μg anti-CD3 and is shown in Figure 2.8C. DN3, DN4, and DP subsets in RagZAP dKO were 5-20-fold more apoptotic than Rag

after 10 μg anti-CD3 (Figure 2.8C). This difference decreased to ~2-fold in all subsets at greater antibody concentrations. Thus, unlike cell cycle, the effect of ZAP-70 on survival was proportional to the pre-TCR signal strength but appeared to be threshold dependent. The cell cycle and apoptosis results suggest that ZAP-70 affects sustained pre-TCR signaling at all stages between DN3 and DP. We attempted similar experiments using *rag1*$^{-/-}$ and *rag1*$^{-/-}$*syk*$^{-/-}$ reconstituted animals (into lethally irradiated *rag1*$^{-/-}$ hosts) but could not achieve reproducible levels of cellular production even within mice reconstituted with *rag1*$^{-/-}$ stem cells. This may be due to radiation damage to the vascular system of the host mouse (Abtahian et al., 2003) and inefficient absorption of the anti-CD3 antibody.

Inhibiting distinct components of the pre-TCR/TCR signaling pathway blocks the DN4 to DP transition

Our anti-CD3 treatment of *rag1*$^{-/-}$ mice and competitive repopulation data suggest that a "sustained" pre-TCR signal is necessary for normal progression from the transitory DN4/ISP stages to the DP stage, but direct evidence is lacking. We reasoned that if ZAP-70-driven sustained pre-TCR/TCR signaling was necessary for stages between DN3a and DP, it might be possible to block DP generation by blocking TCR signaling components within DN4 cells that are fated to become DP cells. Purified DN4 cells transition into DP cells when cultured in isolation *ex vivo* for 12-24 hours (Petrie et al., 1990). This *in vitro* development, in the absence of exogenous growth factors or supporting stromal cells has provided strong evidence that DN4 cells do not require exogenous signals and are direct

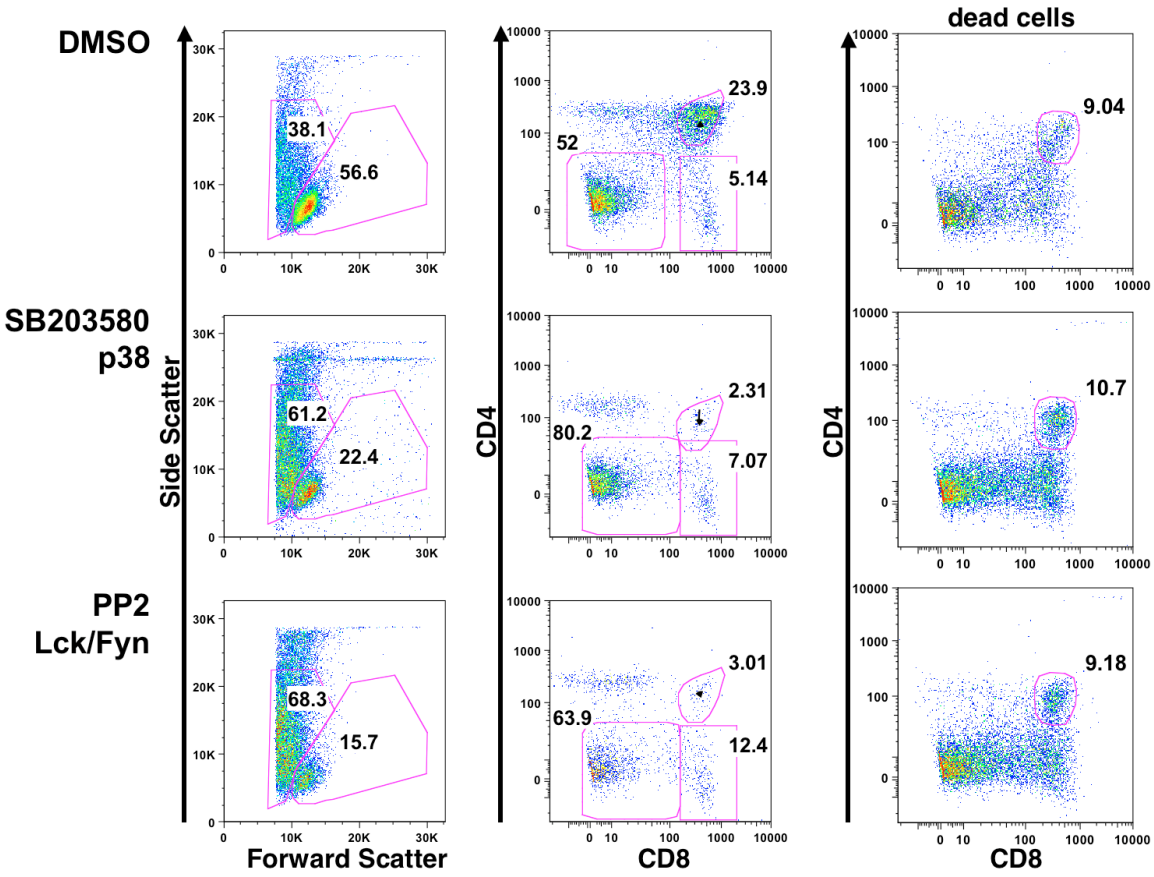
precursors to DP thymocytes. We decided to isolate DN4 cells and test whether blocking pre-TCR components would also block DP development when cultured overnight.

A previous report indicated that p38 MAPK, a downstream effector of TCR signaling, is critical for the DN4 to DP transition (Diehl et al., 2000). Inhibition of p38 using the specific inhibitor SB203580 completely blocked the development of DP thymocytes from purified "double-negative" (DN1-4) cells within fetal thymic organ cultures (FTOC), suggesting a role for p38 function at the DN4 stage (Diehl et al., 2000). We isolated DN4 cells by magnetic bead negative selection and cultured overnight in media as shown in Figure 2.9. The p38 SB203580 inhibitor was used as a control to block DP development. As shown in the middle row of Figure 2.9, inhibiting the MAPK p38 completely blocked DP development from purified DN4 cells, in agreement with the previous report using FTOCs (Diehl et al., 2000). Coculturing DN4 cells with the DMSO vehicle did not impair DP development, as a full ~25% of cells had become DP in 15 hours. Blocking Src-familly kinase activity (SFK) using PP2 also completely blocked DP development. Blocking distinct components of pre-TCR signaling did, however, result in an increased percentage of cells falling within the smaller "dead" lymphocyte flow cytometric gate (Figure 2.9, right-most column). These dead lymphocytes had abumdant percentages of ISP and DP cells, suggesting that blocking pre-TCR signaling lead to increased death of ISP and DP thymocytes.

Thus, inhbiting proximal (SFK) as well as distal (p38) components of the pre-TCR/TCR signaling complex can completely block DP development from DN4 cells. These data are highly supportive of the idea that DN4 thymocytes require a "sustained"

Figure 2.9. Inhibiting distinct components of pre-TCR/TCR signaling blocks the DN4 to DP transition

Purified WT DN4 cells were cultured overnight in media that included either vehicle control (DMSO) or SB203580 or PP2 (top, middle, bottom rows, respectively). Left-most column depicts forward/side scatter to separate larger "live" cells from smaller "dead" cells. Second column depicts phenotypic profile, including DP development, within "live" gated events. Third column depicts phenotypic profile of "dead" cell gate.


DMSO
SB203580
p38
PP2
Lck/Fyn
Side Scatter
Forward Scatter
CD4
CD8
dead cells
38.1
56.6
23.9
52
5.14
9.04
61.2
22.4
2.31
80.2
7.07
10.7
68.3
15.7
3.01
63.9
12.4
9.18

pre-TCR signal to efficiently transition to the DP stage of development. They are also consistent with the idea that ZAP-70 function at DN4 (in competitive repopulation and anti-CD3 treated *rag1*$^{-/-}$ mice) is primarly downstream of pre-TCR/TCR signaling and not in some other pathway that correlates with this stage of development.

ZAP-70 protein is robustly upregulated only after DN3 cells have signaled through the pre-TCR

Since ZAP-70 is quickly upregulated within DN4 cells, we speculated that ZAP-70 might be a direct target or indicator of pre-TCR signaling. As noted before, DN3 thymocytes are a mixture of two populations; a small, quiescent TCRβ unrearranged DN3-E (DN3a) cells and large, cycling TCRβ rearranged DN3-L (DN3b) cells that are beginning the progression toward DP. Our RNA and initial flow cytometry studies conflicted on whether ZAP-70 was first expressed within the DN3 stage. We noticed DN3 cells had a reproducible positive shoulder of ZAP-70 protein but not Syk (Figure 2.10A), suggesting subpopulations have differential ZAP-70 expression.

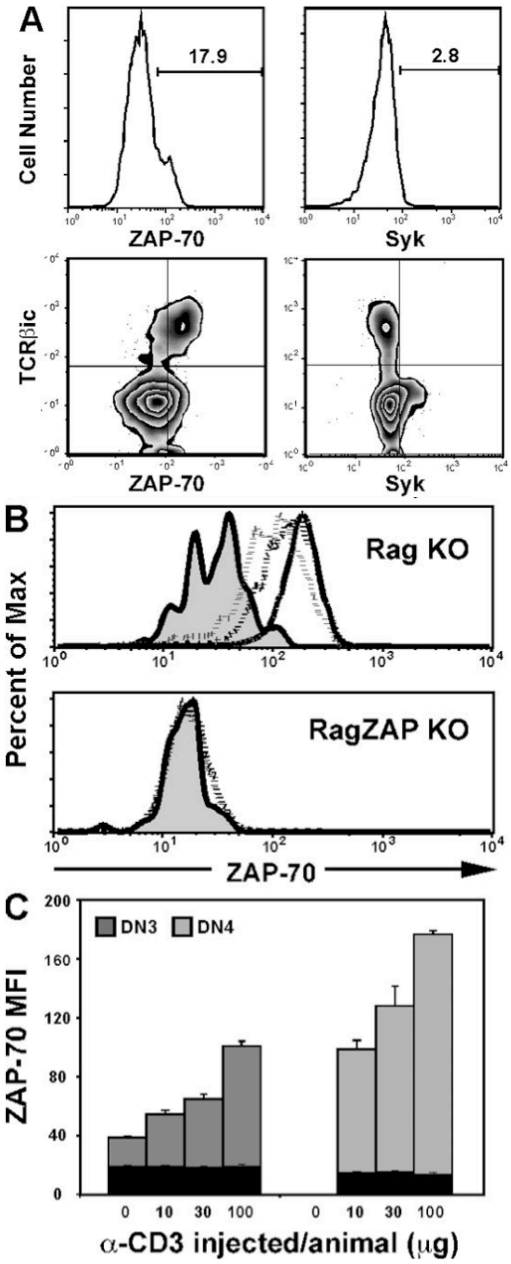
We focused on CD25^{+} DN2/3 thymocytes and assayed for simultaneous intracellular expression of TCRβ and either ZAP-70 or Syk protein to investigate the earliest detectable TCRβ selected cell. TCRβic^{+} cleanly separated a ZAP-70$^{lo/-}$ from a ZAP-70^{+} population. Thus, TCRβ selection correlates with ZAP-70 protein upregulation. The increase in expression was about 3-fold. The antibody specificity was confirmed by staining equivalent *zap70*$^{-/-}$ CD25^{+} DN cells (unpublished data). ZAP-70 correlated with cell size, which also correlated to TCRβic^{+} expression (Figure 2.6 and 2.10A). We also

Figure 2.10. ZAP-70 protein is upregulated after pre-TCR signaling

(A) Top panel indicates ZAP-70 and Syk expression in all DN3 cells. Lower panels show ZAP-70 protein compared to intracellular TCRβ chains within DN3s.

(B) Top panels indicate dose-dependent increased ZAP-70 expression in DN4 from *rag1*$^{-/-}$ mice 5 days after *in vivo* anti-CD3 injection. Shaded histogram is background DN4 from PBS treated mice. Other histograms show 10 μg (dotted line), 30 μg (dashed line), and 100 μg (unshaded) of anti-CD3 treatment. Bottom panel shows ZAP-70 staining in control treated *rag1*$^{-/-}$*zap70*$^{-/-}$ dKO.

(C) Bars depict quantitation of mean +/-SD of ZAP-70 expression as shown in (B) and from the DN3 and DN4 subsets 5 days after *in vivo* anti-CD3 treatment in *rag1*$^{-/-}$ mice. Three mice were included for each condition. Overlaid black bars show mean +/-SD of ZAP-70 expression in similarly treated *rag1*$^{-/-}$*zap70*$^{-/-}$ dKO. This experiment was repeated twice with similar results.


A
Cell Number
17.9
2.8
ZAP-70
Syk
TCRβic
ZAP-70
Syk
B
Percent of Max
Rag KO
RagZAP KO
ZAP-70
C
ZAP-70 MFI
DN3
DN4
200
160
120
80
40
0
0 10 30 100
0 10 30 100
α-CD3 injected/animal (μg)

found that ZAP-70^{+} cells corresponded to CD27^{+} DN3b cells (Figure 2.6 and (Taghon et al., 2006)).

These results suggested that ZAP-70 is upregulated after TCRβ is rearranged and expressed. Therefore, the upregulation of ZAP-70 may be a consequence of initial pre-TCR signaling itself. To test this, we assayed ZAP-70 expression in Rag KO mice as they are completely blocked in pre-TCR signaling and arrested at DN3. We injected increasing amounts of anti-CD3 mAb into Rag KO mice and examined ZAP-70 expression in DN3, DN4 and DP cells. ZAP-70 protein increased in a dose-dependent manner after anti-CD3 treatment as shown for DN4 in Figure 2.10B. Treated RagZAP dKO mice confirmed the specificity of the signal (Figure 2.10B and 2.10C). ZAP-70 protein is almost completely absent in resting Rag KO DN3, consistent with a requirement of an initial pre-TCR signal for robust ZAP-70 upregulation. ZAP-70 expression increased in DN3, DN4 and DP cells (Figure 2.10C and unpublished data) after anti-CD3 injection in a dose-dependent manner. In addition, we assayed mice where TCRβ rearrangement still occurs yet no signal is generated (*lat*$^{-/-}$ and *lck*$^{-/-}$*fyn*$^{-/-}$) and found no upregulation of ZAP-70 or CD27 and therefore no correlation with intracellular TCRβ. Collectively, our data suggest that ZAP-70 is expressed at low levels in DN3-E thymocytes and is upregulated only after initial pre-TCR signaling in DN3-L. Thus ZAP-70, along with CD27, is among the earliest targets or indicators of pre-TCR signaling. Developing thymocytes have the unique feature where initial Syk-dependent pre-TCR signaling at DN3-E quickly upregulates ZAP-70 which then replaces Syk during sustained pre-TCR/TCR signaling during DN3-L, DN4, ISP and DP stages.

Discussion

We have examined the expression patterns and *in vivo* functions of the Syk-family kinases, Syk and ZAP-70, during early stages of thymocyte development. *Syk*$^{-/-}$ thymocytes are impaired in transitioning from DN3 to DN4 during competitive repopulation of the thymus. Analyses of mRNA abundance and intracellular flow cytometry indicated that ETPs express high levels of Syk and little or no ZAP-70 and this remains true as cells commit to the T lineage pathway and become DN2s. After TCRβ selection has occurred, Syk is necessary in quiescent DN3-E (DN3a) cells for initial pre-TCR signaling and optimal entry into cell cycle and transition to DN3-L (DN3b). ZAP-70 appears to have a preferred function during sustained pre-TCR signaling from DN4 through DP stages and aids in the normal expansion and differentiation of thymocytes despite concurrent Syk expression (through the ISP stage), after which positive selection occurs. *In vivo* stimulation with anti-CD3 mAbs in Rag mice confirms that ZAP-70 uniquely functions downstream of the pre-TCR/TCR after the DN3-E stage and before DP selection. Loss of ZAP-70 mostly increases apoptosis levels but also decreases cell cycle progression, especially at the DP stage. ZAP-70 expression is induced by the pre-TCR and is quickly upregulated, coincident with CD27 when newly formed pre-T cells transition from DN3-E to DN3-L. Our data suggest the pre-TCR is a dynamic signaling module where the signaling machinery is fundamentally altered during development. We propose a model where Syk-dependent pre-TCR signaling is replaced with ZAP-70-dependent pre-TCR signaling.

Previous work on the role of Syk in T cell development has been conflicting. While the original knockout papers suggested Syk had little role in development (Cheng

et al., 1995; Turner et al., 1995), later reports argued for either a unique role in TCRγδ development (Mallick-Wood et al., 1996) or a more quantitative role in general T cell development (Colucci et al., 2000). Our work is consistent with and expands on the results of Colucci and colleagues (Colucci et al., 2000). That study found that DN3s were found at higher frequencies in *syk*$^{-/-}$ reconstituted mice, although they never competed *syk*$^{-/-}$ thymocytes versus WT within a given mouse. Our studies allow direct comparison of the *in vivo* fitness of *syk*$^{-/-}$ versus WT and *syk*$^{-/-}$ versus *zap70*$^{-/-}$ thymocytes within the same mouse, allowing us to assess a quantitative impairment of the block and to conclude that the impairment is cell intrinsic. Further still, we demonstrate that ZAP-70 protein is poorly expressed within the DN3-E thymocytes, providing a logical explanation for *syk*$^{-/-}$ impairment.

Clearly, the pre-TCR has several certain well-defined functions (Michie and Zuniga-Pflucker, 2002). However, it is not established when these functions are employed. Rescue from apoptosis may be necessary at the DN3-E stage, but may also be necessary at every stage from DN3-L to DP for normal population expansion. One can imagine continuous pre-TCR signaling enabling maximal cell cycle progression at all stages. In contrast to this, decreasing Rag activity and enforcing allelic exclusion of the TCRβ locus might only be necessary at the DN3-E stage. Some of these distinct functions have been shown to diverge downstream of the pre-TCR (Kruisbeek et al., 2000). We propose they also diverge temporally during differentiation and expansion. ZAP-70-may provide continuous pre-TCR/TCR signaling during DN3-L, DN4, ISP, and/or DP stages that is needed for optimal development. Indeed, our data from *in vivo* stimulated Rag mice demonstrate a strong correlation with anti-CD3 titration and ZAP-70

protein expression in all subsequent stages and absolute DP generation. A similar function has recently been proposed for Slp-76 (Maltzman et al., 2005). Continuous or constitutive pre-TCR signaling has been previously suggested. Studies of *c-cbl*$^{-/-}$ thymocytes suggest a continuous pre-TCR signal as these cells have pre-TCRs that fail to be continuously internalized (Panigada et al., 2002). DN4 cells are shown to activate NF-κB and NFAT downstream of the pre-TCR (Aifantis et al., 2001). A recent report demonstrated temporal separation of Egr3 and RORγ induction after pre-TCR signaling, both necessary for proper DP generation (Xi et al., 2006).

Other signaling components are also known to promote proliferation and survival of DN and DP stages. Early stages of TCRβ selection require activation of Notch1 (Ciofani et al., 2004), p53 (Haks et al., 1999), FADD (Newton et al., 2000) and NF-κB (Aifantis et al., 2001; Voll et al., 2000) pathways among others, many of which are likely to be affected downstream of the pre-TCR. Since these molecules exert their effects at or immediately after the DN3 stage, they may be optimally activated by a Syk-dependent pre-TCR signal. However, Bcl-X, RORγ and TCF/LEF all affect the survival of DP cells (Staal and Clevers, 2003; Sun et al., 2000); and they may be preferentially influenced downstream of a ZAP-70-dependent pre-TCR signal.

Notch and E2A activities precede/activate a host of T lineage genes such as CD3 and Lck as early as in DN1 (Ikawa et al., 2006; Olszowy et al., 1995; Wilson and MacDonald, 1995). Therefore, it is surprising that ZAP-70, a largely T-lineage specific protein, is not highly expressed until after the first T lineage commitment checkpoint. One teleological explanation may be that Syk is better able to transduce the initial pre-TCR signal. This could either be quantitative or a qualitative difference. Quantitatively,

Syk is a more active kinase *in vitro*, causes more basal activation in T cells and is less Src-kinase dependent in T cell lines (reviewed in (Chu et al., 1998b)) which makes it less coreceptor dependent. In fact, use of Syk for initial pre-TCR signaling may explain why the Lck knockout is more leaky at pre-TCR signaling than at positive selection. Qualitatively, Syk may activate/inactivate specific genes. After initial pre-TCR signaling, ZAP-70 may be better able to transduce continuous pre-TCR signals that are necessary for DN to DP transitioning. A previous study addressed whether Syk can substitute for ZAP-70 during positive selection, by transgenic expression of Syk in a ZAP-70 deficient mouse strain (Gong et al., 1997). These thymocytes over-expressed Syk after β-selection and through the DP stage, suggesting that if Syk were over-expressed it could replace ZAP-70 during continuous pre-TCR signaling and DP expansion. However, the efficiency of DP generation was not determined, as these thymocytes were not tested by competitive repopulation leaving open the question of whether Syk could comparably replace all ZAP-70 functions if its expression was maintained from DN3 to DP stages. A recent study shows that Syk-expressing B cells require the traditional MEK pathway for p38 activation while ZAP-70-expressing T cells can also activate p38 in a novel MEKK4/7-independent manner (Salvador et al., 2005). Thymocytes require that p38 not be activated during initial pre-TCR signaling (Diehl et al., 2000) and Syk may suffice. However, p38 becomes necessary for normal transitioning from the DN4 to the DP stage (Mulroy and Sen, 2001) and thus may be largely induced by ZAP-70 based pre-TCR signaling. We are currently testing these possibilities.

It is certainly possible that the differential fitness of Syk and ZAP-70-deficient thymocytes may, at least in part, be reflective of the distinct expression levels of each

kinase during these stages and not due to a unique function of the given kinase at a certain stage. To formally test this would require a model system involving the competition of thymocytes that express comparable levels of either ZAP-70 or Syk throughout thymic development on a null background for both kinases. Additionally, as TCRγδ cells diverge from TCRαβ cells at this DN3 stage due to purported difference in signal strength, the balance of γδ versus αβ T cell may altered (Lauritsen et al., 2006). A relative over-expression of ZAP-70, Syk or both could alter this ratio by increasing the strength of the signal generated, thereby increase relative numbers of TCRγδ. Our comparative studies detail for the first time the extent to which thymocytes differentially express both Syk-family kinases and that such differential expression/and or function results in differential cellular fitness during early development, where a Syk-family driven pre-TCR signal is necessary for normal DP expansion.

Interestingly, one unusual feature that distinguishes TCRα selection from the analogous Ig light chain selection is that TCRα chains are not allelically excluded (Khor and Sleckman, 2002; Meade et al., 2004). Although the mechanisms leading to allelic exclusion are not well understood, Syk may be uniquely suited to better transduce these signals in both lineages. While ZAP-70 can perform some Ig heavy chain allelic exclusion (Schweighoffer et al., 2003), Syk is mostly responsible for this. It is unknown whether Syk or ZAP-70 differentially effect allelic exclusion of the TCRβ chain.

In summary, our studies identify a specific role for Syk during β-selection and cell cycle entry and uncover an unexpected relative requirement for ZAP-70 during early thymocyte development. This occurs despite overlapping expression of both kinases within DN3-L and DN4 and ISP cells. Our data are also consistent with a model where

Syk-dependent pre-TCR signaling induces ZAP-70 expression, which then replaces Syk for ZAP-70-dependent pre-TCR signaling as thymocytes progress toward the DP stage.

Materials and methods

Mice

Zap70$^{-/-}$ mice were backcrossed at least seven times onto the B6 background. *Syk*$^{+/-}$ mice were carried as heterozygotes due to their homozygotic perinatality and were backcrossed at least eleven generations to B6. *Syk*$^{+/-}$ was later crossed to the BoyJ (B6 mice with CD45.1^{+} congenic marker, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) background for one generation. B6 and *rag1*$^{-/-}$ mice used for breeding were originally from Jackson Laboratories. B6 mice used as hosts for competitive repopulation were from Charles River. BoyJ mice were from Taconic and Jackson Laboratories. For BrdU studies, 1-2 mg of BrdU (Sigma) at 20 mg/ml was injected IP 1-2 hr before sacrificing the animal. All animals were housed in specific pathogen-free facility at UCSF according to University and National Institutes of Health (NIH) guidelines.

Real-Time RNA Quantitation

RNA was extracted using TRIzol according to the manufacturer's instructions (Invitrogen). cDNA was created using the Sensiscript protocol (Qiagen). Primers and probes used for quantitative PCR span introns and were as follows. For murine *ZAP70*, forward primer 5'-GCATGCGCAAGAAGCAGATT-3', reverse primer 5-GGGCCTCTCGCATCATCTC-3 and probe [6-FAM]CTTTGTCGGCCTTCTCTGTGCCCTG[BHQ1~Q] were used. For murine *Syk*, forward primer 5'-CTGGTTCCATGGCAACATCTC-3', reverse primer 5-TGGCCCTGATCAGGAATTTTC-3 and probe [6-

FAM]TGACCCTATGAGGACCGTCTGCTCTGAT[BHQ1~Q] were used. *HPRT* primers and PCR conditions were described previously (Grogan et al., 2001).

Immunoblotting

Cell lysates were prepared using a 1% NP-40 lysis buffer with inhibitors and transferred to membranes for SDS-PAGE as previously described (Sieh et al., 1993). The A20 B cell line, Jurkat and P116 (ZAP-70 deficient) and pre-T cell lines Sciet27 and SCB29 were used as ZAP-70 and Syk expressing lymphoid cell lines. Lysates were corrected for protein content before loading. Anti-actin mAb was from Sigma. Lanes were quantitated using a Kodak Image Station 440DF and Kodak ID image analysis software 3.5.

Competitive Repopulations and Chimeras

For competitive repopulation assays, FL vs. FL or BM vs. BM cells were injected IV into lethally irradiated B6 or BoyJ hosts and reconstituted thymii were analyzed 4-8 weeks later. FL was from timed pregnancies at E15.5-16.5 and BM was from adults 8-12 weeks old. *Syk-/-* embryos were identified visually and confirmed by either PCR or flow cytometry of reconstituted blood. For some experiments, *syk-/-* FL was used to generate chimeric mice and the resulting BM was used for competing or reconstituting irradiated hosts. No differences were seen using these BM compared to FL. WT recipients were irradiated with 2 doses of 600 rads 3-5 hours apart. Mixed FL chimeras were injected IV at 1:0, 0:1, 1:3, 3:1, 1:9 or 9:1 ratios. Each FL was resuspended in 1.0 ml media and 200 μl (mixed or not) was used per mouse. For BM, 5 X 10^6 total cells were injected IV into each mouse. Males were used as recipients for most experiments and females for some

with no qualitative differences. Within an experiment all hosts were sex and age matched and were between 5-10 weeks.

Antibodies and Flow Cytometry

Thymi were separated to single-cell suspensions using glass slides. 1-3x10^6 cells were used for each stain. mAbs against CD3, CD4, CD8, CD11b, CD19, Gr-1, were used to exclude lineage-committed cells and delineate DN thymocytes and were conjugated to either PE or APC (BD Biosciences and eBioscience). Biotin-DX5, –TCRγδ and -CD69 (BD Biosciences) with SA-APC or –PE were used to amplify the signal and/or exclude these cells. For Syk and ZAP-70 quantification in thymocytes subsets, DN1 was defined as Lin^- $CD44^+CD117^+CD25^-$. T and B cells were from LN and spleen and defined by $CD3^+CD19^-$ for T cells and $CD3^-CD19^+$ for B cells. CD25, CD44, CD117, CD45R, TCRβ (BD Biosciences) were conjugated to PE, PE-Cy5.5 or PerCP-Cy5.5 and FITC and further characterized DN subsets. mAbs against CD45.1 and CD45.2 were conjugated to FITC, PE or APC (BD Biosciences and eBioscience). mAb against mouse/human ZAP-70 1E7 (Qian et al., 1997) and mouse Syk 5F5 (Chu et al., 1999) were conjugated to AlexaFluor-488 (Ax488) or Ax647 to a fluorophore:protein ratio of 6.5-7:1 or 5:1, respectively using the manufacture's instructions (Invitrogen-Molecular Probes). Anti-BrdU was from Invitrogen-Molecular Probes. Annexin V-FITC was from Caltag. Fc binding was blocked using anti-CD16/32 2.4G2 (Harlan). For intracellular staining, cells were surfaced stained, fixed in Fix and Perm Medium A (Caltag), stained for intracellular ZAP-70, Syk, TCRβ, BrdU, or Lck with Fc block for 30-60 min in Medium B (Caltag), washed with Perm/Wash (BD Biosciences) and fixed before running on the flow

cytometer. BrdU staining also included 4.1mM $CaCl_2$ and 20 U of DNAseI (Roche). For phospho-ZAP/Syk analysis, cells were stimulated with 20 μg/ml anti-CD3 (2C11) followed 20s later by 50 μg/ml goat anti-hamster (Jackson Immunoresearch) secondary cross-linker and fixed to terminate stimulation. Cells were then permeabilized using 90% ice-cold methanol for 30 min, stained with 2 μl phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody (Cell Signaling) for 1 hr, stained with surface markers and donkey-anti-rabbit-PE (Jackson Immunoresearch) for 1 hr, then fixed. Data were collected on a FACSCalibur, LSR II (BD Biosciences) or CyanADP (Dako) and analyzed using FlowJo software (TreeStar). For RNA and immunoblot analysis, cellular subsets were sorted using a MoFlo (Dako).

***In vitro* generation of DP thymocytes from DN4 precursors**

WT DN4 thymocytes were purified by negative selection using magnetic beads using the CD8 T cell Isolation Kit (Miltenyi). This kit uses a cocktail of lineage specific fluorescently labeled antibodies to remove CD4+ T cells and most all other hematopoietic cell types. Anti-CD8, -CD25 and -CD44 specific antibodies were added to this kit to completely remove all DP, SP, DN1, DN2/3 and other lineages, leaving only DN4 cells. Cells were cultured overnight in 10% FBS DMEM in 96-well flat bottom plates at 40,000 cells/well. SB203580 was dissolved in DMSO used at 10 μM and was diluted 1:256 into the overnight culture medium from the original stock solution (Calbiochem). PP2 was dissolved in DMSO and used at 20 μM and was diluted 1:1000. The DMSO vehicle control well had DMSO diluted 1:256 into media. Cells were cultured overnight at 37C, and then stained with anti-CD4 and anti-CD8 antibodies before flow cytometric analysis.

Anti-CD3 Injections

Rag1-/- and *rag1-/-zap70-/-* at 8-12 weeks were used for *in vivo* anti-CD3 treatment. mAb anti-CD3 2C11 was injected IP at 10, 30, or 100 μg/animal diluted to 500 μl in PBS and mice were killed and analyzed 5 days later. All experiments were repeated at least twice with similar results and data is represented at mean +/- SD. For *rag1-/-* and *rag1-/-syk-/-* experiments, chimeric mice were made from FL and were used 5-6 weeks after reconstitution.

Chapter 3

ZAP-70 and CD45 both contribute to sustained pre-TCR signaling

Abstract

The receptor-like tyrosine phosphatase CD45 is a critical positive regulator of T-cell receptor (TCR) signaling. The major function of CD45 is to positively regulate the Src-family kinase Lck, which positively regulates the Syk-family tyrosine kinase ZAP-70. Similarly, ZAP-70 is also critically required for TCR signaling. Recently, we described a novel role for ZAP-70 during early thymocyte development and showed that ZAP-70 is required for normal generation of DP cells before positive selection occurs. The role of CD45 during DP generation has not been investigated and whether CD45 is necessary upstream of ZAP-70 during this process is also not clear. We found that combined genetic loss of ZAP-70 and CD45 resulted in a profound loss of overall thymic cellularity by a magnitude that was greater than the summation of each single-gene knockout, suggesting a synergistic impairment. This reduction was largely representative of reduced numbers of DP thymocytes and was characterized by vastly increased apoptosis. These data indicate that ZAP-70 and CD45 are both necessary for continuing pre-TCR signaling that generates DP thymocytes.

Introduction

Thymocyte development requires signaling through the antigen receptor at very specific stages of early development (Cantrell, 2002). Two major checkpoints are well described and both uniquely serve to ensure faithful selection of two competent protein chains that make up the T cell receptor (TCR). The first major checkpoint is TCRβ-selection, which serves to select for a randomly rearranged in-frame TCRβ chain and excludes other rearrangements (to avoid multiple TCRs on the same cells). Successful rearrangement also rescues cells from apoptosis and initiates cell proliferation and differentiation to the second critical stage of development (Kruisbeek et al., 2000; von Boehmer et al., 1999; Zuniga-Pflucker and Lenardo, 1996). The second major checkpoint is thymic selection, which serves to eliminate autoreactive and non-reactive cells while selecting for useful cells that might become activated upon encounter with foreign antigen (Hogquist et al., 2005; Palmer, 2003). The early stages are described at "double-negative" (negative for both CD8 and CD4) or DN and are separated into four major subsets based on surface expression of the markers CD25 and CD44. DN1-4 thymocytes are distinguished as having $CD44^{+}CD25^{-}$ (DN1), $CD44^{+}CD25^{+}$ (DN2), $CD44^{-}CD25^{+}$ (DN3), and $CD44^{-}CD25^{-}$ (DN4). DN4 is transitory stage and these cells are destined to further upregulate CD8 and eventually CD4 expression to become $CD4^{+}CD8^{+}$ "double-positive" or DP cells and then undergo thymic education as noted above.

We recently proposed that a pre-TCR/TCR-based sustained signal is necessary for progression between these two previously described stages (Palacios and Weiss, 2007). ZAP-70 was shown as uniquely required to properly transduce these sustained TCR-based signals among the Syk-family kinases. While *zap70-/-* TCRβ-selected DN4 cells

still progress to become DP in substantial numbers, their efficiency to do so is significantly impaired compared to wild-type (WT) cells. This was shown to be due to a decrease in survival and a reduction in cell cycle progression in multiple stages between β-selection and thymic selection. Conversely, *syk-/-* thymocytes are only impaired at the earliest β-selection stage and not at any point after, as determined by competitive repopulation assays versus WT or *zap70-/-* thymocytes. The preference for using ZAP-70 between these two major checkpoints occurs despite overlapping protein expression of both kinases during these stages. Thus, only ZAP-70 seems uniquely required for properly transducing this sustained pre-TCR signaling that promotes DP generation.

ZAP-70 is positively regulated upstream by CD45, a plasma membrane-spanning tyrosine phosphatase that is critical for canonical TCR signaling (Hermiston et al., 2003) and is also important for thymocyte development. Mechanistically, this has been proposed to occur via the following simplified regulatory pathway: CD45 largely serves to positively regulate the Src-family kinase (SFK) Lck, which serves to positively regulate the activity of ZAP-70 (Palacios and Weiss, 2004). Biochemically, a major function of CD45 is to maintain Lck in a "primed" or "open" conformation by dephosphorylating a negative regulatory phosphotyrosine (Lck Y505) (Hermiston et al., 2003). Dephosphorylation of this tyrosine changes the conformation of Lck, ostensibly allowing it to become activated after sufficient clustering and transphosphorylation. Activated Lck then serves to phosphorylate ZAP-70 within its regulatory "interdomain B" domain at Y315 and Y319 allowing it to become open and activated (Brdicka et al., 2005). Genetic loss of CD45 in mouse models has shown that CD45 is not required for progression past the initial TCRβ-selection checkpoint, since there is significant

progression past this stage of development (Byth et al., 1996; Sato et al., 1998). While CD45 may not be critical, Src-family kinase (Groves et al., 1996; van Oers et al., 1996b) and Syk-family kinase mice (Cheng et al., 1997) activity is absolutely required. A critical role for CD45 was shown for positive selection signaling during thymocyte education as no thymocytes are positively selected in *cd45-/-* mice (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). No reported studies of CD45 function have, however, tested whether CD45 regulates kinase function and thymocyte development during sustained pre-TCR signaling and DP generation.

One unusual feature that distinguishes Syk and ZAP-70 kinases is their differential dependence on CD45 and Lck for activation, as shown in the model human T lymphoid Jurkat cell line (Chu et al., 1996). ZAP-70 is highly dependent on CD45 and Lck activity in Jurkat T cells. In contrast, Syk can transduce TCR signals in these cell lines in the absence of CD45 or Lck. One possible explanation is that Syk may be utilizing other SFKs (not Lck/Fyn) that do not depend on CD45 for activation. Expression of all other SFKs has not been reported in these cell lines. Another explanation is that Syk is truly less dependent on Src-family kinases and therefore CD45 for activation. Considering only the mild TCRβ-selection phenotype of CD45-deficient thymocytes together with $CD45^{-}Syk^{+}$ Jurkats, one might speculate that Syk is likely providing optimal pre-TCR signals at DN4, ISP and DP stages during DP generation. However, this is in conflict with our previous observation that ZAP-70, when compared to Syk, seems better suited for trasducing signals that promote DP generation (Palacios and Weiss, 2007). Thus, we have observed that only ZAP-70-deficient thymocytes are fitness impaired at DN4, ISP and DP stages (Palacios and Weiss, 2007) and can speculate

that in this case, sustained pre-TCR signaling is likely mediated by residual Syk kinase which should not be dependent on CD45 activity.

To test whether residual Syk function depends on CD45 presence during TCRβ-selection, we mated *cd45-/-* to *zap70-/-* mice. We hypothesized that remaining Syk function during early thymocyte development would be unaffected by loss of CD45 as occurs in Jurkat T cell lines. Since each individual gene-deficient mouse has previously been characterized as grossly "normal" in TCRβ-selection and DP generation in addition to the previously well-characterized block in positive selection, we expected the resulting *zap70-/-cd45-/-* (ZAPCD45) thymus to be only mildy impaired. This would be consisitent with Syk redundantly replacing ZAP-70 in a CD45-independent manner.

We analyzed thymocytes from ZAPCD45 mice for phenotypic composition and viability. We found that while each individual "knock-out" of ZAP-70 or CD45 had some reduced overall cellularity, the combined ZAPCD45 had markedly reduced cellularity as a result of impaired β-selection and also impaired DP generation. Cell viability was greatly reduced in ZAPCD45 thymus compared to either single knockout or WT. DN4 cells that had previously completed β-selection expressed reduced levels of CD27, a marker of signaling at the β-selection stage. DP cells had reduced levels of CD5 on their cell surfaces, an indicator of decreased signal strength through development, consistent with reduced intensity of sustained pre-TCR signaling. Thus, combined loss of both ZAP-70 and CD45 leads to increased impairments at multiple stages of early thymocyte development, including initial pre-TCR signaling at the β-selection stage and in sustained pre-TCR signaling at DN4 and later stages leading to DP generation. This confirms our previous report of ZAP-70 function in sustained pre-TCR signaling and further suggests

that residual Syk function in this process is highly dependent on the presence of CD45, contrary to what was expected from T cell line studies.

Results

Gross phenotype of ZAPCD45 dKO thymus

ZAPCD45 mice were born healthy and at Mendelian ratios. Thymi from dKO mice were visibly smaller than either single knockout, suggesting a compound impairment in thymocyte development. Absolute counts of total thymocytes confirmed this (Figure 3.1). To accurately enumerate total cellular counts, we counted cells from ZAPCD45 and control mice that were all between 5.0 and 5.7 weeks of age and had approximately equal numbers of males and females represented. Our analysis of control mice gave results that were comparable to previous reports (Kishihara et al., 1993; Negishi et al., 1995b). CD45-deficient thymus was approximately one-half as populated as WT ($2.16x10^8 \pm 3.1x10^7$ for WT versus $1.31x10^8 \pm 2.7x10^7$ for *cd45-/-*) and *zap70-/-* were only about one-third as much as WT ($0.80x10^8 \pm 1.4x10^7$). Overall thymic cellularity from ZAPCD45 mice was $0.125x10^8 \pm 4.7x10^6$. Thus, combined deficiency of both CD45 and ZAP-70 resulted a profound loss of thymic cellularity with overall levels reaching only 6% that of WT. This impairment is almost 4-fold greater than would be predicted if the effects of CD45 and ZAP-70 were on different pathways and merely additive, and suggests loss of a specific function that either molecule can provide.

Previously, we reported that ZAP-70 provides a distinct survival advantage to developing thymocytes as they expand to fill the DP pool before thymic selection occurs (Palacios and Weiss, 2007). To investigate whether survival was affected in thymocytes from ZAPCD45 animals directly *ex vivo*, we assessed viability of unfractionated thymocytes after minimal bench-top manipulation (see Material and Methods). As shown in Figure 3.2, viability of thymocytes directly measured *ex vivo* was drastically impaired

Figure 3.1 Overall thymic cellularity of ZAP45 dKO mice is vastly reduced. Bar graphs represent the mean +/- SD of overall thymocyte counts from WT, *zap70-/-*, *cd45-/-*, and ZAP45 dKO. Each group was represented by 7-11 mice which were between 5.0 and 5.7 weeks old and cells were counted by a Coulter cell counter after minimal manipulations *ex vivo*.

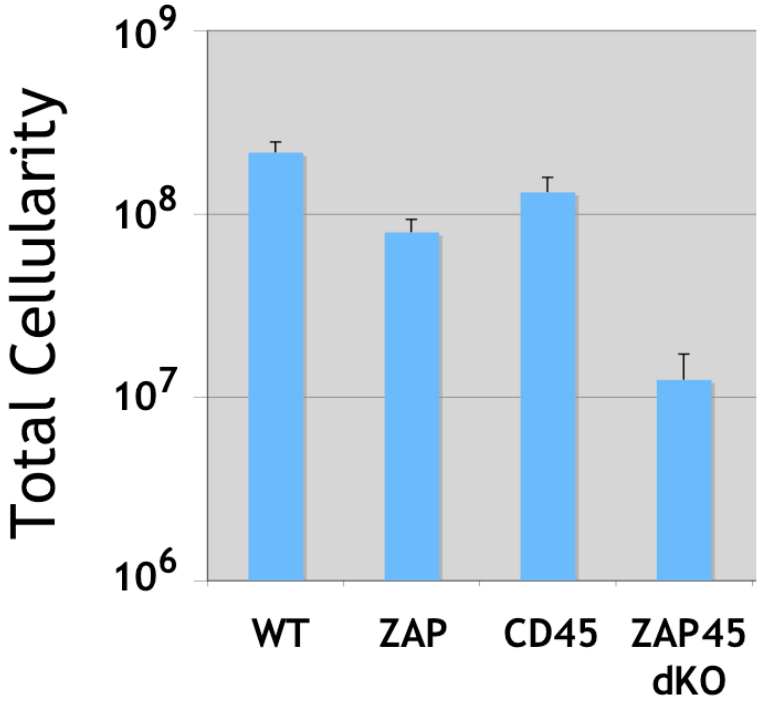
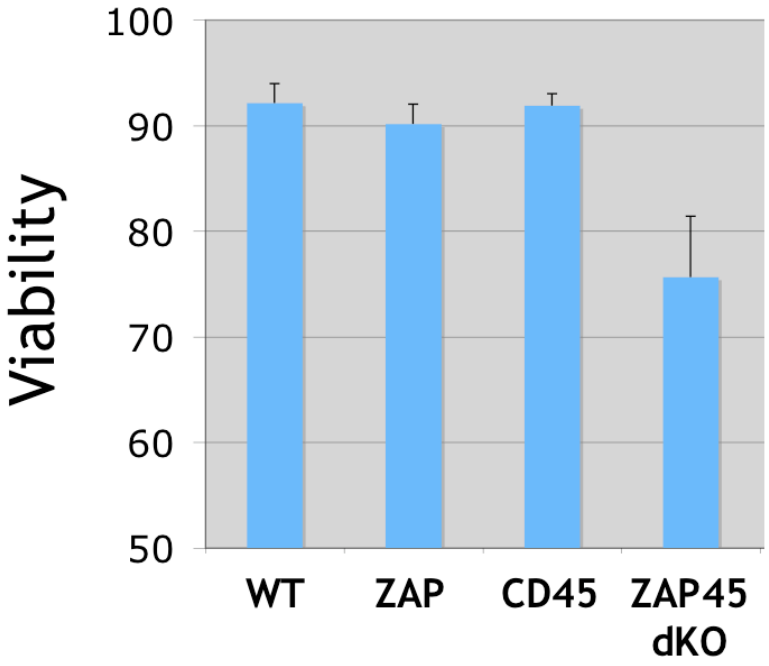

Total Cellularity
10^9
10^8
10^7
10^6
WT
ZAP
CD45
ZAP45 dKO

Figure 3.2 Viability of thymocytes is greatly reduced. WT, *zap70-/-*, *cd45-/-*, and ZAP45 dKO thymii were minimally manipulated as described in Figure 3.1 and Material and Methods, and viability was assessed by trypan blue exclusion as determined automatically by the Coulter cell counter. Data are represented as mean +/- SD of percent trypan blue negative of all lymphocytes.


Viability
100
90
80
70
60
50
WT
ZAP
CD45
ZAP45 dKO

in ZAPCD45 (92.1%±1.9 WT versus 75.7%±5.8 ZAPCD45). While *zap70-/-* and *cd45-/-* likely have individually lesser effects on survival, we were not able to measure significant differences in these noncompetitive assays as we had using competitive repopulation assays for *zap70-/-* (Palacios and Weiss, 2007).

Effects of CD45 and ZAP-70 deficiency on DN subsets

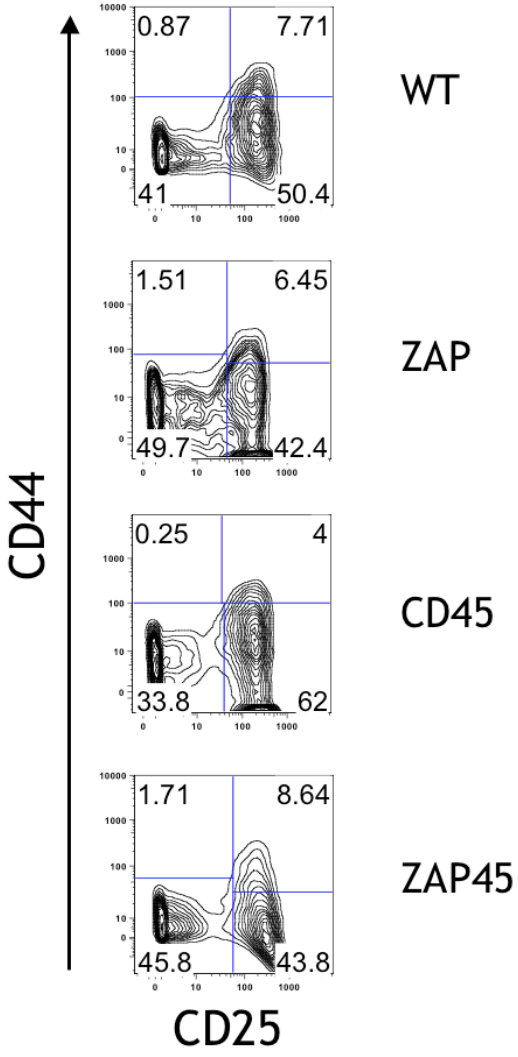
We previously reported that ZAP-70 is first upregulated in the earliest TCRβ selected cells within the DN3 subset during early thymocyte development (Palacios and Weiss, 2007). However, in that study, we did not detect a developmental fitness impairment in *zap70-/-* thymocytes until the DN4 stage. Since CD45-deficient mice are reported to be partially impaired in TCRβ selection, we asked whether combined deficiency of CD45 and ZAP-70 proteins impaired the development cells just undergoing β-selection. The frequencies of the various DN subsets are shown in a representative flow cytometric plot in Figure 3.3. While no significant changes (i.e., accumulations or losses) were observed in any particular subset as measured by frequency of all DNs or conversely as a ratio of DN3 to DN4 (Figure 3.3B), we found that the absolute counts of all DNs, reflecting mostly DN3s and DN4s, the earliest cells expressing newly formed TCRβ chains, were significantly reduced compared to all other genotypes (Figure 3.2). While *cd45-/-* thymocytes were impaired (an accumulation) at the earliest TCRβ-selection stage (DN3) and *zap70-/-* had a slight impairment at the DN4 stage (Figure 3.2) ZAPCD45 were reduced in both subsets of cells when measured on an absolute scale.

The TNF-family receptor CD27 has been shown to be upregulated immediately after pre-TCR signaling and its upregulation can be used as a marker of pre-TCR

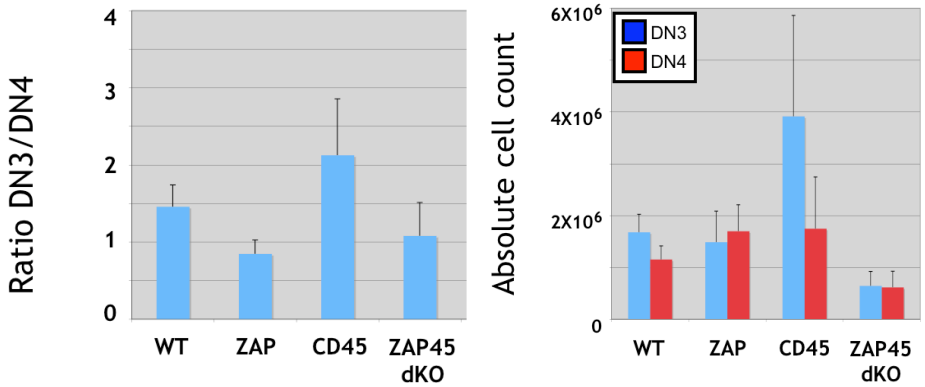
Figure 3.3 Phenotypic profile of CD4+CD8+(Lin-) DN thymocytes of ZAP45 dKO mice. (A) WT, *zap70-/-*, *cd45-/-*, and ZAP45 dKO thymocytes were analyzed by flow cytometry for CD44 & CD25 expression to determine DN2-4 distribution. Typically representative flow cytometric plots are shown for each mouse strain and are representative of at least 7 mice analyzed per genotype. Numbers indicate percent represented in that quadrant. See text for description of subsets.

(B) Left bar graph indicates mean +/-SD of the ratio of DN3 CD44-CD25+ to CD44-CD25- DN4 subsets. Right bar graph indicates mean +/-SD of absolute enumeration of the DN3 and DN4 subsets.

A


0.87
7.71
41
50.4
WT
1.51
6.45
49.7
42.4
ZAP
0.25
4
33.8
62
CD45
1.71
8.64
45.8
43.8
ZAP45
CD44
CD25

B

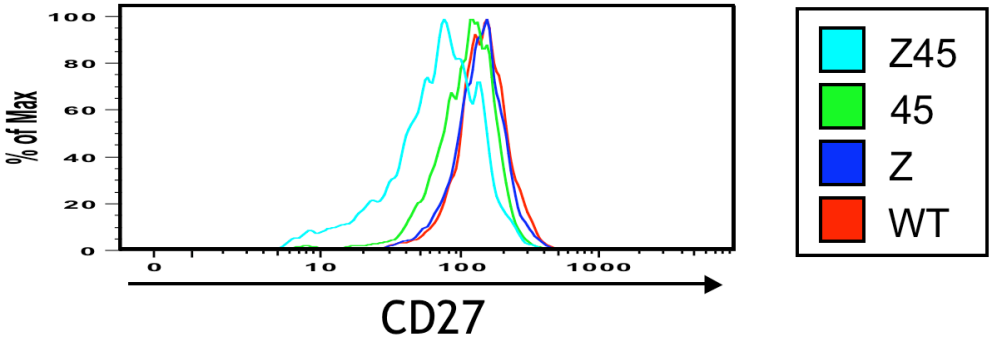

Ratio DN3/DN4
4
3
2
1
0
WT
ZAP
CD45
ZAP45 dKO
Absolute cell count
6X10⁶
4X10⁶
2X10⁶
0
DN3
DN4
WT
ZAP
CD45
ZAP45 dKO

signaling (Gravestein et al., 1996; Taghon et al., 2006). We found that levels of CD27 expression on DN4 subsets correlated well with ability to passage through the DN3 TCRβ-selection stage and could be therefore used as surrogate marker for signal strength (Figure 3.4). ZAP-70-deficient DN4 thymocytes are not impaired at DN3 progression and expressed levels of CD27 similar to WT DN4 cells. CD45-deficient thymocytes, however, are impaired at DN3 progression (Figure 3.3) (Byth et al., 1996; Sato et al., 1998) and showed a decrease in CD27 expression on DN4s (Figure 3.4). DN4 from ZAPCD45 mice have even lower levels of CD27 (Figure 3.4), consistent with a greater impairment in pre-TCR signal strength beginning at DN3.

Effects of CD45 and ZAP-70 deficiency on DP and SP subsets

We next wished to address whether DP and SP development is uniquely affected in ZAPCD45 dKO mice. Both *cd45-/-* and *zap70-/-* mice show drastic reductions in peripheral T cell populations, largely due to severe blocks in positive selection within the thymus. The surface marker CD5 is thought to be a negative regulator of TCR signaling and its expression correlates with TCR signal strength in DP thymocytes and in positive selecting thymocytes (Tarakhovsky et al., 1995). Bulk DP thymocytes from *cd45-/-* and *zap70-/-* mice expressed significantly lower levels of CD5 on their surface compared to WT, an indication of decreased signal strength through that stage of development as shown in Figure 3.5. ZAPCD45 dKO DP thymocytes expressed even lower levels of CD5, consistent with reduced TCR signal strength in unselected DP thymocytes (Figure 3.5). These results confirm our previous findings that ZAP-70 is required for normal

Figure 3.4 Expression of CD27 on DN4 thymocytes from ZAP45 dKO mice. DN4 from WT, *zap70-/-*, *cd45-/-*, and ZAP45 dKO thymocytes were defined by flow cytometry as Lin-CD44-CD25- (see materials and methods) and analyzed for CD27 expression. Overlayed histogram depicts representative CD27 profile of DN4 cells within each mouse strain. Bar graph depicts mean +/-SD of geometric mean fluorescence intensity (MFI) of CD27 using the same fluorescently-labeled antibody over several experiments and each bar represents at least 7 mice.


% of Max
100
80
60
40
20
0
0
10
100
1000
CD27
Z45
45
Z
WT

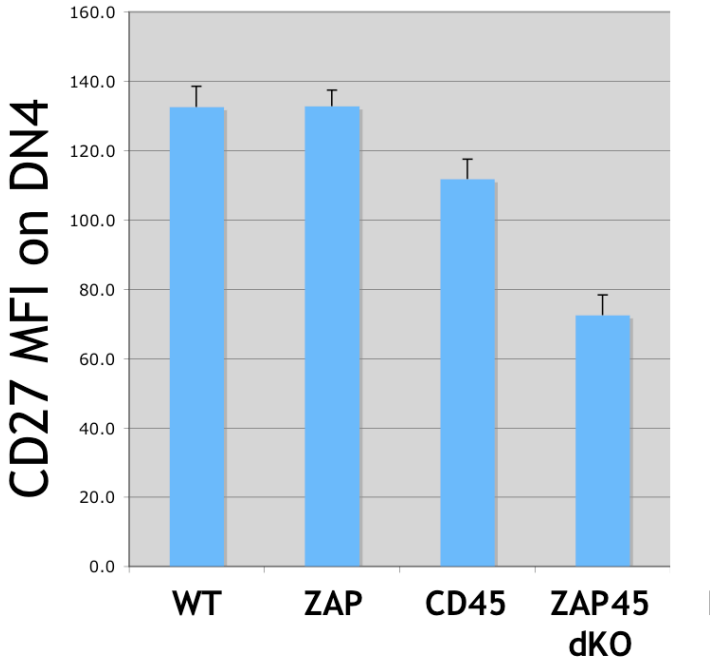
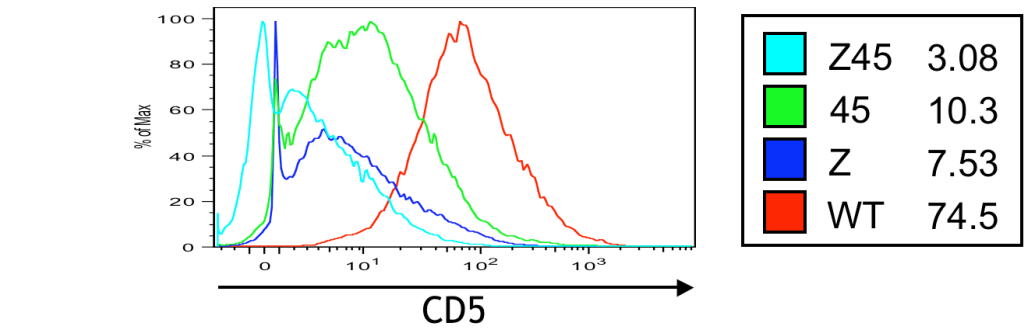
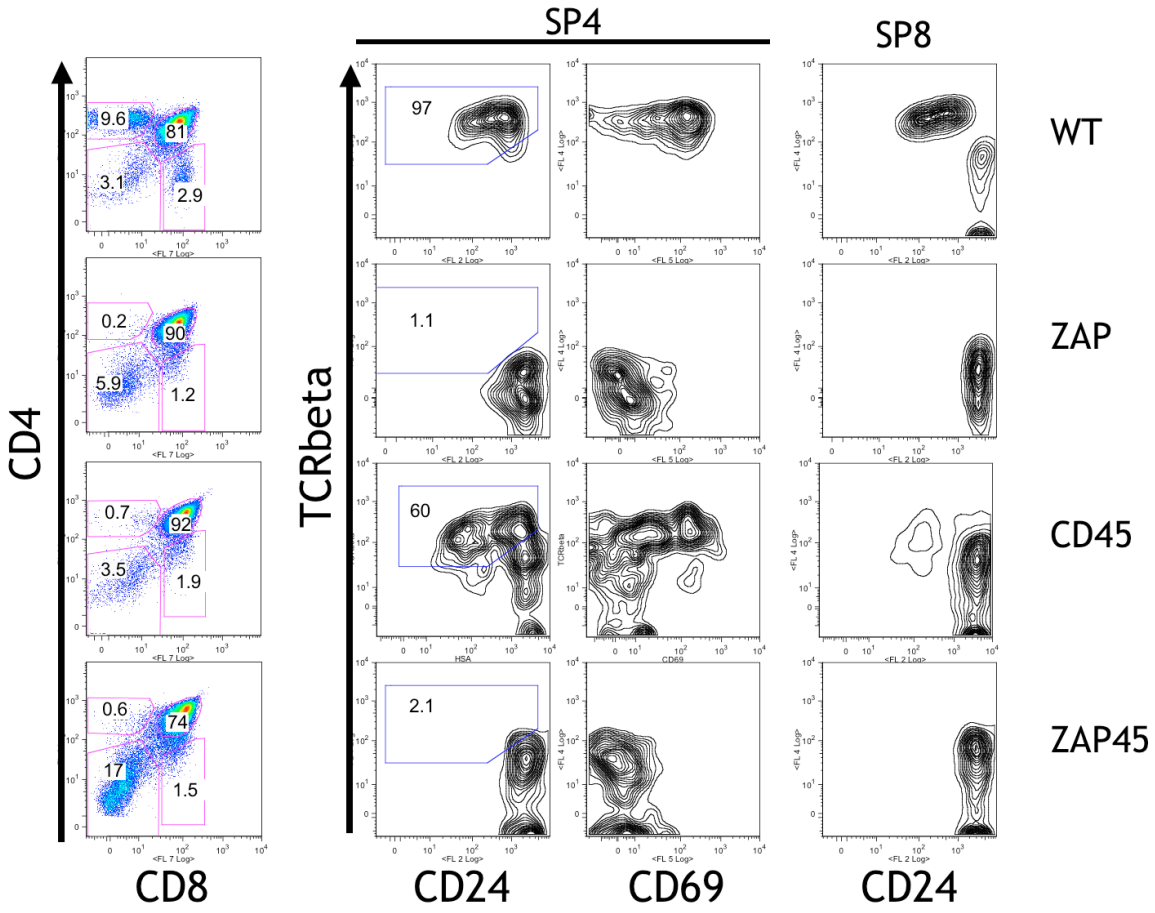

CD27 MFI on DN4
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
WT
ZAP
CD45
ZAP45 dKO

Figure 3.5 Phenotypic profile of DP and SP thymocytes from ZAP45 dKO mice. Thymocytes were analyzed for CD4 & CD8 expression and percentages assessed. Overlayed histogram depicts CD5 expression on ungated DP thymocytes as shown in the pseudo-colored plots below. Typically representative flow cytometric plots are shown for each mouse strain and are representative of at least 7 mice analyzed per genotype. Numbers next to each genotype name indicates the MFI of CD5 on the DP population. Contour plots illustrate expression of TCRβ, CD24 and CD69 on SP4 cells and of TCRβ and CD24 on SP8 cells.


% of Max
CD5
Z45 3.08
45 10.3
Z 7.53
WT 74.5


SP4
SP8
CD4
TCRbeta
CD8
CD24
CD69
CD24
WT
ZAP
CD45
ZAP45
9.6
81
3.1
2.9
0.2
90
5.9
1.2
0.7
92
3.5
1.9
0.6
74
17
1.5
97
1.1
60
2.1

generation of DP cells even before positive selection and further suggest that CD45 and ZAP-70 both contribute to continuous pre-TCR/TCR signaling.

Mature SP thymocytes are characterized by increased surface expression of TCRβ chain and by loss of CD24 and differential expression of CD69 (Sebzda et al., 1999). Thymocytes from *zap70-/-* mice show a nearly absolute block in SP development as measured by $TCR\beta^{hi}CD24^{lo}$ expression, confirming the critical role of ZAP-70 in positive selection (Figure 3.5). *Cd45-/-* thymocytes are, by comparison somewhat leaky (Figure 3.5), consistent with a previous report (Trop et al., 2000). The combined loss of CD45 and ZAP-70 proteins phenocopies the ZAP-70 single knockout with respect to SP development. Thus, the small amount of CD45-deficient positive selection observed is completely blocked by the additional deletion of ZAP-70, confirming the model that ZAP-70 kinase functions downstream of CD45 during thymic selection.

Discussion

We have examined the combined effects of removing both the tyrosine kinase ZAP-70 and the tyrosine phosphatase CD45 on all the major stages of thymocyte development. ZAPCD45 dKO mice are born viable and grossly normal. However, thymus cellularity is vastly reduced when compared to either single deletion alone or to WT thymus. A major feature of ZAPCD45 thymocytes is the large increase in apoptotic cells when assayed directly *ex vivo* and compared to either WT or the single deletion. The absolute number of DN cells, including the major subsets DN3 and DN4, is significantly reduced within ZAPCD45, suggesting a synergistic impairment in development and progression through these stages. A major decrease in DP production from DN precursors largely explains the >17-fold reduction in overall thymic cellularity. Consistent with this, DP cells from ZAPCD45 mice have almost no CD5 expression. SP development in ZAPCD45 mice phenocopies the absolute block seen in *zap70-/-* mice, suggesting that any positively selecting signals transduced independent of CD45 in the *cd45-/-* mouse must still travel through ZAP-70.

Effects on early thymocyte development

TCRβ selection is reported to be partially impaired in the absence of CD45, while ZAP-70 is first upregulated within DN3-L cells that have just undergone TCRβ selection, and therefore may promote progression from the DN3-L to the DN4 stage. However, *zap70-/-* thymocytes seem to have little defect at this stage (Figure 3.2 and (Palacios and Weiss, 2007) perhaps owing to sufficient Syk remaining that can perform this function. *cd45-/-* thymocytes are partially impaired at this early stage of TCRβ selection as evidenced by a

relative increase in the percentage of DN3 among the DN subsets within *cd45-/-* thymocytes (Figure 3.2 and (Byth et al., 1996; Sato et al., 1998). The DN compartment of ZAPCD45 thymocytes suggested no impairment in either DN3 or DN4 stages since both percentages appeared similar to WT, also indirectly suggesting that ZAP-70 may counteract the effects of CD45 at the DN3 stage. Instead, we propose that both the DN3 and DN4 are similarly impaired, thus explaining the WT-like ratio of DN3 to DN4 observed in ZAPCD45 mice. The overall decrease in absolute numbers of both DN3 and DN4 is consistent with this interpretation.

We find that CD27 is a surrogate marker for pre-TCR signal strength when measured at the DN4 stage among the genotypes examined in this study. The level of CD27 may be reflective of the pre-TCR signal strength at any stage between DN3 and DN4, but might be mostly a result of the initial DN3 pre-TCR signal. This is supported by the observation that *zap70-/-* thymocytes do not impair at DN3 (instead at DN4) and CD27 appears the same as WT, yet only produce about 1/3 as many DP cells, which likely reflects impairments at DN4 and later stages. Consistent with this, CD45 thymocytes have a relative impairment at DN3 (accumulation) and a reduced level of CD27 on DN4 cells and this results in roughly ½ as many DP cells generated. Thus, *zap70-/-* and *cd45-/-* individually impair at different stages and both result in a relative loss of DP generation, and CD27 expression supports a specific DN3 impairment for *cd45-/-* only.

Similar analysis of ZAPCD45 mice reveals a further reduction in CD27 expression on DN4 cells. This would suggest a greater impairment in initial DN3 pre-TCR signaling and a role for ZAP-70 during DN3 pre-TCR signaling. When CD45 is

absent, initial pre-TCR signaling at DN3 evidently depends on ZAP-70. Therefore, while the resulting DN4 cells had obviously signaled at DN3, the strength of the signal was further reduced in ZAPCD45.

Effects on DP generation

Our results further underscore our previous observations that ZAP-70 functions to promote sustained pre-TCR/TCR signaling between the DN4 and DP stages. Any residual signaling is clearly quite little, since DP cellularity is at least 17-fold reduced. Moreover, Syk is likely functioning in place of ZAP-70 in the *zap70-/-* thymocyte. And this pre-TCR/TCR Syk-dependent function is highly dependent on CD45 activity. This is contrary to what might have been predicted from studies in T cell lines where Syk was able to transduce TCR signaling independent of CD45, unlike ZAP-70 (Chu et al., 1996). Preliminary studies in our laboratory suggest combined deletion of CD45 and Syk do not result in such a dramatic paucity of DP thymocytes (data not shown), implying ZAP-70 might have some functionality without CD45 in a developing thymocyte, in contrast to Syk. However, it is also possible that some other phosphatase partially compensates for CD45 activity in thymocytes such as PTPα or CD148 (Lin et al., 2004; Pallen, 2003).

The reduced level of CD5 on the surface of DP thymocytes from ZAPCD45 mice is also consistent with a reduced intensity of pre-TCR/TCR signaling at the DP stage or perhaps earlier. It is unclear exactly what function CD5 provides to developing T cells, though most studies support a role in negative regulation of TCR signal strength (Azzam et al., 2001; Bhandoola et al., 2002; Pena-Rossi et al., 1999; Tarakhovsky et al., 1995), providing a logical explanation for increasing expression through development. Since

cd45-/- thymocytes are partially leaky through positive selection, at least as measured by percentages of DP and SP, it was possible that absence of CD45 might allow some legitimate signal to be transduced, either using a different phosphatase or via a different mechanism of Src and Syk-family kinase activation. Careful analysis of *zap70-/-* thymocytes shows that absence of ZAP-70 provides a near-absolute block in positive selection. The effect of removing both proteins, CD45 and ZAP-70 shows that ZAP-70 is epistatic to CD45 function. That is, any TCR-mediated positively selecting signal that occurs independently of CD45 must still travel downstream through ZAP-70. Thus, any signal generated without CD45 is still likely to be activating Lck/Fyn and ZAP-70. CD45 is thought to exert its positive regulatory function on TCR signaling by dephosphorylating the inhibitory tyrosine of Src-family kinases, Lck and Fyn, thereby promoting the SFK open conformation and allowing it to become more easily activated. This is counterbalanced by the kinase activities of Csk, which phosphorylates the inhibitory tyrosine. Thus, a requirement for CD45 would strictly depend on the local and relative activity of Csk, PAG (which binds Csk), Fyn and Lck. How all of these proteins are recruited and activated, both in the basal and activated state, is still not well understood.

We have shown loss of two positive regulators of TCR-signaling, ZAP-70 and CD45, when combined result in a severe defect in progression through early thymocyte development. Loss of either protein alone results in a mild reduction in DP generation and cell viability, yet both evidently converge on the same specific function of cell survival. We previously reported a measurable defect in survival of *zap70-/-* thymocytes, but this was only obvious within the context of *in vivo* anti-CD3-stimulation of *rag1-/-*

thymocytes, likely due to the high rate of apoptotic cell clearance in the thymus. In contrast, apoptosis rates of ZAPCD45 thymocytes are so high they are easily measured directly *ex vivo*. It is possible that CD45 and ZAP-70 function in entirely different pathways, each resulting in cell survival. We cannot formally rule this out since it is possible that CD45 can regulate other pathways, such as Jak activation (Irie-Sasaki et al., 2001; Trigueros et al., 2003). We feel this is a less plausible explanation because cytokine pathways are not proven to be functionally important at this stage (Van De Wiele et al., 2004; Yu et al., 2004) and our data on CD5 levels on DP cells from *cd45-/-* and ZAPCD45 suggest progressively reduced levels of TCR-based signals up to and including the DP stage, before positive selection. Our data also suggest that CD45 and Syk function to propagate sustained pre-TCR signaling within *zap70-/-* thymocytes and that Syk is highly dependent on CD45, contrary to expectations from cell line studies. Thus, confirming and expanding on our previous report, we find that ZAP-70 drives sustained pre-TCR signaling by augmenting cell survival and that CD45 can positively regulate this pathway in the absence of ZAP-70.

Materials and methods

Mice

Zap70$^{-/-}$ mice were backcrossed at least seven times onto the B6 background. *Cd45exon6*$^{-/-}$ mice were backcrossed onto B6. *Zap70*$^{-/-}$*cd45exon6*$^{-/-}$ mice appeared grossly normal but lacked peripheral T cells. B6 (originally from Jackson Laboratories) and CD45.1^{+} B6 (BoyJ, originally from Taconic and Jackson Laboratories) were both used as WT controls with no differences noted. For assessment of thymic output, all animals were killed at 5.0-5.7 weeks of age and processed similarly and stained with the same cocktail of antibodies to identify phenotypic profile. For each genotype, approximately equal numbers of females and males were used. All animals were housed in specific pathogen-free facility at UCSF according to University and National Institutes of Health (NIH) guidelines.

Antibodies and Flow Cytometry

To accurately measure levels of viability directly *ex vivo*, we minimized and standardized our experimental manipulations as follows. First, mouse thymus was harvested within 20 minutes of killing the animal. Next, each thymus was put into 5 ml ice-cold 2%FBS PBS and separated to single-cell suspensions using glass slides, then counted for concentration and viability using an automated cell counter (Coulter Vicell). 1-3x10^6 cells were used for each stain. For DN subsets, mAbs against CD3, CD4, CD8, CD11b, CD19, Gr-1, DX-5, TCRγδ (Lin^{+}) were used to exclude lineage-committed cells and delineate DN thymocytes and were conjugated to biotin (BD Biosciences and eBioscience). Steptaviden-conjugated to either APC, Pacific Blue (PB) or Pacific Orange (PO)

(Invitrogen) was used to detect the Lin^+ cells for exclusion. Anti-CD27 (BD Biosciences)-PE was used to assess expression on DN subsets. For multicolor flow cytometry on a CyanADP, the following fluorescent dyes were used in combination, FITC/AlexaFluor 488, PE, PE-Texas Red (PETR), PE-Cy5.5, PE-Cy7, PO, PB, and allophycocyanin (APC)/AlexaFluor 647. PO-conjugated CD8 was purchased from BioLegend. PB-conjugated antibodies were purchased from either BioLegend or BD Biosciences. PE-CD27, PE-Cy5.5-TCRβ, PETR-CD25, PE-Cy7-CD44 and APC-Lin+ were used to characterize DN cells. For SP and DP characterization, FITC-CD5, PE-CD24, PE-Cy5.5-TCRβ, PE-Cy7-CD69, PB-CD4, and AlexaFluor 647-CD8 were used. Fc binding was blocked using anti-CD16/32 2.4G2 (Harlan). Intracellular staining was described in the "Materials and methods" section of Chapter 2. Data were collected on a FACSCalibur, LSR II (BD Biosciences) or CyanADP (Dako) and analyzed using FlowJo software (TreeStar).

Chapter 4

Concluding remarks and future directions

Over the years it has become clear that both SFK and Syk-family kinase signaling is critical for T-lineage development and function. Antigen receptor signaling, such as BCR, TCR, and Fc signaling are all dependent on the SFK and Syk-family kinase 2-step ITAM-dependent mechanism of signaling initially characterized by this lab for T cells. Other unappreciated functions also depend on antigen receptor function. For example, homeostatic maintenance and lymphopenic expansion, both require signals that are propagated through the antigen receptors and these have been shown to require at least SFK signaling. A requirement for Syk-family kinase signaling has not been reported to date for these functions. The major checkpoints stages of thymocyte development were also shown to be critically dependent on SFK and Syk-family signaling. But until now, it has remained unknown whether there are requirements of antigen receptor signaling for stages between the DN and DP stages. Moreover, while the role of SFK signaling has been grossly determined at the initial pre-TCR and DP thymic selection stages, the specific roles of Syk-family kinases has only been clearly defined for the DP thymic selection stage, where only ZAP-70 is shown to be critical. Previous work on the roles of Syk-family kinase signaling at the initial pre-TCR DN3 stage has not examined whether ZAP-70 or Syk are preferentially utilized by thymocytes.

Work in this thesis has shown that the Syk family kinases, Syk and ZAP-70 are differentially regulated through early thymocyte development and that loss of either kinase results in distinct developmental impairments of the given thymocyte. We've provided evidence that pre-TCR signaling continues past the DN3 stage and that this sustained signal induces and then depends on ZAP-70 up-regulated expression. Thus, we

propose a revised model based on the one previously shown (Figure 1.1) that now incorporates our novel findings (Figure 4.1).

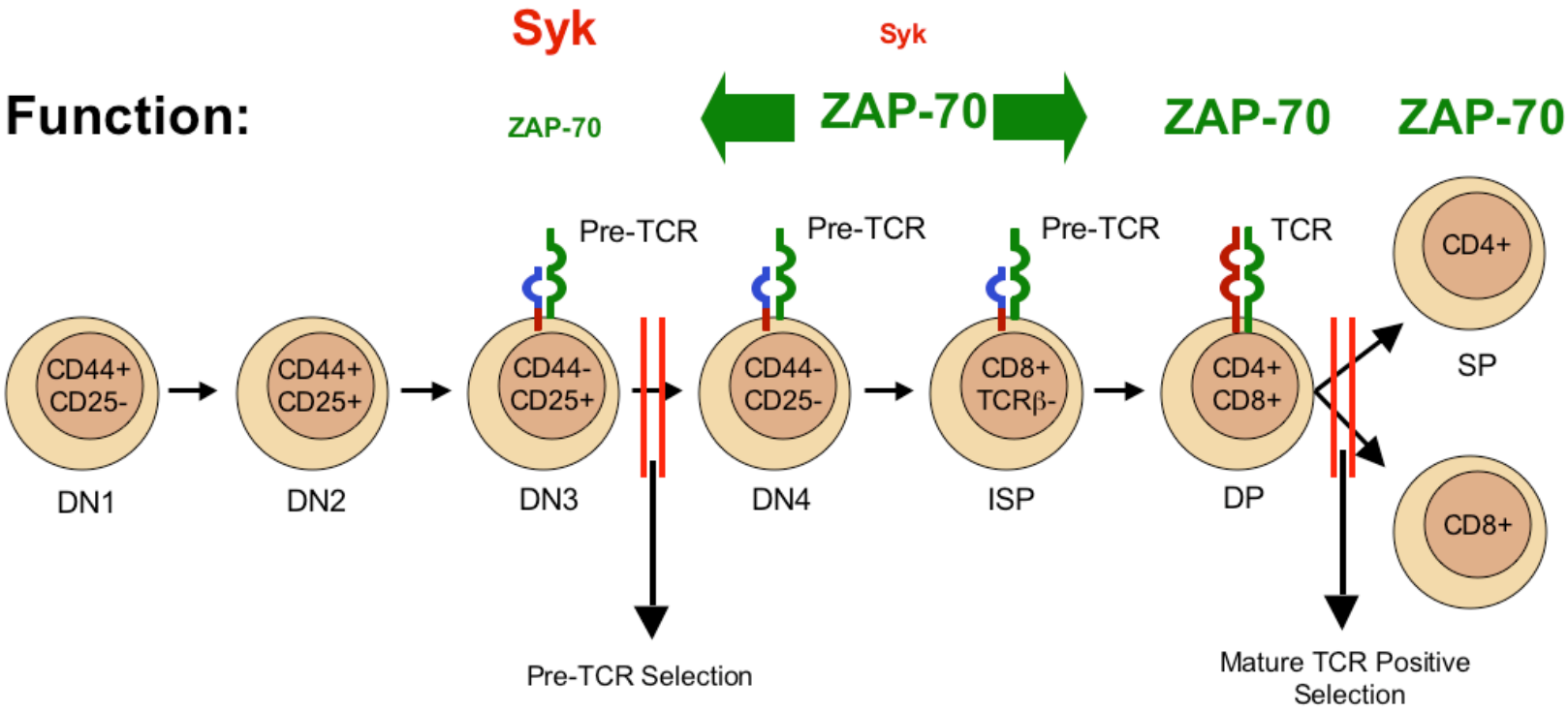
Expression patterns of Syk and ZAP-70 during thymocyte development

Contrary to the cursory assumptions made from previous studies, we have found that ZAP-70 and Syk are not similarly expressed through all stages of early thymocyte development. Instead, we found that ZAP-70 is hardly detectable in the earliest thymocytes and not robustly expressed until well after cells have committed to the αβT pathway, which occurs at the DN3 stage. Since TCR rearrangements do not depend on Syk-family kinase expression, the absolute block seen in the double-knockout mouse is therefore a block in initial pre-TCR signaling. Syk is most highly expressed in the earliest committing thymocyte, the $CD117^{+}CD44^{+}CD25^{-}$ DN cell and likely sooner. Syk is likely to also be expressed in precursor common lymphoid progenitor (CLP) cells that seed the thymus and may be expressed earlier. One can imagine a function for Syk in hematopoietic stem cells at these stages. However, we saw no difference in colonization of thymus between *syk*$^{-/-}$ and WT stem cells under our experimental conditions. However, a different study (Colucci et al., 2000) indicated that irradiating mice with varying amounts of radiation could differentially "clear" out the stem cell niches within the bone marrow. Use of *rag1*$^{-/-}$ that were also $\gamma c^{-/-}$ showed *syk*$^{-/-}$ stem cells could reconstitute all T lineage cells, whereas they could not in mice that were only *rag1*$^{-/-}$, presumably because loss of the cytokine common γ chain reduced the amount competing stem cells within the bone marrow after stem cell transfer. Thus, it remains unknown, if under different

Figure 4.1 Proposed model of pre-TCR/TCR signaling stages and Syk-family kinase function throughout development. “Expression” depicts schematic of relative expression patterns now established for Syk-family kinases for all thymocyte stages. “Function” indicates proposed requirement for initial/sustained pre-TCR signaling and which Syk-family kinase is preferentially used. The initial pre-TCR signal is propagated as soon as a TCRβ chain is properly expressed is largely Syk-dependent. Syk is important for proper cell cycle entry as Syk-deficient DN3 cells that have properly rearranged and expressed a TCRβ chain fail to “blast” properly and do not initiate DNA synthesis normally. This signal normally immediately up-regulates ZAP-70 expression (while down-regulating Syk), effectively altering the receptor’s own signaling machinery. Beginning at least at DN4, ZAP-70 now drives sustained pre-TCR signaling. Residual expression of Syk can partially compensate for lack of ZAP-70 and this activity is highly dependent on CD45 function, presumably through SFK activation of Syk. This continuous pre-TCR/TCR signal is necessary for normal expression of CD4 and CD8 and expansion of the DP compartment prior to positive selection.

Expression:

Function:



circumstances, Syk might be important for overall fitness of stem cell or of lymphoid precursor.

Another unappreciated function for Syk in T cell development might be in a subset of DP cells. We find that Syk mRNA is easily detectable within DP thymocytes, whereas protein is not. We do not know when this disconnect between message RNA and protein occurs. It is possible that Syk protein is upregulated within a subpopulation of DP cells, possibly either in positively or negatively selecting cells or even in some other undefined subset. Our attempts to reproducibly detect a Syk^{hi} protein expressing subpopulation by flow cytometry within DP thymocytes did not yield an identifiable population (not shown). There is one report that Syk protein expression depends on the coexpression of OCA-B, a B-lineage cofactor for transcription factors (Siegel et al., 2006). OCA-B is reported to be upregulated in DP, T cells and Jurkat T cells after TCR stimulation (Sauter and Matthias, 1997; Zwilling et al., 1997), suggesting a role in stabilizing and thereby increasing Syk protein expression in a subset of DP thymocytes. Thus, Syk protein may have an important function in a subset of DP thymocytes.

ZAP-70 expression is sharply upregulated within TCRβ selected cells. One intriguing question is whether ZAP-70 is upregulated within TCRγδ selected cells. Recent work suggests that TCRαβ and TCRγδ divergence is largely determined by the strength of signal delivered by the early expressed receptor (Hayday et al., 1999), where weaker signals lead to TCRαβ differentiation and stronger signals lead to TCRγδ. Thus, one might expect that ZAP-70 protein should, at least transiently, be expressed at higher levels in newly selected TCRγδ cells, since we have shown that ZAP-70 expression

correlates with strength of the pre-TCR signal, although there may be an upper limit to this expression pattern.

Additionally, the fact that ZAP-70 is up-regulated ~5X from the DN3 to the DN4 stage is, *a priori*, suggestive of some function at this stage. There are few, if any, signaling proteins that have been reported increased this much at the DN4 stage. ZAP-70 has few established functions beyond antigen receptor signaling, suggesting a novel role for ZAP-70 and an appreciated role for pre-TCR signaling in development

Syk is preferentially employed during initial pre-TCR signaling

We have shown that thymocytes specifically express and depend on Syk protein for pre-TCR signaling at the DN3 stage. Multiple measurements suggest that DN3 cells newly expressing TCRβ chains and lacking Syk fail to signal properly and are impaired in entering cell cycle or up-regulating TCRβ protein expression. It remains unknown if thymocytes evolved to use Syk at this stage instead of ZAP-70 for some specific purpose. Biochemical difference between Syk and ZAP-70 are documented in this lab and others; Syk is less SFK dependent and is a more active kinase. Teleologically, one could envision thymocytes require the most sensitive signaling apparatus possible to rescue resting DN3 cells from apoptosis as soon as a competent TCRβ chain is expressed, or to shut down further TCRβ or TCRγδ rearrangements and prevent multiple TCRs on the same cell. The most definitive way to test these possibilities is to create a mouse that expresses *zap70* mRNA under the regulatory constraints of the *syk* gene and to express this within a $syk^{-/-}$ background. This mouse would express ZAP-70 within the earliest thymocyte progenitors, up to and including the resting DN3-E stage when initial

signaling occurs and cell cycle status, TCRβ and CD27 status could be assessed. This could be accomplished by "knocking-in" approaches, where *zap70* cDNA is inserted into the upstream portion of the *syk* locus by homologous recombination or by recombineering techniques where a bacterial artificial chromosome containing the entire *syk* locus is recombined to express *zap70* instead of *syk* and the entire region of DNA is inserted into the genome non-homologously (Copeland et al., 2001). Syk-deficiency is all lineages results in mice that die soon after birth and it would be interesting to test if substituting ZAP-70 protein instead of Syk would allow for rescue of animal viability in addition to rescue of initial pre-TCR signaling at the DN3-E stage.

We do not know if loss of Syk protein would affect allelic exclusion at the TCRβ locus. Syk activity correlates with allelic exclusion at the IgG heavy chain pre-BCR, pre-TCR and Ig light chain selection stage. Thus, loss of Syk in αβT cell development is correlated with lack of allelic exclusion at the TCRα locus. Ideally, crossing a $syk^{-/-}$ and *TCRβ*-transgenic mouse might directly answer whether Syk is required for TCRβ allelic exclusion, a process that requires discrete signaling pathways downstream of the pre-TCR. Using a *TCRβ*-transgenic instead of a TCRαβ might preserve "normal" pre-TCR signaling as many transgenic TCRs are highly expressed early in development, unlike the endogenous pre-TCR. It might also be informative to cross the *TCRβ*-transgenic mouse to the ZAP-70 "knock-in" proposed earlier to directly test if Syk provides a unique allelic exclusion function that ZAP-70 cannot.

Another study provided evidence that DN3-L cells that having signaled through Syk (wild-type cells) initiate different programs when differentiating toward αβT or γδT cell pathways (Taghon et al., 2006). Lack of Syk could alter these programs. A genomics

analysis using cDNA arrays of *syk*$^{-/-}$ DN3-L might yield interesting clues of whether Syk signals drive expression of a specific program.

Thymocyte use ZAP-70 for sustained pre-TCR signaling between DN3 and DP stages

Our data argues that pre-TCR signaling is continuous after the DN3-E stage and up until the DP stage. This appears to be is necessary for expansion and generation of DP thymocytes. ZAP-70 expression is coordinately up-regulated during these stages. To directly address whether a pre-TCR signal is actually required for progression from the DN4 to DP stage would require halting all pre-TCR signaling at this stage. It is technically difficult to terminate pre-TCR/TCR signaling within the transient DN4 stage of development *in vivo*. Any attempt to use lineage-specific gene deletion (of e.g. LAT, Lck/Fyn, Slp-76, ZAP-70/Syk) would be subject to "leakiness" artifacts, because even if gene deletion appeared complete at the right stage by PCR, delayed loss of protein would likely be a concern. Even small amounts of signaling proteins could confound results, but rigorous assessment of protein levels within single cells could address this issue. Another avenue of research that might shed further light on this question is to culture isolated DN4 thymocytes *in vitro* and culture them overnight in media for 24-48hr as they differentiate to the DP stage (Petrie et al., 1990; Wilson et al., 1989). As we have demonstrated, one can use TCR-pathway inhibitors to block DP development. We have provided evidence that blocking SFK signaling completely blocks development at the DN4 and ISP stages and no DP thymocytes develop, as is also seen after blocking MAPK p38 function ((Mulroy and Sen, 2001) and Chapter 3). Inhibitors of Syk-family kinases,

calcineurin and the ras-pathway may also block development at this stage, but NF-AT and ras activation are considerably downstream of the pre-TCR/TCR. Signals that promote cell cycle progression, or survival or CD4/CD8 upregulation may diverge prior to calcineurin and ras-pathway activation. Thus, some inhibitors might prevent CD4/CD8 upregulation, while others might impair survival, while others may specifically impair cell cycle progression. All inhibitors could be tested with the various genetic mutants in combination to yield interesting information. For example, if ZAP-70 is important at this stage and since commercial Syk inhibitors are available, one could use *zap70*$^{-/-}$ and WT and inhibit either Syk or SFK using PP2 and test for DP development.

Just as for signaling during the DN3-E stage, we do not formally know if thymocytes evolved to replace Syk with ZAP-70 for sustained pre-TCR signaling for a specific purpose. Is there some function that ZAP-70 provides at this stage that Syk cannot? One piece of evidence suggests this could be the case. Work from the laboratory of Jonathan Ashwell provides the strongest evidence of *in vivo* functional differences between Syk and ZAP-70 (Salvador et al., 2005). T cells expressing ZAP-70 were shown to differentially activate p38 via an MKK-independent pathway, where p38 is activated via tyrosine phosphorylation by Lck or ZAP-70which then leads to autophosphorylation on its own activation loop threonine-tyrosine motif. Differentially regulated targets of p38 were not investigated. Since p38 activity is required for DP generation, DN4 thymocytes could require that p38 be alternatively activated and this would require ZAP-70 instead of Syk. To directly test for this, one could cross-link the pre-TCR on isolated DN4 cells from WT and *zap70*$^{-/-}$ mice and immunoprecipitate p38 and perform an *in vitro* autophosphorylation assay. If ZAP-70 is required for this activation, p38 from purified

WT DN4 cells should be able to autophosphorylate after pre-TCR crosslinking. Similarly, stimulated DN4 from *zap70*$^{-/-}$ thymus should be unable to autophosphorylate. The numbers of cells required for this kind of experiment might be prohibitive, given their paucity in mice. Alternatively, a monoclonal antibody of sufficient sensitivity could be developed that would recognize the characteristic PY323 of p38 that is phosphorylated by ZAP-70 and be detected by flow cytometry. Individual cells could be analyzed for alternative p38 activation by assaying PY323 positivity after stimulation through the pre-TCR of DN4 cells from WT and *zap70*$^{-/-}$ mice. Another possibility would be to create a "knock-in" mouse expressing a p38 molecule that is mutated at Y323 to phenylalanine. Competing the p38 knock-in thymocytes with WT thymocytes in irradiated chimeric mice as they repopulate the thymus would ideally test this.

Inasmuch as one could ideally replace ZAP-70 with Syk in the genome of a mouse as mentioned previously in this discussion, one might consider the converse experiment: Swapping ZAP-70 with Syk within the context of a knock-in mouse. ZAP-70-like expression of Syk within DN4 would allow testing of whether ZAP-70 has been evolutionarily selected to perform a particular function within DN4, ISP and DP that Syk might not. Chan and colleagues made a rough approximation of this experiment by transgenically (over) expressing Syk protein within a *zap70*$^{-/-}$ mouse (Kong et al., 1995). This grossly rescued all thymocyte development, but developmental fitness was never addressed. A mouse featuring Syk knocked into the ZAP-70 locus might yield a different result. Moreover, if peripheral T cells were produced, one could test the effects of replacing ZAP-70 with Syk in peripheral T cell function and TCR signaling. This was

difficult to analyze in Jurkat cell lines, as $Syk^{+}ZAP\text{-}70^{-}$ Jurkats did not grow well (Williams et al., 1998).

Several experiments would be possible with the creation of $Syk^{+}ZAP\text{-}70^{-}$ peripheral T cells. Proliferation assays in response to TCR- or to superantigen stimulation would indicate whether Syk could substitute for ZAP-70 in TCR signal transduction. Routine biochemical analysis of major components within the TCR signaling pathway could easily be address by performing *in vitro* stimulations and immunoprecipitating and performing immunoblot analysis for key phosphorylation residues within Lck, Fyn, TCRξ, Syk, LAT, Slp-76, phospholipase C γ 1 (PLCγ1), Tec kinases and MAPK pathways. One might expect results similar to those found when Syk replaces ZAP-70 in Jurkats, where increased Erk phosphorylation and higher basal Ca^{2+} were found. However, this could easily not be the case, since regulation of basal level signals are likely to be very different in a developed T cell versus a Jurkat cell. Since Syk is considered to be a more "active" kinase than ZAP-70 or at least less SFK dependent, one might expect T cells are more responsive to antigen challenge and perhaps more prone to autoimmune induction. Experiments inducing experimental autoimmune encephalomyelitis (EAE) might indicate if $Syk^{+}ZAP\text{-}70^{-}$ T cells are more likely to contribute to CD4+ mediated T cell destruction of CNS tissues. Challenging the animals with pathogens that elicit strong T cell responses such as LCMV or *L. monocytogenes*, might help elucidate clues to a deeper evolutionary question: why did T cells evolve to use ZAP-70 at all when virtually all other hematopoietic cells use Syk? Challenging the mouse with a well-characterized model infectious agent could be very informative.

Effects of CD45 on ZAP-70 kinase function in lymphocyte development

Our studies of combined deletion of CD45 and ZAP-70 *in vivo* has underscored the importance of ZAP-70 function during expansion of DP thymocytes and progression through the DN4 and ISP stages. It was unknown if CD45 would have any effect at all on this progression and we have shown that loss of both proteins produces a synergistic decrease in thymocyte production, largely reflecting impaired DP generation. The major DN subsets, DN3 and DN4 in WT thymus are all decreased in numbers indicating impairment in survival or production of both subsets. Analysis of either single knockout suggest that *zap70*$^{-/-}$ DN cells are largely impaired at progression through DN4 while *cd45*$^{-/-}$ DN cells block at DN3, since these populations accumulate in the respective mouse. Loss of both proteins suggests that at DN3, ZAP-70 may be functioning in concert with CD45 to promote progression; possibly at the DN3-L stage after ZAP-70 is first up-regulated. At the DN4 stage, residual Syk function is likely allowing *zap70*$^{-/-}$ thymocytes to progress, but this function seems highly dependent on CD45, since loss of both CD45 and ZAP-70 yields more than a 17-fold reduction in DP cells.

That there is any progression at all would suggest that Syk does have some CD45 independent function as shown in T cell lines. Preliminary *in vitro* studies culturing WT DN4 cells using PP2 to inhibit SFK would suggest this is not the case, as PP2 can prevent virtually all DP generation. These studies are preliminary and are subject to being *in vitro* artifacts, but they are rather clear. If CD45 exerts its entire positive regulatory activity by dephosphorylating SFK and "priming" it for activation (see previous section *Model of regulation of SFK kinases*), then any DP generation within ZAP45 dKO mice would have to result from Syk function that is independent of CD45. This is clearly not very efficient,

since these mice produce about $1.25x10^7$, which is still roughly only 3X more than *rag1*$^{-/-}$ produce. This ultimately bears on the question of whether there is an absolute requirement for pre-TCR signaling to progress past this stage. The PP2 data would argue there is, but this needs to be confirmed. Also, previous work in this laboratory suggested thymocytes may express other SFKs besides Lck and Fyn and the function of all these kinases with respect to CD45 regulation and downstream function are not established. SFK could affect other pathways and some could simply be less dependent on CD45 and still positively regulate Syk within DN4, albeit inefficiently.

DP cells from *zap70*$^{-/-}$ thymus express significantly lower levels of CD5 than *cd45*$^{-/-}$ DP cells, yet both are quite impaired at positive selection, with some leakiness in the *cd45*$^{-/-}$. And the combined ZAP45 DP cell expresses even less CD5. This suggests that the level of CD5 on bulk DP thymocytes has nothing to do with positive selection and might instead reflect the strength of the sustained pre-TCR/TCR signal at the DN4 and ISP stage.

Complete inhibition of SP development within ZAP45 thymus provides good evidence that any CD45 independent signal that allows a few SP to develop are still required to travel through ZAP-70. This underscores previous work that argued that ZAP-70 is required for positive selection. Whether the low level of SP production seen in the CD45exon-6-deficient mouse is an artifact of residual CD45 expression has not been clarified.

This thesis has shown that careful analysis of *in vivo* development can reveal novel conclusions about how signaling molecules function in development. We analyzed tyrosine kinase-deficient thymocytes and the results led to the proposal that a novel

mechanism is important for amplification of DP thymocytes. Previous work has given clues that sustained pre-TCR signaling may occur in developing pre-T cells, but work presented in this thesis establishes that the Syk-family kinases are central to regulating this signal. This research has shown that the pre-TCR signal is qualitatively distinct at different stages of development and that this initial pre-TCR signal actually directs changes to its own signaling machinery. That is, the initial pre-TCR uses Syk to efficiently transduce signals, and then shuts off Syk expression, while at the same time up-regulating ZAP-70 expression. ZAP-70 then transduces sustained pre-TCR signals that are necessary for normal production of DP cells. In support of this conclusion, loss of ZAP-70 results in impaired production of DP cells. Further loss of CD45, far more severely impairs DP generation, underscoring a requirement for sustained pre-TCR signaling in normal thymocyte development.

Appendix

Lck regulation of Syk-family kinases through development

Abstract

Lymphocyte-specific kinase (Lck) is a tyrosine kinase expressed primarily in T lymphocytes and has been shown to be a critical proximal regulator of T cell receptor (TCR)-based signals. Lck is the direct upstream activator of Syk-family kinases in T cells. In Jurkat T cell lines, the Syk-family tyrosine kinases, Syk and ZAP-70 are shown to differentially depend on Lck for activation after TCR-stimulation. The effect of Lck activity on Syk-family kinases has not been investigated in primary T lineage cells. To test whether Syk or ZAP-70 differentially depend on Lck for functional activity in primary T lymphoid cells, we bred *lck-/-* mice to *zap70-/-* or *syk-/-* mice and assessed thymocyte development. We found that ZAPLck double-knockout thymus had more DP and SP cells than Lck-deficient thymus. SykLck double-knockout thymus appeared similar to Lck-deficient thymus. Thus, we unexpectedly find that loss of two purportedly positive regulators of thymocyte development, Lck and ZAP-70, actually results in some rescue of lymphocyte development at two critical checkpoints, TCRβ-selection and positive selection. This suggests either Lck/ZAP-70 have some previously unknown negative regulatory function at this stage or that Fyn/Syk may be rescuing some pre-TCR/TCR signals that ZAP-70 cannot perform without Lck expression.

Introduction

Lck is a SFK and a major tyrosine kinase expressed in T lymphocytes whose function in proximal TCR signaling is well established (see Chapter 1). Briefly, Lck serves to phosphorylate the TCRζ chain after receptor activation induced by coclustering of the TCR with or without the CD4 or CD8 coreceptor. Phosphorylated TCRζ ITAMs serve as docking sites for the Syk-family kinases, Syk and ZAP-70. Lck further serves to phosphorylate critical residues within the interdomain B domain of ZAP-70 thereby "opening" it up for eventual full activation.

Previous work in this lab (see Chapter 1 and references therein) reported that Lck is required for full ZAP-70 kinase activation but not for Syk activation. In fact, gross examination of the activated TCR signaling pathway appears normal in Lck-deficient T cell lines when Syk is expressed but not when ZAP-70 only is expressed. Thus, loss of Lck may affect the function of Syk and ZAP-70 differently.

Genetic studies of Lck have underscored the critical nature of Lck in all stages of thymocyte development. Lck is required for TCRβ-selection, positive selection, peripheral naïve T cell survival and lymphopenic expansion of T cells (Molina et al., 1992a). The requirement for Lck during TCRβ–selection is not absolute as Fyn is shown to compensate for some progression through this early checkpoint in the absence of Lck (Groves et al., 1996; van Oers et al., 1996b). Loss of Lck resulted in a severe block in TCRβ–selection such that the entire thymus cellularity was about $10x10^6$ total cells (compared to ~$100x10^6$ for WT) and approximately half of the *lck-/-* thymocytes were DP cells. Thus, the absolute number of DN cells did not change, but due to the vast reduction in DP cells, DN composition went from the normal ~2% to approximately

~50%. Positive selection appeared even more profoundly affected since SP generation was 1% that of WT. Thus, loss of Lck was reported to block TCRβ–selection to 10% that of normal numbers of total thymocytes while positive selection was reduced to 1% that of WT.

We have previously shown that Syk and ZAP-70 differentially contribute to early thymocyte development by propagating initial pre-TCR and sustained pre-TCR signaling, respectively. Lck is expressed at high levels at all stages (DN2-SP) after αβT cell commitment is initiated and is likely to affect Syk-family kinase activity throughout development (Buckland et al., 2000). Lck may also affect sustained pre-TCR signaling since one report provided evidence that *lck-/-* thymocytes had reduced ISP (DP precursor cells) numbers and survival . Lck may uniquely affect the function of Syk and/or ZAP-70 in developing thymocytes as they progress through initial and sustained pre-TCR signaling. To test these possibilities we mated *lck-/-* mice to *zap70-/-* mice to obtain ZAPLck double-knockout mice and then analyzed thymocyte development. We also analyzed SykLck double-knockout mice as controls for some experiments.

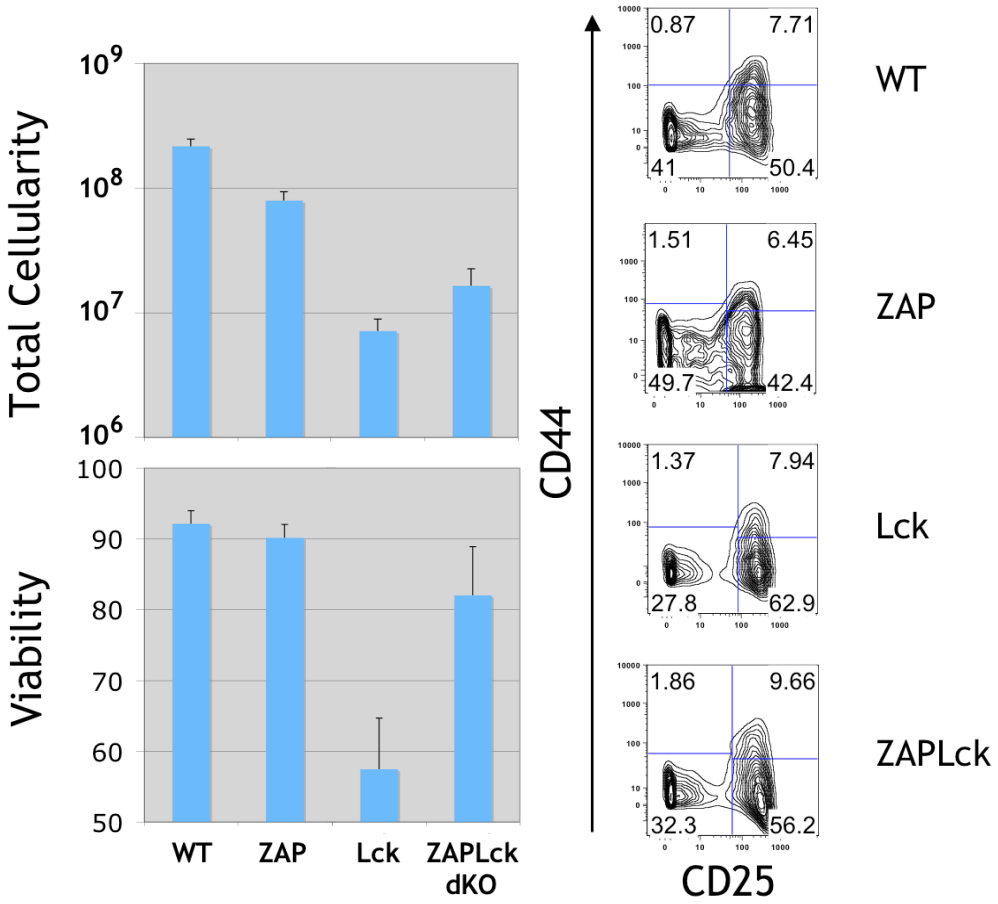
Results and Discussion

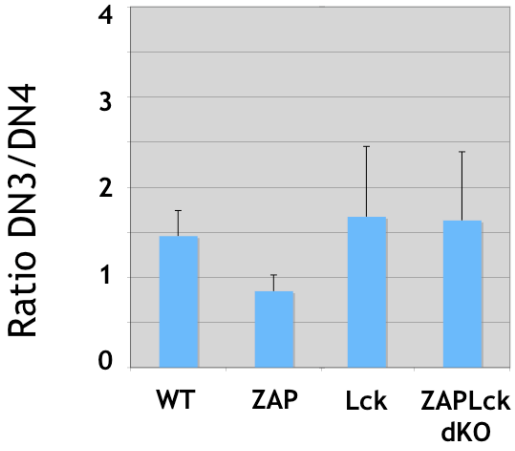
Overall phenotype and DN development in ZAPLck and SykLck thymi

We assessed total thymocyte cellularity and viability from WT, *zap70-/-*, *lck-/-* and ZAPLck mice that were all aged between 5.0 and 5.7 weeks, as was done in Chapter 3, and the results are shown in Figure A.1. The data for WT and *zap70-/-* are the same as those shown in Figure 3.1. We found total cellularity of *lck-/-* thymocytes was $7.2x10^8 \pm 1.7x10^6$, which was in agreement with the original reported phenotype (Molina et al., 1992a). ZAPLck thymus consistently produced about 2.3-fold more ($1.6x10^7 \pm 6.1x10^6$) thymocytes than *lck-/-* alone (P=0.0029, student's T-test).

Thymocyte viability and total cellularity is partially rescued in the ZAPLck mouse as compared to the *lck-/-* mouse. This is unexpected since loss of two positive regulators of thymocyte development (pre-TCR/TCR signaling components), ZAP-70 and Lck, would be expected to further impair development more than either single knockout. Rescue of development in ZAPLck is supported by multiple parameters. In addition to increased overall survival and total cellularity, ZAPLck thymocyte have increased levels of CD27 on their DN4 cells and a greater percentage of DP (and relatively reduced fraction of DNs) when compared to *lck-/-*. We previously showed that CD27 seems to correlate with initial pre-TCR signal strength in DN4 cells and ZAPLck DN4 express CD27 similar to WT DN4s (see Figure 3.4), suggesting that additional loss of ZAP-70 allows *lck-/-* impaired DN3 cells to signal via the pre-TCR at near-WT levels at least through this early stage. Clearly, ZAPLck thymocytes are still much impaired in DP generation, since cellularity is far below that of WT mice but this may be due to impairments at stages after DN3, possibly in sustained pre-TCR signaling.

Figure A.1 Cellularity and viability of all thymocytes and CD25CD44 phenotypic profile of DNs from ZAPLck thymocytes. Top two bar graphs represent mean +/-SD of total cellularity and viability as assessed for WT, *zap70*$^{-/-}$, *lck*$^{-/-}$, and ZAPLck dKO thymocytes by Coulter cell counter after minimal *ex vivo* manipulation. Thymocytes were analyzed by flow cytometry for CD44 & CD25 expression to determine DN2-4 distribution. Typically representative flow cytometric contour plots are shown for each mouse strain and are representative of 7-11 mice analyzed per genotype. Control strains WT and *zap70*$^{-/-}$ were from the same analysis as that done on ZAP45 dKO shown in Chapter 3. Numbers indicate percent represented in that quadrant. See text for description of subsets. Bottom bar graph indicates mean +/-SD of the ratio of CD44-CD25+ DN3 to CD44-CD25- DN4 subsets.


Total Cellularity
10^9
10^8
10^7
10^6
Viability
100
90
80
70
60
50
WT
ZAP
Lck
ZAPLck dKO
CD44
CD25
0.87
7.71
41
50.4
WT
1.51
6.45
49.7
42.4
ZAP
1.37
7.94
27.8
62.9
Lck
1.86
9.66
32.3
56.2
ZAPLck


Ratio DN3/DN4
4
3
2
1
0
WT
ZAP
Lck
ZAPLck dKO

At least two possibilities could explain this phenomenon. First, in addition to the well-described positive regulatory role ZAP-70 has in TCR-based signaling, ZAP-70 may also exert a minor negative regulatory role that is not revealed until Lck is removed. One speculative guess is that Lck/ZAP-70 may transduce classic SFK-Syk-family kinase signals in early development but ZAP-70 may also, perhaps when not complexed to Lck, facilitate a low-level downregulating signal that may terminate signaling or even enhance cell death. Thus, in *lck-/-* mice, the severe block in thymocyte development may be due to, in addition to lack of Lck positive regulation, active impairment of thymocyte development by ZAP-70. Another explanation might be that removing both Lck and ZAP-70 allows the remaining SFK and Syk-family kinases, Fyn and Syk to couple together to rescue some development. Since Fyn and Syk are far more widely expressed in hematopoietic and other tissues, it is feasible that they are more evolutionarily ancient and pre-date Lck and ZAP-70 existence and function. Thus, removing Lck and ZAP-70 may allow a more ancient albeit less efficient coupling of Fyn and Syk that can propagate some pre-TCR signals that are slightly stronger than Fyn and ZAP-70 (in *lck-/-*) at that stage of development. Since DN3 and DN4 cells are few in these mice, biochemical testing of these possibilities will require better antibodies that can allow single-cell analysis of Lck, Fyn, ZAP-70 and Syk activation status among the various genotypes.

For LckSyk studies, because Syk-deficiency causes perinatal death, we reconstituted lethally irradiated *rag1-/-* mice and analyzed the resulting thymus as shown in Figure A.2. It should be noted that the results from WT and *lck-/-* shown in Figure A.1 and A.2 are different because the first set of data are of an analysis of normally viable

Figure A.2 Cellularity and viability of all thymocytes and CD25/CD44 phenotypic profile of DNs from SykLck thymocytes. Top two bar graphs represent mean +/-SD of total cellularity and viability as assessed for WT, *syk*$^{-/-}$, *lck*$^{-/-}$, and SykLck dKO thymocytes by Coulter cell counter after minimal *ex vivo* manipulation. Mice were created by reconstitution of lethally irradiated (500 rad x 2) *rag1*$^{-/-}$ with fetal liver from genotyped E13.5-15.5 embryos. Cells were analyzed as shown in Figure A.1 and are representative of at least 4 mice analyzed per genotype and the entire reconstitution performed twice with similar results. Control strains WT and *lck*$^{-/-}$ were reconstituted in parallel.

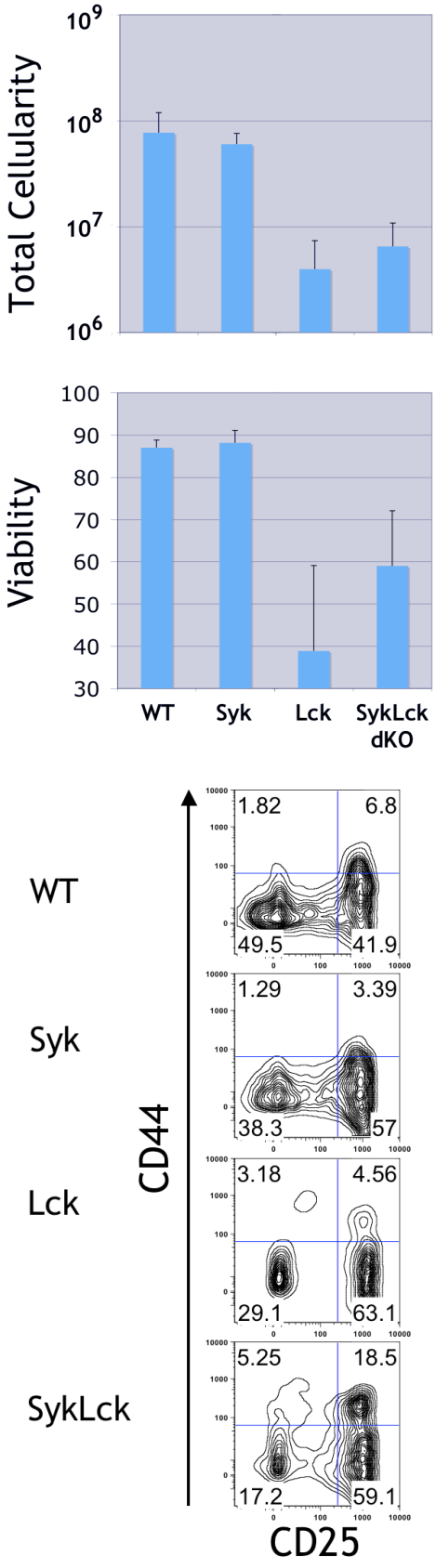
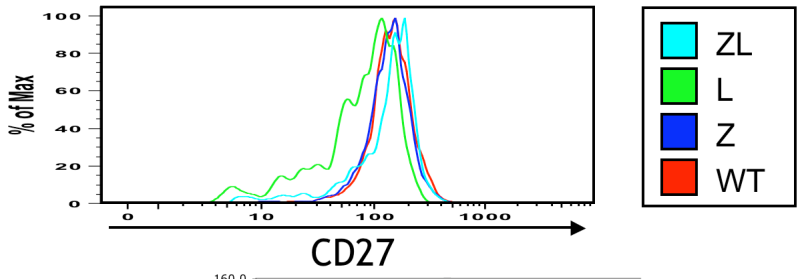
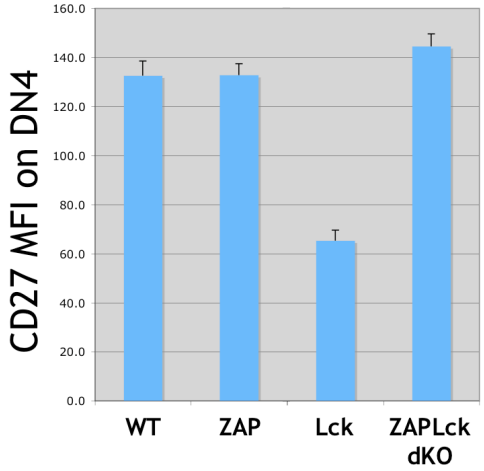
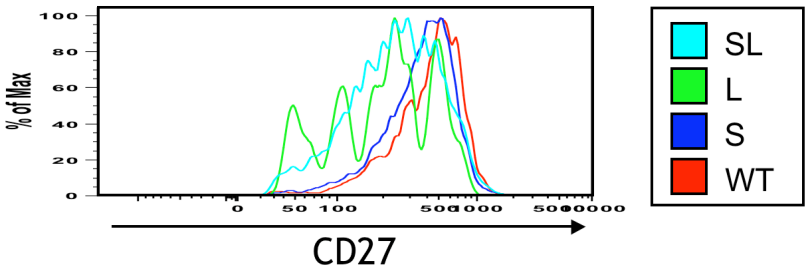
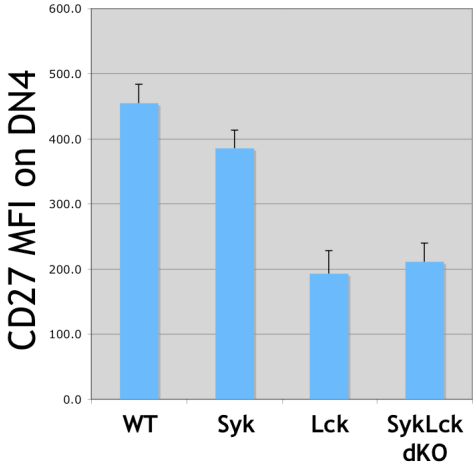

Total Cellularity
10^9
10^8
10^7
10^6
Viability
100
90
80
70
60
50
40
30
WT
Syk
Lck
SykLck dKO
WT
Syk
Lck
SykLck
CD44
CD25
1.82
6.8
49.5
41.9
1.29
3.39
38.3
57
3.18
4.56
29.1
63.1
5.25
18.5
17.2
59.1

Figure A.3 Expression of CD27 on DN4 thymocytes from ZAPLck and SykLck dKO mice. DN4 from WT, *zap70*$^{-/-}$, *lck*$^{-/-}$, ZAPLck and *syk*$^{-/-}$and SykLck dKO thymocytes were defined by flow cytometry as Lin-CD44-CD25- (see materials and methods) and analyzed for CD27 expression. Overlayed histogram depicts representative CD27 profile of DN4 cells within each mouse strain. Bar graph depicts mean +/-SD of geometric mean fluorescence intensity (MFI) of CD27 using the same fluorescently-labeled antibody over several experiments for WT, *zap70*$^{-/-}$, *lck*$^{-/-}$, ZAPLck mice and each bar represents at least 7 mice. For SykLck analysis, WT, *lck*$^{-/-}$, *syk*$^{-/-}$and SykLck mice bars are representative of at least three mice and two separate experiments and they were also reconstituted in parallel.


% of Max
100
80
60
40
20
0
0
10
100
1000
CD27
ZL
L
Z
WT


CD27 MFI on DN4
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
WT
ZAP
Lck
ZAPLck dKO


% of Max
100
80
60
40
20
0
0
50
100
CD27
SL
L
S
WT

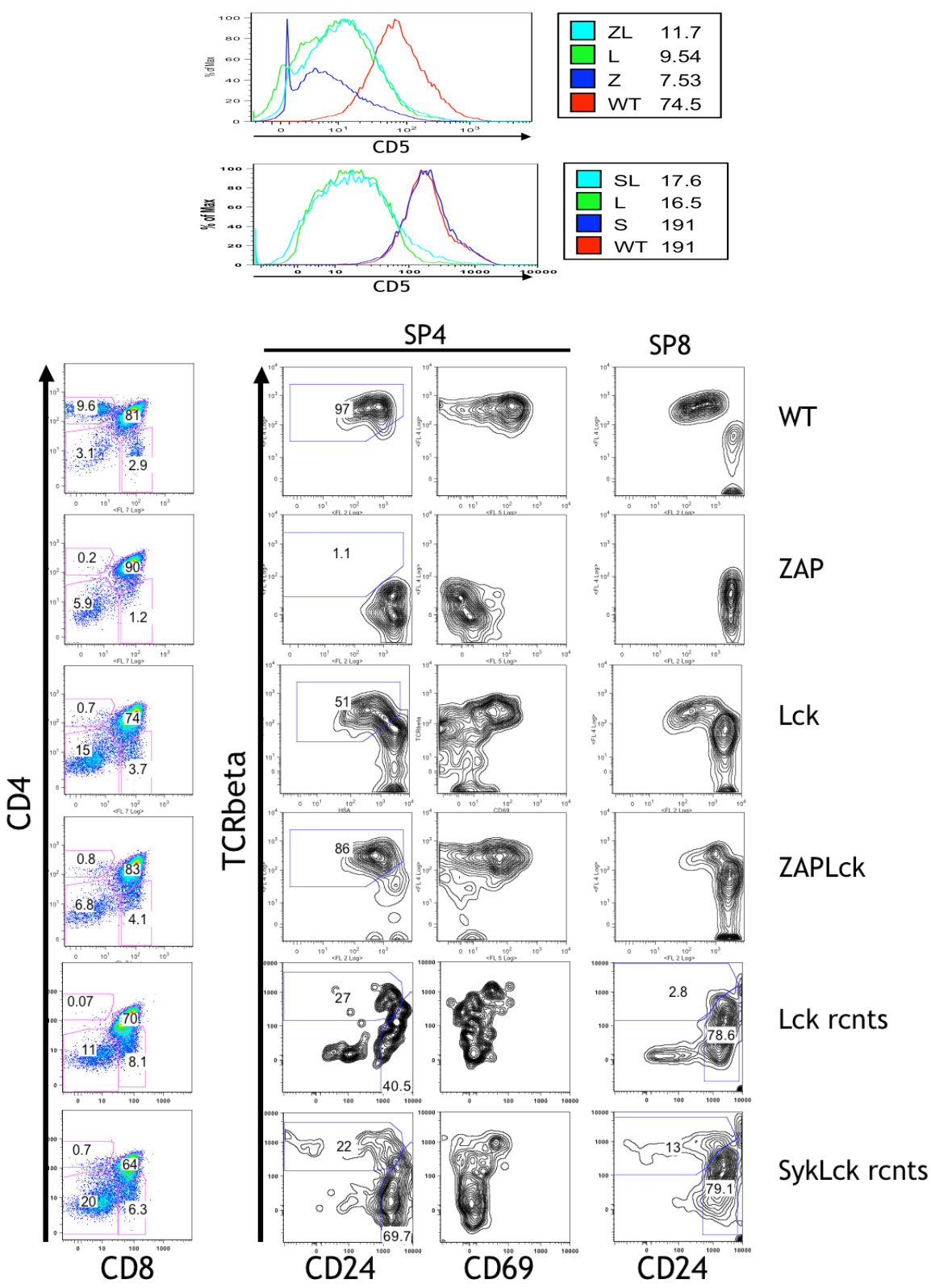

CD27 MFI on DN4
600.0
500.0
400.0
300.0
200.0
100.0
0.0
WT
Syk
Lck
SykLck dKO

mice and the second set of data represents an analysis of mice reconstituted at 5-7 weeks with the given hematopoietic genotype. More variability is seen in SykLck cellularity and viability counts than in the ZAPLck studies, likely owing to the limitations of making reconstituted mice. SykLck thymocyte cellularity appears similar to *lck-/-* (P=0.34, $4.0x10^6 \pm 3.4x10^6$ for *lck-/-* versus $6.5x10^6 \pm 4.3x10^6$ for SykLck), suggesting no obvious impairment or rescue when Syk is additionally deleted in the Lck-deficient background. This suggests that Fyn might couple with both Syk and ZAP-70 in the *lck-/-* background, but loss of Syk does not enhance the function of Fyn interacting with ZAP-70. Perhaps within the *lck-/-* background, Fyn might activate more Syk, but ZAP-70 might be more accessible to it, possibly by competing better for relevant ITAMs. The levels of CD27 on DN4 cells from SykLck suggest no improvement in initial pre-TCR signaling compared to *lck-/-*.

DP and SP generation in ZAPLck and SykLck thymi

The generation of DP and SP cells is also enhanced in ZAPLck when compared to *lck-/-* thymocytes and is shown in Figure A.4. DP cells are increased as noted by an increase in their percentage and the overall increase in thymic cellularity. The *lck-/-* thymus contains a small number of bona-fide mature SP cells, suggesting some residual function from Fyn and ZAP-70/Syk during positive selection. The *zap70-/-* thymus, however, appears to be characterized by a near-absolute block in SP generation (as discussed in Chapter 3). Surprisingly, combined loss of Lck and ZAP-70 appears to rescue more SP generation. The increase is modest, but their $CD24^{lo}TCRb^{hi}$ (and the increase in this fraction compared to immature $CD24^{hi}$ cells) phenotype suggests there

Figure A.4 Phenotypic profile of DP and SP thymocytes from ZAPLck and SykLck dKO mice. Thymocytes were analyzed for CD4 & CD8 expression and percentages assessed. Overlayed histogram depicts CD5 expression on ungated DP thymocytes as defined in the pseudo-colored plots below. Typically representative flow cytometric plots are shown for each mouse strain and are representative of at least 7 mice analyzed per genotype. Number next to genotype names indicate MFI of CD5 on DP populations. Contour plots illustrate expression of TCRβ, CD24 and CD69 on SP4 cells and of TCRβ and CD24 on SP8 cells For SykLck (SykLck rcnts) analysis, WT was also analyzed by reconstitution and was qualitatively similar to the one show here that was not reconstituted. Numbers within contour plots indicate percent within that gate.


ZL 11.7
L 9.54
Z 7.53
WT 74.5
CD5
SL 17.6
L 16.5
S 191
WT 191
CD5
SP4
SP8
WT
ZAP
Lck
ZAPLck
Lck rcnts
SykLck rcnts
CD4
CD8
TCRbeta
CD24
CD69
CD24

are more SP cells being positively selected. Moreover, levels of CD5 are increased on DP cells of ZAPLck when compared to *zap70-/-* thymocytes, which are extremely low. It is difficult to interpret exactly what CD5 on DP cells reflects, i.e. whether it is a measure of the strength of signal at previous stages or at the given stage is still not clear.

DP and SP generation in SykLck mice is more difficult to interpret but not likely to be much different than in *lck-/-* and is shown in Figure A.4. The percentage of DP in SykLck and *lck-/-* was quite variable due to the limitations of creating reconstituted mice and did not appear consistently different. The levels of CD5 seem similar in both genotypes and were reproducible across different mice and experiments. Of note, the small amount of SP generation seen in *lck-/-* mice in Figure A.3 seems almost absent in reconstituted *lck-/-* mice as shown in Figure A.4. The reason for this difference is unclear but may also be a limitation of reconstitution with an irradiated stroma of an adult mouse.

Conclusions

While the data regarding SykLck are either negative or difficult to interpret, the results seen in ZAPLck mice are reproducible and intriguing. Thymocyte development in ZAPLck mice is somewhat rescued when compared to Lck-deficiency alone, which is a severely impaired in development. Loss of ZAP-70 allows more Lck-deficient pre-T cells to progress past the DN3 stage and probably more to progress from the DN4 stage to the DP stage. Whether DN4 cells from ZAPLck mice are better able to progress to the DP stage is a question that could be directly tested by employing competitive repopulation assays of the thymus using stem cells from ZAPLck and *lck-/-* mice. It remains unknown

whether sustained pre-TCR signaling is improved in ZAPLck thymocytes, but it might be reasonable to speculate that it is.

Our data suggest that survival rates are rescued in ZAPLck thymocytes when compared to *lck*$^{-/-}$ alone. It would be productive to assay levels of apoptosis in DN4 and ISP cells that are expanding to fill the DP niche. It is likely that pathways downstream of sustained pre-TCR signaling diverge, vis a vi functions for proliferation, gene expression, and survival. Thus, if ZAPLck cells are primarily rescued by increases in cell survival, specific pathways such as Akt, NF-kB or Bcl-xL may be activated, yet the MAP kinase pathway may be unaffected (Kruisbeek et al., 2000). In addition to eventually using optimized activation specific antibodies to test for these possibilities, one could at least assay for *ex vivo* apoptotic rates and also for spontaneous apoptotic rates *in vitro* after overnight culture (Sun et al., 2000; Voll et al., 2000). Additionally, interrogating the level of cell cycle distribution among all relevant thymocyte subsets could suggest a mechanism for improved development.

Regarding the improved level of positive selection, it would be interesting to test whether DP cells from ZAPLck mice are better able to signal, *in vitro*, in response to TCR stimulation as a surrogate for positive or negative selection signaling. At a cursory level, this could at least be measured by assaying for Ca2+ mobilization and Erk phosphorylation (Gong et al., 2001; Neilson et al., 2004). At this stage, the MAP kinase pathway might be partially rescued, which may explain the increase in SP development and positive selection. Furthermore, SP development is also characterized by active rescue of the selected cells, which is largely dependent on upregulation of the anti-apoptotic factor, Bcl-2 (Veis et al., 1993). This can be measured by flow cytometry in

newly selecting cells at the DP and SP stages and might be higher in ZAPLck mice than *lck-/-* mice.

Combined *in vivo* deletion of ZAP-70 and Lck has revealed unexpected complexities of the functions of these kinases. While difficult to analyze due to the low level of overall cellular output, these mice provide an interesting paradox of results. How can loss of two strong positive regulators result in a partial rescue in development? We have already discussed some avenues of research that may shed light on this. Other long-term experiments that might yield clues regarding mechanisms might be to cross these mice to a TCR transgenic mouse, such as the HY system to directly test for positive and negative selection (Simpson et al., 1997). Since these mice are clearly compromised in TCR signaling, they may also be selecting for more self-reactive T cells. Aging mice and assaying for T cell driven autoimmunity by searching for evidence of T cell infiltrates in non-hematopoietic tissues and organs, as has been done for AIRE-deficient and *skg* mice, could be revealing (Anderson et al., 2002; Sakaguchi et al., 2003).

Materials and methods

Mice

Zap70$^{-/-}$ mice were backcrossed at least seven times onto the B6 background. *Cd45exon6*$^{-/-}$ and *lck*$^{-/-}$ mice were backcrossed onto the B6 background. *Zap70*$^{-/-}$*lck*$^{-/-}$ mice appeared grossly normal but lacked peripheral T cells. *Syk*$^{+/-}$ mice were bred to *lck*$^{-/-}$ mice were carried as *syk*$^{+/-}$*lck*$^{-/-}$ mice due to perinatal death of Syk-deficient mice. B6 (originally from Jackson Laboratories) and CD45.1^{+} B6 (BoyJ, originally from Taconic and Jackson Laboratories) were both used as WT controls with no differences noted. For assessment of thymic output, *zap70*$^{-/-}$*lck*$^{-/-}$ mice (and controls) were processed as detailed in Chapter 3. Crossing *syk*$^{+/-}$*lck*$^{-/-}$ mice to each other generated *syk*$^{-/-}$*lck*$^{-/-}$ embryos. *Rag1*$^{-/-}$ (Jackson Laboratories) mice were used as recipients for transfers and were irradiated with 1000 Rag (500 Rads X 2 doses, 3-5 hrs apart) before being injected with 1/5 of one fetal liver equivalent that had been previously frozen for genotyping. Two to five fetal livers were pooled before injection. See Chapter 2 for additional stem cell transfer protocols. Irradiated animals were given antibiotic water and antibiotic-supplemented pellets and thymi were harvested 5-7 weeks later. Animals were housed in specific pathogen-free facility at UCSF according to University and National Institutes of Health (NIH) guidelines.

Antibodies and Flow Cytometry

All thymic manipulations and flow cytometric assessments were performed as described in Chapter 3.

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