University of Alberta

REGULATION OF THYMOCYTE DEVELOPMENT BY CD45

by

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ABSTRACT

The protein tyrosine phosphatase, CD45, is crucial for proper T cell development and activation. CD45 contains a large glycosylated and alternatively spliced external domain that is developmentally regulated, thus, it was hypothesized that the external domain of CD45 could regulate thymocyte development. Engagement of the external domain of CD45 by specific antibody treatment in fetal thymic organ culture resulted in impaired development of thymocytes at specific stages, which correlated with annexin V staining. CD45 deficient mice exhibited increased annexin V staining of adult thymocytes and T and B lymphocytes, as well as exhibited disrupted thymus and spleen morphology. The thymocyte developmental defect was found to be intrinsic to the thymocytes, not a result of a CD45 deficient environment, and was not a result of impaired phagocytic clearance of apoptotic thymocytes. Collectively these studies demonstrate a lymphocyte intrinsic role for CD45 in regulating thymocyte development, and maintaining healthy lymphocyte populations.

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LIST OF ABBREVIATIONS

7AAD	7-amino-actinomycin D
Ab	antibody
Ag	antigen
AIRE	autoimmune regulator of gene expression
AnV	annexin V
Apaf-1	apoptotic protease activating factor-1
APC	antigen presenting cell
Bak	Bcl-2 homologous antagonist-killer
Bax	Bcl-2 associated X-protein
Bcl-2	B-cell lymphoma protein-2
Bcl-xL	B-cell lymphoma protein-xL
BCR	B cell receptor
Bid	Bcl-2 like inhibitor of death
Bim	Bcl-2 interacting mediator of cell death
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CD	cluster of differentiation
CD45-/-	CD45 deficient
CTL	cytotoxic T lymphocyte
DN	double negative
DNA	deoxyribonucleic acid

DP	double positive
DTAF	dichlorotriazinylaminofluorescein
EDTA	ethyenediamine tetraacetic acid
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
FCS	fetal calf serum
FITC	fluoroscein isothiocyanate
FTOC	fetal thymic organ culture
GII	glucosidase II
hr	hour
hrp	horseradish peroxidase
ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KO	knock-out
LAT	linker for activation of T cells
LTβR	lymphotoxin β receptor
mAb	monoclonal antibody
MAPK	mitogen activated protein kinases
MBL	mannose binding lectin
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
min	minute

NFAT	nuclear factors of activated T cells
NF-κB	nuclear factor kappa-B
NK	natural killer cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
PI3K	phosphatidylinositol 3-kinase
PLC	phospholipase C
PS	phosphatidylserine
PTPase	protein tyrosine phosphatase
PVDF	Polyvinylidene fluoride
RAG	recombinase activating gene
$S1P_1$	sphingosine-1-phosphate receptor 1
SA	streptavidin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	src-homology 2 domain
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
SMAC	supramolecular activation complex
SOCS-1	suppressor of cytokine signaling-1
SP	single positive
STAT	signal transducers and activators of transcription
TCR	T cell receptor
Tg	transgenic

Th	T-helper cell
TMRE	tetramethylrhodamine ethyl ester perchlorate
TNFR	Tumor necrosis factor receptor
Wt	wildtype
ZAP-70	zeta-chain associated protein kinase 70kDa
zVAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

CHAPTER 1 – INTRODUCTION

1.1. Basics of the Immune System

The immune system provides defense against a vast array of pathogens. It does so by orchestrating a complex and multifaceted response against the presence of foreign entities, eliminating them and preventing re-infection. The immune system is divided into two main, highly interrelated branches, the innate and the adaptive immune responses. The innate immune response consists of physical barriers, chemical inflammatory mediators, soluble serum proteins, and cellular components such as granulocytes, phagocytes, and natural killer cells. These components can elicit damage directly to pathogens or infected cells, or can act to opsonize and enhance the clearance of foreign organisms or molecules by phagocytes. The innate defenses are immediate and respond to a broad range of pathogens. The adaptive response, on the other hand, requires time to develop into a response specifically tailored to the infecting pathogen. The main components of the adaptive immune response are T and B lymphocytes. Depending on the usage of the T cell receptor genes, T cells can be classified into the αβ or γδ lineage. The role of αβ T cells in adaptive immunity is well defined, whereas, γδ T cells are more innate-like and their functions are not well characterized. Therefore, future reference to T cells will refer to that of the αβ lineage.

T and B lymphocytes express receptors (T cell receptor, TCR; or B cell receptor, BCR) that are specific for a particular peptide or antigen, and hence upon activation after specific Ag recognition, clonal expansion of these cells enables a large scale response specifically targeted against a particular antigen of a pathogen. There are two major types of αβ T cells, T helper cells (Th, $CD4^+$) that release various cytokines upon

activation involved in lymphocyte activation, and cytotoxic T lymphocytes (CTLs, $CD8^+$) which respond to and kill infected cells and tumor cells by degranulating toxic mediators. B lymphocytes produce immunoglobulin, known as antibodies (Abs) specific to invading pathogens which can act to prevent pathogen colonization and enhance their clearance by phagocytic cells. In addition, both T and B lymphocytes have the potential to differentiate into long term 'memory' cells which retain their antigenic specificity, but are much faster responders upon subsequent infection thereby preventing recurrent infections by the same pathogen.

The immune system functions as a whole with each component playing an important and interdependent role in order to effectively eliminate a large range of pathogens. A defect in one component, such as critical innate pathogen recognition receptors (Janeway et al. 2002), or of T or B lymphocytes (de Villartay et al. 2003), results in immunodeficiency and susceptibility to certain pathogens. Alternatively, if not tightly regulated the immune system can cause a massive assault against the host resulting in autoimmune disease. For these reasons tolerance mechanisms are in place to prevent the generation and activation of autoreactive lymphocytes. Lymphocytes that recognize host expressed antigens during development are eliminated, resulting in what is referred to as central tolerance. Mechanisms also exist to prevent activation of, or to remove, potentially autoreactive lymphocytes in the periphery that escaped central tolerance. These peripheral tolerance mechanisms include regulation by regulatory T cells ($CD4^+CD25^+$) and tolerogenic dendritic cells.

Antigen presentation to T cells

Professional antigen presenting cells (APCs) are the bridge between the innate and adaptive immune responses. APCs include dendritic cells, macrophages and B cells. They engulf Ag which if recognized as foreign through the use of broad range pathogen-recognition receptors (Janeway et al. 2002), leads to APC activation. Once activated, APCs then process and present antigen to T lymphocytes along with the expression of co-stimulatory molecules. Ag is presented to T lymphocytes as peptides bound to major histocompatibility complex – class II (MHC-II) molecules in the case of $CD4^+$ T cells, or MHC-I, for $CD8^+$ T cells. T cells recognize foreign peptide in the context of MHC through their T cell receptor (TCR) complex in conjunction with the co-receptors CD4 or CD8. A second signal is generated through the engagement of the T cell co-stimulatory receptor CD28 by B7 molecules expressed upon activated APCs (Germain et al. 1999). These two signals induce T cells to proliferate and differentiate into effector Th cells (if $CD4^+$) or CTLs (if $CD8^+$). Activated Th cells can then interact with B cells (which also express MHC-II) within lymph node and spleen and aid in B cell activation through the production cytokines and expression of co-stimulatory molecules. Activated CTLs are then able to target infected cells in the periphery which express foreign peptide MHC-I.

T cell activation

TCR engagement by peptide-MHC induces a cascade of intracellular signaling events. The TCR complex is comprised of the TCR$\alpha\beta$ chains, which recognize the antigen/MHC complex, and the CD3 $\varepsilon\delta\gamma\zeta$ chains, comprising the signaling components. The CD3 chains contain immunoreceptor tyrosine-based activating motifs (or ITAMs) in

their cytoplasmic tails, which upon TCR engagement become phosphorylated by the src family kinase, lck (Weiss et al. 1994). This phosphorylation recruits the syk family kinase ZAP-70 to bind the phosphorylated ITAMs (Chan et al. 1992) through its SH2 domains. Upon phosphorylation and activation of ZAP-70 by lck, it then phosphorylates adapter proteins such as LAT and SLP-76 (Wardenburg et al. 1996). Subsequent events involve the activation of a number of downstream signaling pathways including phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), and mitogen activated protein kinase (MAPK) pathways. Downstream signaling leads to the activation of specific nuclear transcription factors such as NFκB, and NF-AT which lead to cellular proliferation, upregulation of survival genes, and induction of genes required to elicit effector functions (Germain et al. 1999). See Figure 1-1.

1.2 Thymocyte development

T cell development occurs in the thymus where thymocytes are selected for their ability to bind MHC molecules through their TCR, so that they can be effectively activated once mature in the periphery. The TCR α and β-chains are generated during thymocyte development by gene rearrangement events elicited by the recombination-activating-gene (RAG) proteins (Bassing et al. 2002). Specific usage and joining of different gene segments within the antigen recognition region of the receptor enables a huge diversity of receptor specificities and the ability to detect a wide range of pathogenic antigens. However, this randomly generated diversity also has the potential to generate TCR specificities to molecules expressed by the organism itself (referred to as 'self'). Thus, the thymus must educate developing thymocytes and select for only those

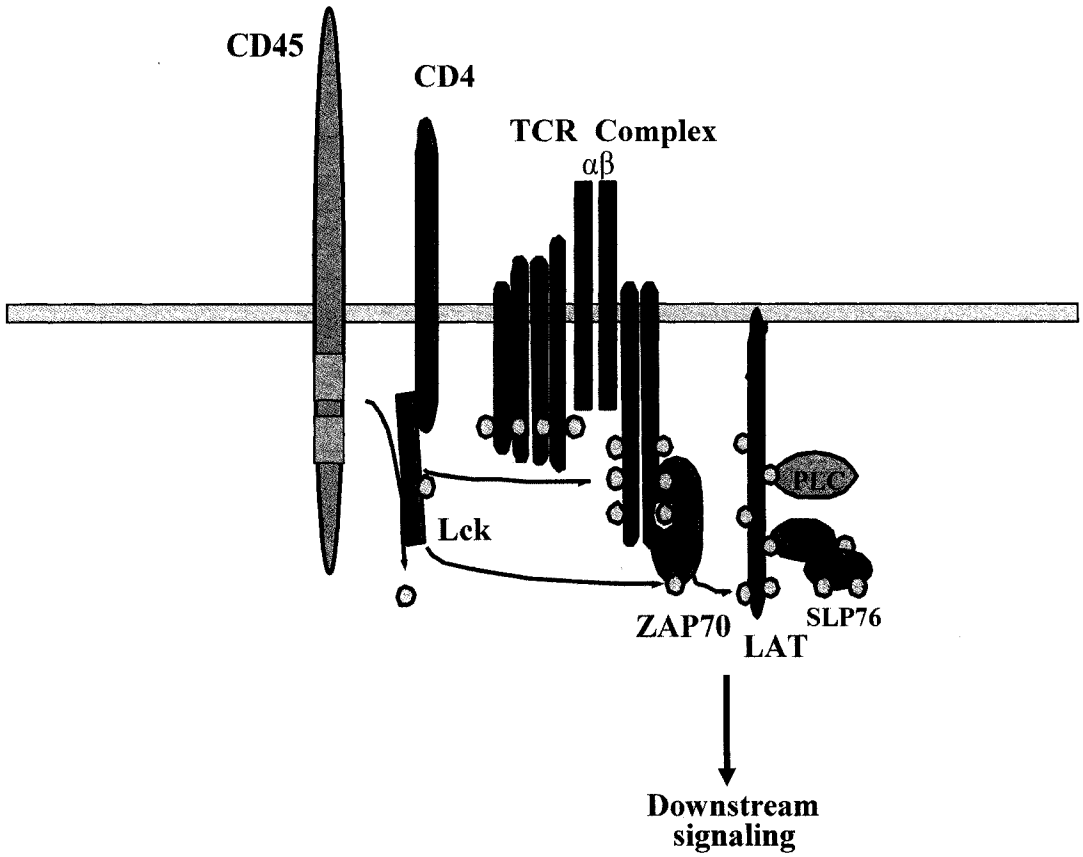

Figure 1-1. T cell signaling: TCR proximal events.

Upon TCR engagement, the Src kinase lck becomes activated and phosphorylates ITAMs within the TCR associated CD3 chains. This recruits ZAP-70, which binds to the phosphorylated ITAMs of the ζ-chains, becomes activated and phosphorylates the adapter molecules LAT and SLP-76. The multiple phosphorylation sites on the adapter proteins recruits a number of signaling molecules, including PLC. This leads to downstream signaling pathways resulting in the activation of specific transcription factors involved in T cell effector functions, proliferation, and survival. Phosphorylation is denoted by yellow circles. Lck is regulated by the protein phosphatase, CD45. CD45 dephosphorylates lck at a negative regulatory site, thereby allowing lck to become activated upon TCR ligation.

thymocytes expressing TCRs which are able to bind MHC molecules (MHC restriction), but are not specific for host derived self peptides (central tolerance).

T cell development is characterized by the sequential expression of the T cell receptor β and α –chains, as well as the expression of the co-receptors CD4 and CD8 (Fig. 1-2). Thymocyte precursors originating from the bone marrow (or fetal liver) enter the thymus, commit to the T cell lineage, and migrate to the outer region of the thymus known as the cortex. At this stage thymocytes lack the expression of both CD4 and CD8, and this is known as the double negative stage (DN). As DN thymocytes mature they undergo a number of selection and differentiation events, mediated through interactions with the thymic stroma of the cortex region (Takahama 2006). There are four major stages of DN development characterized by differential expression of CD44 and CD25; DN1 ($CD44^+$, $CD25^-$), DN2 ($CD44^+$, $CD25^+$), DN3 ($CD44^-$, $CD25^+$), and DN4 ($CD44^-$, $CD25^-$). The TCRβ gene is rearranged and expressed at the DN3 stage in conjunction with a surrogate pre-Tα chain. Productive β-chain expression allows the thymocyte to pass the 'β-selection' checkpoint and mature to the DN4 stage (Sebzda et al. 1999). Subsequent expression occurs of both CD4 and CD8, which is referred to as the double positive (DP) stage. TCRα chain rearrangement then occurs resulting in the expression of a clonotypic αβTCR. The DP thymocyte is then scrutinized by both positive and negative selection processes for proper TCR expression and non-self reactivity, and then maturation into $CD4^+$ or $CD8^+$ single positive (SP) T cells occurs in the inner medullary regions of the thymus (Germain 2002; Hollander et al. 2006)

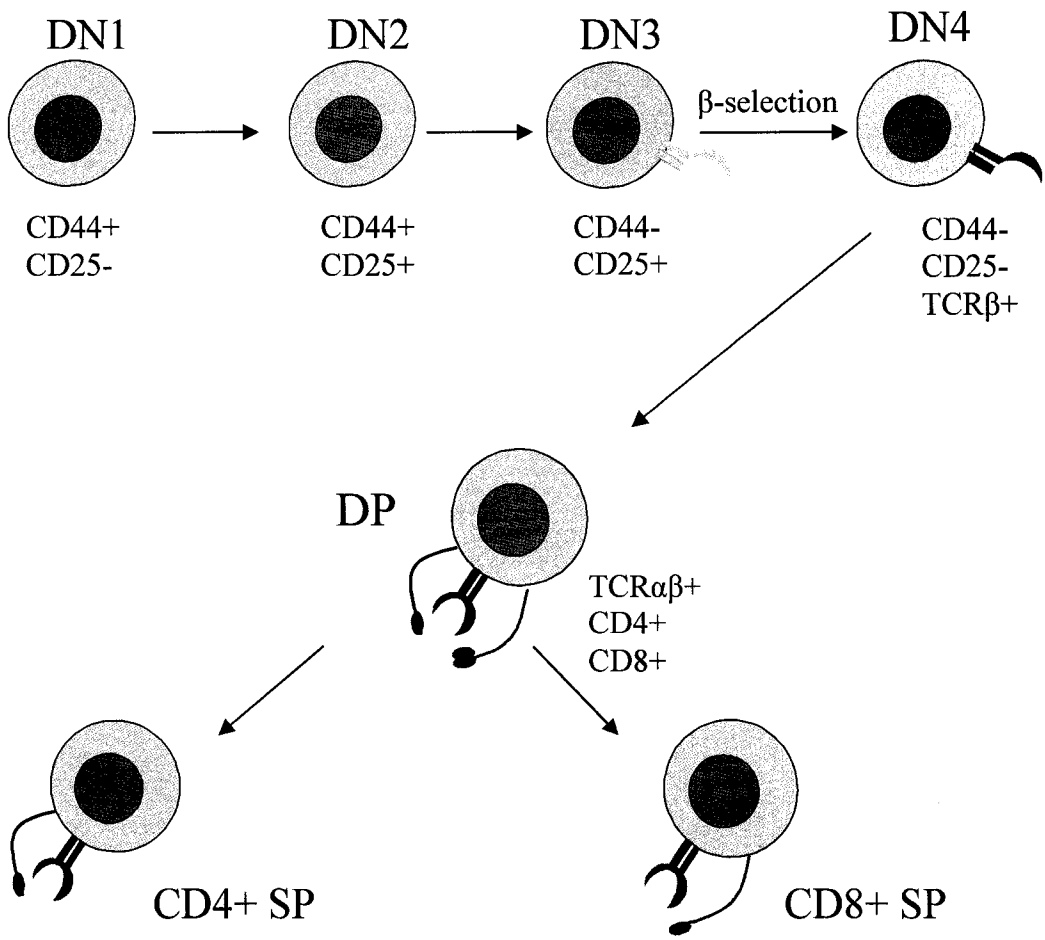

Figure 1-2. Thymocyte development

Thymocyte development is characterized by the sequential expression of the TCR-β and -α chains, and the co-receptors CD4 and CD8. Thymocyte precursors lack the expression of both co-receptors and a TCR and are termed double negative (DN). The DN stage is characterized by four stages, defined by the expression of CD44 and CD25. Rearrangement of the TCR-β genes occurs at the DN3 stage followed by β-selection which promotes transition to the DN4 stage. The co-receptors CD4 and CD8 are then both expressed and TCR-α chain is rearranged as the thymocyte progresses to the DP stage. DP thymocytes then undergo selection events and mature into CD4+ single positive (SP) or CD8+ SP thymocytes.

Thymocyte Selection

Thymocytes undergo extensive positive and negative selection processes which select for proper TCR fit to MHC, but non-self reactivity to host derived peptides, respectively. This distinction is widely accepted to occur based on TCR avidity for peptide-MHC within the thymus (Ashton-Rickardt et al. 1994; Sebzda et al. 1994; Sebzda et al. 1999; Zamoyska et al. 2004). Thymocytes which exhibit low avidity TCR interaction with MHC molecules are positively selected to survive. Clonal deletion occurs, on the other hand, of thymocytes expressing TCRs that engage MHC with high avidity interactions, through the process of negative selection. Thymocytes expressing TCRs that fail to engage self-MHC ligands on thymic stromal cells also die, through a process referred to as "death by neglect" (Zamoyska et al. 2004). Effectively this selects for thymocytes expressing TCRs capable of binding host expressed MHC molecules (known as MHC restriction) but without strong reactivity to self peptides, thereby reducing the potential for autoimmune reactivity. This is a very complex series of events and results in the selection, and release into the periphery of only 1-3% of thymocytes generated (Takahama 2006).

The localization of these different selection events remains controversial. Immature DN thymocytes mature to DP thymocytes in the outer cortex region of the thymus where they interact with selecting MHC expressed on cortical epithelial cells (Takahama 2006). Migration of selected DP and SP cells towards the medulla then occurs, where interaction with medullary epithelial cells is required for effective establishment of central tolerance to peripheral antigens (Kurobe et al. 2006). Medullary epithelial cells can mediate clonal deletion of self-peptide reactive T cells through the

expression of tissue-specific antigens (Derbinski et al. 2001). Gene expression of tissue-specific antigens occurs through the specialized expression of the transcription factor autoimmune regulator of gene expression (AIRE) and likely other specialized transcription factors. Expression of peripheral antigens mediated by AIRE is critical to the establishment of self tolerance as its deficiency results in multi-organ autoimmune disease (Zuklys et al. 2000; Liston et al. 2003; Derbinski et al. 2005).

Determination of positive versus negative selection is likely a reflection of the overall signal induced by TCR engagement. Differential avidity would influence the strength of proximal signals which would then determine distinct downstream signaling outcomes (Mariathasan et al. 1999; Love et al. 2003). In support of this, proximal signaling proteins have been found to be important for both positive and negative selection events. Experiments using a dominant negative form of lck demonstrated its requirement for both positive and negative selection (Hashimoto et al. 1996). Other proximal signaling proteins such as ZAP-70 (Negishi et al. 1995; Sakaguchi et al. 2003), and LAT (Sommers et al. 2001) have also been shown to play a role in both selection processes. Notable differences in signaling between positive and negative selection emerge within the distal signaling pathways, one main difference being the duration and strength of Erk/MAPK versus the JNK/p38 pathway depending upon the strength of selecting ligands (Alberola-Ila et al. 1996; Starr et al. 2003; Zamoyska et al. 2004). The signaling involved in thymocyte selection is still, however, quite controversial and appears much more complex than simple TCR avidity and may involve different co-receptor interactions and thymocyte localization (Mariathasan et al. 1999; Sebzda et al.

1999; Baldwin et al. 2005). The ultimate outcome of the differential signaling is either induction of survival, by positive selection, or death, by neglect or by negative selection.

Thymocyte survival and death.

Developmental fate of thymocytes is regulated by the balance of survival versus death inducing signals mediated by either positive or negative selection, respectively. Death of developing thymocytes due to neglect or negative selection is mediated through apoptotic processes. Apoptosis is an active and controlled form of cell death characterized by morphological hallmarks including cell shrinkage and blebbing of the plasma membrane, externalization of phosphatidylserine residues to outer plasma membrane, nuclear and chromatin condensation, and DNA degradation (Marsden et al. 2003). Apoptosis can be mediated by two distinct pathways, the extrinsic pathway which involves engagement of cell death receptors, such as the FasR and TNFR, and is primarily initiated by caspase activation, or via intrinsic pathways which are regulated at the level of the mitochondria by members of the Bcl-2 family (Marsden et al. 2003). Caspases are members of the aspartate cysteine protease family and are well characterized effector proteins that mediate apoptosis of both pathways through proteolytic cleavage of specific substrates (Siegel 2006), although caspase-independent apoptotic cell death mechanisms have been described (Borner et al. 1999; Kitanaka et al. 1999; Bidere et al. 2001; Jaattela et al. 2003).

The Bcl-2 family consists of both anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and pro-apoptotic members, including Bid, Bax, Bak, Bad, and Bim (Marsden et al. 2003). Members of the pro-apoptotic family promote the formation of pores within the

mitochondrial membrane by multimerization of Bax and Bak complexes, causing a loss of mitochondrial membrane potential, and the release of cytochrome-c into the cytoplasm. Cytochrome-c can then complex with Apaf-1 and caspase-9, forming the 'apoptosome' that can activate the final effector caspase-3 and mediate downstream effector functions of apoptosis (Marsden et al. 2003). Anti-apoptotic Bcl-2 family members function to inhibit and block the action of the pro-apoptotic members in order to maintain mitochondrial membrane integrity.

The mechanisms of apoptosis during thymocyte development are actually not well defined (Starr et al. 2003). There is very little evidence to support a role for death receptors in mediating thymocyte death, whereas involvement of members of the intrinsic mitochondrial pathway has been demonstrated (Palmer 2003). Bcl-2 is known to be important for mediating survival of DN populations, and is up regulated along with Bcl-xL upon positive selection of DP thymocytes, to promote their survival (Linette et al. 1994; Ma et al. 1995; Groves et al. 1997). Over expression of Bcl-2 can prevent death caused by neglect (Strasser et al. 1994) implicating death by neglect as an apoptotic process. Over expression of Bcl-2 has also been shown to reduce negative selection in TCR transgenic (Tg) models (Strasser et al. 1994).

Death induction through negative selection is also not well defined, but a role for the pro-apoptotic member Bim has been described. Mice deficient in Bim show reduced deletion of thymocytes by CD3 engagement, and reduced clonal deletion of thymocytes in TCR Tg mice (Bouillet et al. 2002). The downstream effects of negative selection and Bim induction are unclear since mice deficient for Apaf-1, a member of the downstream apoptosome, do not demonstrate impaired negative selection (Hara et al. 2002). A role

for caspases in negative selection is also very unclear. Activation of effector caspase-3 has been demonstrated in thymocytes undergoing negative selection. However, thymocytes deficient in certain caspases, including caspase-3, exhibit normal thymocyte development (Palmer 2003), although this may be due to redundancies in caspase expression. Transgenic expression of caspase inhibitors has revealed inconsistent results; one study demonstrated reduced negative selection (Minter et al. 2003), while another found no effect on thymocyte development and negative selection (Doerfler et al. 2000). Thus, the apoptotic processes occurring during thymocyte development remain to be more clearly defined.

Other factors such as IL-7 contribute to thymocyte survival during development. IL-7 signals through the IL-7Rα chain and the common gamma chain (γ_c), causing the activation of the associated Janus kinases, JAK-1 and -3, and subsequent activation of the transcription factors, STAT1 and -5 (Jiang et al. 2005). IL7R-/- mice exhibit dramatically reduced thymocyte numbers but normal CD4/8 ratios (von Freeden-Jeffry et al. 1995). Mice injected with IL-7 or made Tg for IL-7 exhibit increased thymocyte numbers and proliferation (Khaled et al. 2002) demonstrating the importance of IL-7 for thymocyte survival and proliferation.

Thymocyte responsiveness to IL-7 is dynamically regulated during development. IL-7 is important for survival at the DN stages of thymocyte development (Kim et al. 1998) and for thymocyte proliferation after β-selection (Trigueros et al. 2003). IL-7R expression is then down regulated (Hare et al. 2000), and suppressor of cytokine signaling-1 (SOCS-1) (Yu et al. 2006) is upregulated in DP thymocytes resulting in unresponsiveness to IL-7 signaling thereby allowing sensitivity to death by neglect. DP

thymocytes then only become IL-7 responsive after receiving positive selection signals (Hare et al. 2000; Van De Wiele et al. 2004). The effect of IL-7 on thymocyte survival is mediated by upregulating anti-apoptotic members of the Bcl-2 family, and inhibiting pro-apoptotic proteins such as Bax, Bim, and Bad (Jiang et al. 2005). IL-7 also promotes thymocyte proliferation by preventing cell cycle arrest (Li et al. 2006). These studies demonstrate the importance of IL-7 during thymocyte development for survival and proliferation.

The large percentage of dying thymocytes during development is very rapidly and effectively cleared by thymic resident macrophages (Surh et al. 1994). Macrophages express a number of receptors involved in recognition and phagocytosis of apoptotic cells (Savill et al. 2002; Lauber et al. 2004). The externalization of phosphatidylserine on the outer leaflet of the plasma membrane of apoptotic cells is a well characterized marker for stimulating phagocytosis (Fadok et al. 1992) through recognition by a macrophage expressed phosphatidylserine receptor (Fadok et al. 2000; Hoffmann et al. 2001) and potentially other receptors (Williamson et al. 2004). Many other receptors have been identified to be important in promoting phagocytosis of apoptotic cells, such as scavenger receptors (Platt et al. 1996), CD14 (Taylor et al. 2005), and cell surface CD91/calreticulin, which has been shown to promote phagocytosis by interaction with the collectins C1q, mannose binding lectin, and surfactant proteins A and D (Ogden et al. 2001; Vandivier et al. 2002). The importance of clearance of apoptotic cells *in vivo* was demonstrated by mice deficient for complement component C1q which exhibit autoimmune phenotypes consistent with systemic lupus erythematosus (Taylor et al. 2000). The significance of these specific receptors within the thymus *in vivo*, however,

has not been well characterized. The ligands expressed on apoptotic cells for these receptors and mediators also have not yet been identified

Thus, thymocyte development is characterized by dynamic and complex interactions with the thymic stroma and regulated changes in the expression of a number of molecules to mediate proper thymocyte progression, differentiation and survival.

1.3 CD45

CD45, also known as leukocyte common antigen, T200, or Ly5, is a protein tyrosine phosphatase expressed on all nucleated cells of hematopoietic origin (Trowbridge et al. 1994). It is a large, highly abundant transmembrane protein ranging in size from 180-220 kDa. It contains two tandem phosphatase domains in its cytoplasmic tail, D1 and D2, of which only the membrane proximal domain (D1) is active (Desai et al. 1994; Felberg et al. 1998). The external domain of CD45 contains alternatively spliced exons, A, B, and C, and potentially others (Li et al. 2004), resulting in multiple isoforms being expressed. The alternative region is followed by a conserved region consisting of a cysteine rich region, and three fibronectin type III-like domains (Okumura et al. 1996; Symons et al. 1999). In addition, the external domain is highly glycosylated with N-linked carbohydrate throughout and O-linked carbohydrate mainly on the variable regions. The carbohydrate consists predominantly of complex poly-N-acetyllactosamine containing chains, and α-2,6-linked sialic acid (Pulido et al. 1990; Sato et al. 1993; Hermiston et al. 2003). See Figure 1-3.

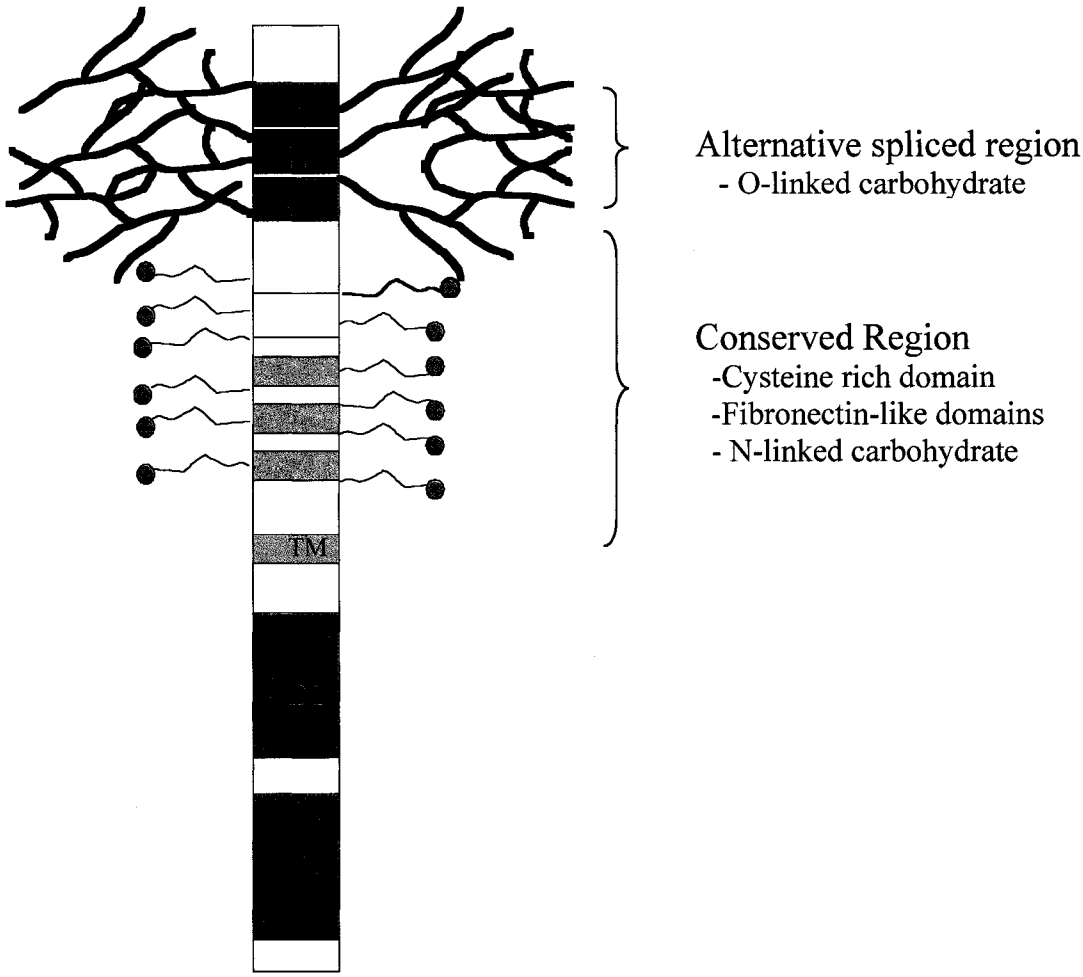

Figure 1-3: Schematic diagram of CD45

The extracellular domain consists of an alternatively spliced region of exons A, B, and C, followed by a more conserved regions consisting of a cysteine rich region, and three fibronectin-like domains. Complex branched O-linked carbohydrate is depicted on the spliced region in dark thick lines, and N-linked carbohydrate is dispersed through the extracellular domain and is depicted by thin lines, terminated in sialic acid depicted as round balls. The transmembrane (TM) domain is followed by the intracellular phosphatase domains, D1 and D2.

CD45 function

CD45 functions as key regulatory molecule in both T and B cell activation. Its role as a positive regulator of lymphocyte signaling was initially demonstrated in CD45 deficient T cell lines and clones, which exhibited impaired intracellular signaling and activation in response to antigen receptor stimulation (reviewed by Trowbridge et al. 1994). These studies were confirmed by the generation of number of CD45 gene knock-out mice (Kishihara et al. 1993; Byth et al. 1996; Mee et al. 1999), which exhibited immuno-compromised phenotypes demonstrating impaired lymphocyte activation, defects in T cell development, and B cell maturation (Byth et al. 1996; Stone et al. 1997; Mee et al. 1999), and recently identified impaired NK cell function (Huntington et al. 2005; Hesslein et al. 2006).

In T cells, CD45 phosphatase activity is responsible for dephosphorylating a negative regulatory site on the src family kinases, lck and fyn, thereby enabling their activation following TCR stimulation (Ostergaard et al. 1989; Mustelin et al. 1990; Mustelin et al. 1992). Lck is regulated by tyrosine phosphorylation at a positive (Y-394) and negative regulatory site (Y-505). Phosphorylation at Y-505 by Csk tyrosine kinase negatively regulates its kinase activity by converting it to an inactive form (Germain et al. 1999). Dephosphorylation of this negative regulatory site, by CD45 causes a conformation change allowing phosphorylation of the positive regulatory site, Y-394, after TCR stimulation by autophosphorylation, thereby converting lck to its active form (Johnson et al. 2000). In essence, CD45 is responsible for maintaining and regulating a 'primed' pool of lck which is ready to respond following TCR stimulation. However, the mechanism of CD45 function is much more complex, as it also demonstrates negative

regulatory functions on TCR signaling and lck activity (Mustelin et al. 1995; Alexander 2000). The activating Y-394 site on lck has also be shown to be dephosphoyrlated by CD45 and CD45 deficient cells have been found to have hyper-phosphorylated lck and increased lck activity (D'Oro et al. 1999), implicating CD45 in negative regulation as well as positive regulation of lck signaling.

CD45 has also been implicated as a negative regulator of cytokine signaling by dephosphorylating Janus kinases (JAKs). CD45 deficient lymphocytes exhibited increased phosphorylation of JAKs and the STAT transcription factors in response to cytokine signaling (Irie-Sasaki et al. 2001). Consistent with this, B lymphocytes isolated from CD45-/- mice were found to exhibit increased responsiveness to IL-7 and increased phospho-STAT5 levels (Fleming et al. 2004).

CD45 and T cell development

Generation of CD45 deficient (CD45-/-) mice revealed a critical role for CD45 in T cell development. Thymocyte development was found to be blocked both at the DN to DP stage and at the DP to SP stages, resulting in increased percentages of DN thymocytes, decreased percentages of DP and SP thymocytes, and very few mature T cells in the periphery (Kishihara et al. 1993; Byth et al. 1996; Mee et al. 1999). CD45 deficient mice exhibited impaired positive and negative selection. CD45-/- mice exhibited reduced positive selection in TCR-Tg mice, which correlated with decreased expression of the selection markers CD69, CD5, and TCR (Mee et al. 1999). Negative selection in CD45 deficient mice was found to be reduced in response to endogenous and exogenous superantigen (Conroy et al. 1996; Mee et al. 1999) and in response to death

induction by CD3 Ab in fetal thymic organ culture (Byth et al. 1996). Consistent with a role for CD45 in mediating proper negative selection, T cells that do mature and exit into the periphery of CD45-/- mice were demonstrated to express self-reactive TCRs (Trop et al. 2000).

The thymocyte developmental defect in CD45-/- mice is primarily thought to be a consequence of impaired lck activation. In support of this, CD3 stimulation of RAG-/- thymocytes which can drive thymocyte development to DP stage, could not in CD45-/- mice (Pingel et al. 1999), suggesting CD3 signaling and downstream lck activity is impaired by CD45 deficiency. Transgenic mice expressing a constitutively active form of lck (Y505F) on a CD45-/- background restored thymocyte development when a transgenic TCR was also expressed (Seavitt et al. 1999). However, others have found that active lck (Y505F) could only rescue development to the DP stage, but not completely to the SP stage (Pingel et al. 1999). Interestingly, lck deficient mice exhibit a major block early on in thymocyte development at the DN to DP progression (Molina et al. 1992), which is in contrast to the dual block exhibited by CD45-/- mice suggesting that CD45 may play an alternate role during development.

Regulation of CD45

The role of CD45 as either a positive or negative regulator of T cell signaling may be determined by its localization spatially and temporally in relation to signaling machinery. CD45 was initially thought to be excluded from lipid rafts, where the majority of signaling molecules reside, hence preventing the dephosphorylation of

activated molecules (Janes 1999). In support of this, expression of a chimeric CD45 molecule containing the cytoplasmic domain of CD45 and the lipid raft targeting amino terminal of lck resulted in impaired TCR signaling (He et al. 2002). However, it has now been shown that small but significant percentages of CD45 molecules are present within rafts and translocate out upon TCR signaling (Edmonds et al. 2002). CD45 within rafts has been shown to antagonize TCR mediated signals, whereas CD45 excluded from rafts functions more as a positive regulator (Zhang et al. 2005). CD45 localization is also dynamic in respect to the regions of contact between a T cell and APC, known as the immune synapse. Confocal fluorescent microscopy studies of T cells on planner bilayer systems found that CD45 was initially excluded from the central regions of TCR engagement, and then is recruited back to the area of sustained TCR engagement (Johnson et al. 2000). However, studies using APC-T cell conjugates have shown that very early events in T cell-APC conjugation involve CD45 localization to the regions of engaged TCR and lck (Freiberg et al. 2002). These studies demonstrate that the localization of CD45 is dynamically regulated during T cell activation and suggest differential roles for CD45 function in relation to its localization.

Dimerization of CD45 has also been suggested to regulate its function (Xu et al. 2002). Induced dimerization of chimeric EGFR-CD45, consisting of the CD45 cytoplasmic domain and the extracellular domain of the epidermal growth factor receptor (EGFR), results in abrogated TCR signaling (Desai et al. 1993). The inhibition induced by dimerization is thought to occur via wedge structure in the cytoplasmic tail of CD45 that may block the catalytic domain upon dimerization. Indeed mutation of the wedge region resulted in a loss of dimerization induced inhibition of signaling, and resulted in a

lymphoproliferative syndrome in CD45 wedge mutant genetic knock-in mice (Majeti et al. 2000), as a result of hyper-responsive B cells (Hermiston et al. 2005). However, recent crystallographic studies on the cytoplasmic domain of CD45 refute the possibility of the wedge blocking the catalytic site (Nam et al. 2005), therefore, the exact mechanism behind the importance of wedge region is unknown.

The External Domain

Isoform and glycoform expression of the external domain of CD45 is regulated by cell type, developmental state, and activation state of the cell. Alternative splicing of exons 4, 5, and 6 lead to the production of different possible exon containing isoforms that are identified by restricted Ab reactivity, termed CD45-RA, -RB, -RC, or –RO, which lacks all three alternatively spliced regions. Protein expression of only four major isoforms was found to occur in T cells derived from the C57Bl/6 mouse, RO, RB, RBC, and RABC (McNeill et al. 2004). Naïve T cells in the periphery express predominantly RB containing isoforms, whereas a switch to the lower molecular weight RO isoform occurs upon T cell activation (Birkeland et al. 1989) and differentiation into memory T cells (Hermiston et al. 2003). Differential expression of CD45 isoforms is also differentiation dependent, as mature $CD8^+$ cells were found to express predominantly RBC, whereas, mature $CD4^+$ cells express predominantly RO (McNeill et al. 2004). Dynamic expression of CD45 isoforms also occurs during thymocyte development. Higher molecular weight isoforms of CD45 are predominantly expressed on DN thymocytes, which then switch to predominately express CD45RO at the DP stage, and then mainly RB is expressed at the SP stage (Hermiston et al. 2003).

Delineating the absolute function of the external domain of CD45 has been difficult since defects in T cell development and activation as a result of CD45 deficiency, have been demonstrated to be reconstituted by transgenic expression of either RABC, RB, or RO isoforms (Kozieradzki et al. 1997; Tchilian et al. 2004). Absolute CD45 expression levels rather than isoform expression appear to be the determinant of CD45 function (Wallace et al. 1997; Tchilian et al. 2004; Dawes et al. 2006), which is important to note considering no transgenic model to date has obtained wildtype levels of CD45 expression. There is some evidence to suggest, however, that that the external domain can regulate CD45 phosphatase activity. Chimeric CD45 molecules consisting of the CD45 cytoplasmic domain fused to the large glycosylated ectodomain of CD43 could reconstitute signaling whereas chimeras with small ectodomains could not (Irles et al. 2003). Studies have demonstrated isoform dependent differences for specific functional outcomes of T cell activation, however, the results were contradictory and difficult to directly compare due to different cell types, stimulation method, and readout of activation used (Chui et al. 1994; Novak et al. 1994; Hall et al. 1999; Czyzyk et al. 2000; Xu et al. 2002; Matto et al. 2005; Stutz et al. 2005).

A role for the large glycosylated external domain of CD45 in regulating its localization with respect to TCR signaling has also been suggested. CD45RO was found to migrate into lipid rafts more rapidly than the larger CD45RB and RA containing isoforms in myeloma cells (Li et al. 2005) Studies have also shown differential association of CD45 isoforms with the TCR/CD4 complex (Leitenberg et al. 1999), suggesting a role for different isoforms in regulating TCR signaling through differential molecular associations. A role for the external domain of CD45 has also been suggested

to play a role in regulating CD45 dimerization. Upon chemical crosslinking, CD45RO isoforms were found to dimerize more efficiently than larger CD45RA containing isoforms which is thought to be due to increased steric hindrance of bulky carbohydrate and negative sialic acid charges (Xu et al. 2002). These data suggest the an important role of the large external domain of CD45 in regulating its function but the exact role of specific isoforms and the mechanism of regulation remains, for the most part, elusive.

CD45 lectin interactions

Although a specific ligand for CD45 has not been identified, a number of proteins have been found to interact with the extracellular domain of CD45 via lectin interactions. The B cell protein, CD22, is a member of the siglec family and has been found to bind to CD45 in *trans* via sialic acid residues (Stamenkovic et al. 1991; Sgroi et al. 1995; Han et al. 2005). CD22 engagement of CD45 was shown to negatively regulate T cell activation *in vitro* (Aruffo et al. 1992), but the effects have yet to be demonstrated to have significance *in vivo.* Members of the galectin family which bind to N-acetylactosamine residues, namely galectin-1 and galectin-3, have also been shown to bind to carbohydrate on CD45. Galectins exhibit immunoregulatory functions and are expressed in a wide range of lymphoid tissues including the thymus (Perillo et al. 1997; Elola et al. 2005). Galectin-1 and -3 interact with a number of cell surface proteins including CD45, CD43, CD7, CD71 and CD2 and function to induce T cells apoptosis (Perillo et al. 1995; Perillo et al. 1997; Stillman et al. 2006). Galectin-1 induced apoptosis does not require CD45 expression, but CD45 and its glycosylation status can regulate death induction (Nguyen et al. 2001). Expression of Core2 O-glycans promotes galectin-1 induced apoptosis

(Nguyen et al. 2001), whereas, expression of α-2,6-sialic acid residues renders cells resistant (Amano et al. 2003). Another secreted galectin-like glycoprotein protein, placental protein 14, has also been shown to bind to CD45 and regulate TCR signaling in a sialic acid dependent manner (Rachmilewitz et al. 2003; Ish-Shalom et al. 2006).

Mannose binding lectin (MBL), a component of the complement cascade, has been shown bind to N-linked glycans of CD45 on thymocytes (Uemura et al. 1996). Previous studies by others in our lab, have demonstrated developmental regulated association of CD45 with MBL, and with the ER protein glucosidase II (GII) (Baldwin et al. 2000; Baldwin et al. 2001). GII associated CD45 was found to be secreted to the cell surface rapidly via a golgi-independent mechanism, resulting in the display of immature mannose rich carbohydrate on the cell surface (Baldwin et al. 2002). The functional consequences of these interactions are unknown, and the physiological significance of CD45-lectin interactions is still not well defined. Therefore, the function of the external domain of CD45 remains to be determined.

1.4 Rationale and Project Objectives:

Dynamic isoform and glycoform usage of the external domain of CD45 is developmentally regulated during thymocyte development. Therefore I hypothesized that the external domain of CD45 would play a fundamental role in regulating thymocyte development. The aims of this study were to 1) determine if the external domain of CD45 contributed to the regulation of thymocyte development by CD45 and what functional consequences occur upon CD45 engagement during development; and 2) to

further examine the effect of CD45 deficiency on primary and secondary lymphoid compartments and the its effect on the clearance of apoptotic cells by macrophages.

Hypotheses:

- Manipulation of the external domain of CD45 would disrupt thymocyte development.
- CD45 deficiency results in decrease lymphocyte survival and defective clearance by phagocytes.

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CHAPTER 2:

CD45 DEFICIENCY AND ANTIBODY ENGAGEMENT OF THE EXTERNAL DOMAIN REGULATE THYMOCYTE SURVIVAL DURING DEVELOPMENT IN FETAL THYMIC ORGAN CULTURE

2.1 Introduction

CD45 is an abundant cell surface protein tyrosine phosphatase expressed on all nucleated cells of hematopoietic origin. The cytoplasmic phosphatase domain of CD45 is responsible for dephosphorylating the src family protein kinases Lck and Fyn at a negative regulatory site allowing for their activation and subsequent downstream signaling events required for T cell activation upon TCR stimulation (Hermiston et al. 2003). The large, and heavily glycosylated external domain of CD45 contains three alternatively spliced exons which lead to the production of different possible exon containing isoforms termed RO, RA, RB, and RC (due to restricted Ab reactivity). In addition, the external domain contains numerous potential N- and O- linked glycosylation sites, thereby enabling the possibility of diverse structure and function. However, the function of this complex external domain remains highly controversial and relatively unknown.

CD45 is known to play a vital role in thymocyte development as exemplified in mice deficient for CD45, which display major defects in T cell development (Kishihara et al. 1993; Byth et al. 1996; Cyster et al. 1996). CD45 knock-out (CD45-/-) mice exhibit a block at the double negative (DN) to double (DP) stage as well as a major block at the DP to single positive (SP) transition (Byth et al. 1996). They have been demonstrated to

exhibit defects in both positive (Byth et al. 1996; Mee et al. 1999) and negative (Ogilvy et al. 2003) selection leading to the production of a very limited and self-reactive T cell population (Trop et al. 2000). Although this requirement is generally attributed to the role of the phosphatase domain, the complex regulation of the external domain during development suggests that this domain may also be important for regulating aspects of development.

CD45 isoform usage is highly regulated during T cell development and activation (Hermiston et al. 2003). Higher molecular weight isoforms of CD45 are predominantly expressed on immature thymocytes at the DN stage, as well as on naïve T cells in the periphery, whereas DP and SP thymocytes and activated/memory mature T cells express the lower molecular weight R0 isoform (Hermiston et al. 2003). Glycosylation of CD45 is also developmentally regulated as determined by lectin binding, including differential binding of mannose binding lectin (Uemura et al. 1996), glucosidase II (Baldwin et al. 2001), and galectin-1 (Baum et al. 1995; Walzel et al. 1999) to thymocytes at specific stages of development. Consistent with a functional role for the external domain during thymocyte development, it has been demonstrated by Benveniste et al. that engagement of the external domain of CD45 with a mAb by injection into adult mice or treatment of fetal thymic organ culture can disrupt thymocyte differentiation and the generation of mature SP thymocytes, but this block was not examined further (Benveniste et al. 1994).

Taken together, these data support a role for the external domain of CD45 in T cell development; however, its exact function remains to be identified. In this study modulation of the external domain of CD45 by mAb treatment of thymocytes developing in fetal thymic organ culture (FTOC) was performed to determine a role for the external

domain during thymocyte development. Fetal thymic organ culture is a very useful ex-vivo model of thymocyte development. Development occurs in isolation, therefore, thymocyte developmental progression can be monitored over time, and the cultures can be easily manipulated. Thymocyte development in FTOC occurs similar to fetal thymocyte development *in vivo*, exhibiting normal gene rearrangement, positive and negative selection, and comparable CD4 and CD8 ratios and markers of development (Jenkinson et al. 1990; Jenkinson et al. 1994). Using the FTOC system in this study, I examined the involvement of the external domain of CD45 during T cell development. The data suggest a role for, or regulation by, the extracellular domain of CD45 in mediating the survival of thymocytes at specific stages of development.

2.2. Materials and Methods

Mice

C57BL/6 (wildtype) and B6.129ptprc$^{tm1\text{-}holmes}$ (CD45Δexon9, CD45-/-) (Byth et al. 1996) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and housed in conventional and viral-antigen-free mouse facilities respectively (Heath Sciences Lab Animal Services, University of Alberta). The lack of CD45 protein expression in the CD45-/- animals was confirmed by FACS analysis. All animal work was performed in accordance with the policies set out by the Canadian Council on Animal Care and approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee.

Antibodies and reagents

Antibodies for FTOC treatment, the pan-CD45 isoform specific antibodies I3/2.3 (rat IgG2b) (Trowbridge 1978) and M1/9 (rat IgG2a) (Coffman 1982), the anti-CD45RA, 14.8 (rat IgG2a) (Johnson et al. 1989), the anti-CD45RB antibodies, MB23G2 and MB4B4 (both rat IgG2a) (Birkeland et al. 1988), anti-Ras (Y13-238), used as rat IgG2a control, and the anti-phosphotyrosine (PY72) used for blotting, were purified from hybridomas as described (Ostergaard et al. 1998). The hybridoma producing SFR3-DR5 (anti-DR5) was used as a source of rat IgG2b isotype control and was obtained from American Type Culture Collection (ATCC, Manassas, VA). Directly coupled antibodies CD3-PE (145-2C11), CD4-PECy5 (RM4-5), CD8β-FITC (53-5-8), CD5-allophycocyanin (APC) (53-7.3), CD25-FITC (7D4), CD44-PECy5 (IM7), CD45-APC (30-F11), CD45RA-PE (14.8), CD45RB-PE (16A), CD45RC-PE (DNL-1.9), CD69-PE (H1.2F3), TCRβ-PE (H57-597), goat anti-rat Ig-PE, annexin V-FITC/PE, 7AAD, APC-BrdU Flow Kit, and anti-phosphoStat5 (PY694) were purchased from BD Pharmingen (BD Biosciences, San Diego, CA). Anti-Lck (clone 28) purchased from BD Transduction Laboratories (BD Biosciences). Fab fragments were generated using ImmunoPure Fab Preparation kit (Pierce, Rockford, IL). Rabbit anti-rat Ig used for crosslinking was obtained from the Jackson ImmunoResearch Laboratories (West Grove, PA). Etoposide was purchased from Sigma (St Louis, MI). zVAD-fmk was purchased from Kamiya Biomedical (Seattle WA).

Fetal Thymic Organ Culture

Fetal thymi were obtained from gestational day 14 (d14) fetal mice extracted from timed, pregnant Wt B6 or CD45-/- mice. Thymic lobes were cultured on Millipore 13 mm round 0.45 μm HA membrane filters (Fischer Scientific, Ottawa, ON) supported on square pieces of 7 mm thick Surgifoam gelatin sponges (Ethicon, Somerville, NJ) in 6 well dishes containing complete RPMI (Invitrogen, Carlsbad, CA) containing 12% FCS (HyClone, Logan, UT), 100 U/ml penicillin and streptomycin (Invitrogen, Carlsbad, CA), and 50 μM β2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Cultures were incubated with CD45 specific antibodies or isotype controls at 40 μg/ml unless otherwise stated as developmental effects were found to be maximal at this concentration (Appendix A1). FTOCs were cultured at 37°C and 7% CO_2 in a humidified incubator for 6 days. Thymi were then harvested by scraping the lobes over a 40 μm nylon cell strainer (BD Biosciences, San Jose, CA) with a 3 cc syringe plunger and then stained for developmental markers using directly coupled antibodies and analyzed by flow cytometry. Total thymic cellularity was determined by hemocytometer counting and trypan blue exclusion. The number of cells in each developmental stage was calculated by multiplying the percentage of thymocytes in each developmental stage, as determined by CD4 and CD8 staining by flow cytometry, by the total thymic cellularity.

Proliferation

Thymocyte proliferation was assessed by BrdU incorporation. BrdU (10μM) was added to FTOC cultures on day 6 for 1hr. Thymocytes were then harvested, stained for

CD4 and CD8β, and stained with anti-BrdU-APC according to manufacturer's instructions (BD Biosciences, San Diego, CA).

Flow Cytometry

Thymocyte suspensions derived from harvested FTOCs were stained in a minimal volume with 5 μg/ml of directly coupled antibodies on ice for 20 min, washed with 1% calf serum in PBS and then were fixed in 2% formaldehyde. Flow cytometry was then performed using a BD FACSCalibur (BD Biosciences, San Jose, CA) using the following approximate settings then fined tuned using unstained and single stained controls: FSC voltage E0, 2.00 amp gain, SSC 500(lin), FL1 700(log), FL2 650(log), FL3 700(log), FL4 750(log). If required during multicolor acquisition, compensation was set at: FL1 0.4% FL2, FL2 30-40% FL1, FL2 0.2% FL3, FL3 20-30% FL2, FL3 10% FL4, FL4 0.2% FL3. Data was collected and analyzed using Cell Quest Pro software (Santa Rose, CA).

TMRE staining

Thymocytes ($1x10^6$ cells) were harvested from FTOC and stained in 200μl of cell culture media with 0.2μM tetramethylrhodamine, ethyl ester, perchlorate (TMRE) for 30 mins at 37°C, 7% CO_2. Thymocytes were then washed and stained with annexinV-FITC. For apoptosis induction, freshly isolated adult thymocytes were treated with 20μM etoposide for 4hrs at 37°C, 7% CO_2, then washed in PBS and stained with TMRE as described above.

SDS-PAGE and Western blot analysis

Thymocytes were lysed in 1% NP40/PBS and loaded at $1x10^6$ cells/well and run on a 12% SDS-PAGE. Proteins were then transferred onto polyvinylidene fluoride (PVDF) Immobilon (Millipore, Bedford, MA). Membranes were blocked in 2% BSA, blotted with PY72 or anti-Lck followed by anti-mouse-hrp. Membranes were treated with Western Lightning Chemiluminescence reagent (Perkin Elmer, Wellesley, MA) and developed using Fuji Super RX film.

Statistical analysis

T-tests were performed assuming unequal variance to generate P values at a 95% confidence level using Microsoft Excel software. P values <0.05 were considered to be statistically significant.

2.3. Results

CD45 specific mAbs disrupt thymocyte development at the DN to DP and at the DP to SP stages in fetal thymic organ culture.

To examine the contribution of the external domain of CD45 in thymocyte development, mAbs specific to the external domain of CD45 were added to FTOC and their effect on thymocyte development assessed by staining for developmental markers. Initially, to control for individual variability, the effects of mAb treatment on T cell development were examined on individual sister thymic lobes. One thymic lobe from an individual fetal mouse was treated with either isotype control or pan-specific CD45

antibodies I3/2 (Trowbridge 1978; Johnson et al. 1989) or M1/9 (Coffman 1982), while the other sister lobe was left untreated (no mAb control). Treatment with I3/2 or M1/9 resulted in a block at the double negative (DN) to double positive (DP) stage as indicated by the over 2-fold increase in the percentage of DN thymocytes and a concomitant decrease in the percentage of DP thymocytes seen compared to the untreated lobe (Fig. 2-1A). CD45 mAb treatment also disrupted development at the single positive (SP) stages, resulting in a 2-fold decrease in the percentage of CD4 SP cells, and an increase in the percentage of CD8 SP cells of 2-3 fold over the untreated control. Rat IgG2a and IgG2b isotype controls showed no difference from the corresponding untreated control lobe. Thus, CD45 specific mAb treatment induced a profound developmental block at the DN to DP stage as well as the SP stage of thymocyte development.

Total thymic cellularity was also found to be reduced by CD45 mAb treatment (Fig. 2-1B). The average individual untreated FTOC lobe contained 2.7 x 10^5 cells compared to 1.2x10^5 cells in I3/2 treated lobes, and 1.8 x 10^5 cells in M1/9 treated lobes. This was found to be a significant reduction ($P < 0.05$) compared to the untreated lobes. Isotype control antibodies had no impact on thymic cellularity. CD45 antibodies can, therefore, disrupt thymocyte development and reduce thymic cellularity.

Antibodies specific for different CD45 isoforms have varying effects on T cell development in FTOC.

To examine the potential role of different CD45 isoforms on fetal thymocyte development, pooled fetal thymi were cultured with antibodies specific for the RA (14.8) (Johnson et al. 1989), or RB (MB23G2, MB4B4) (Birkeland et al. 1988) containing

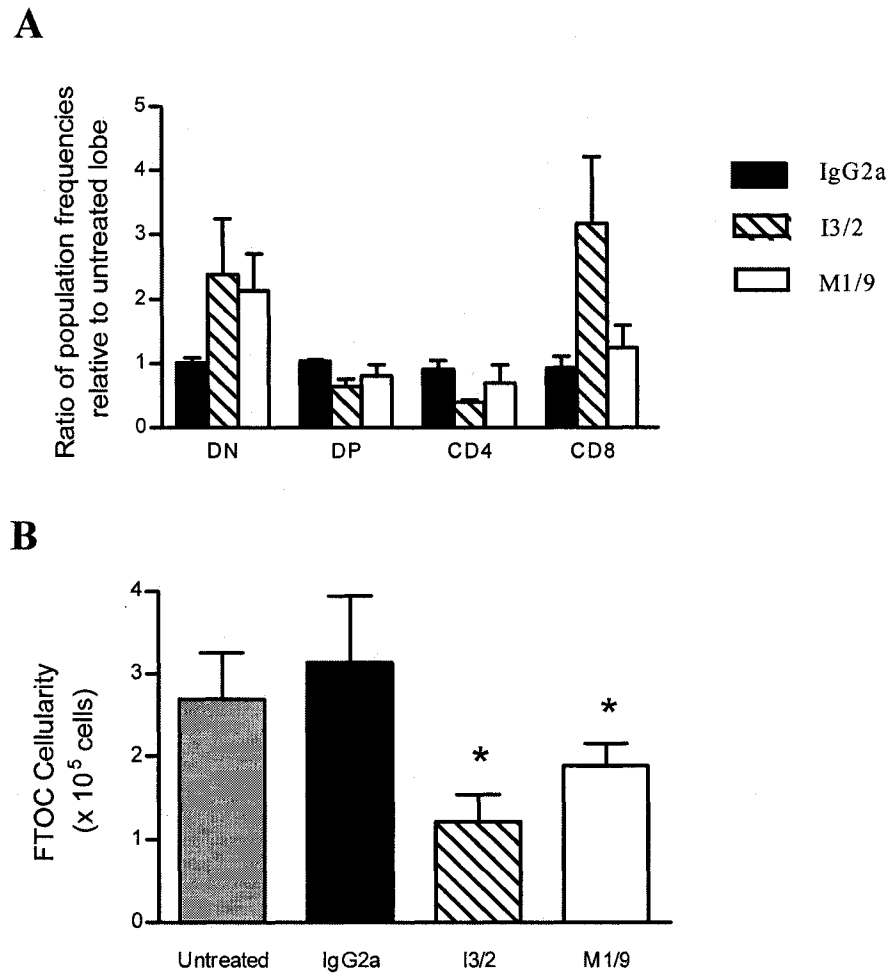

FIGURE 2-1. *CD45 pan-specific mAb treatment blocks fetal thymocyte development at the DN to DP and the DP to SP stages.* Sister lobes from individual wt d14 fetal mice were cultured such that one lobe was left untreated (no mAb) and the other lobe was treated with 40 μg/ml of either I3/2, M1/9 (pan-specific CD45 antibodies), or rat IgG2a isotype control and then harvested after six days in culture. Harvested thymocytes were then examined for developmental progression by fluorescent staining for the co-receptors CD4 and CD8β by flow cytometry. A) The percentage of thymocytes at each developmental stage (DN, DP, CD4, CD8) from Ab treated lobes are expressed as a ratio to the population percentages in the untreated lobe. B) Total cellularity of each individual lobe was calculated as described in the materials and methods. Error bars represent standard error, n=3, * indicates statistical significance at a P value less than 0.05 ($P<0.05$).

CD45 isoforms, or with pan-specific CD45 mAb, I3/2, M1/9. The average percentages of thymocytes at each developmental stage after mAb treatment of FTOCs are shown in Figure 2-2. The untreated control FTOCs yielded average thymocyte population frequencies of 27% DN thymocytes, 53% DPs, 12% CD4 SPs, and 7% CD8 SP thymocytes. I3/2 treatment resulted in an average increase in the percentage of DNs to 48%, a decrease in DPs and CD4 SPs, to 34% and 6.8% respectively, and an increase in the percentage of CD8 SPs to 11%. I3/2 treatment resulted in a highly significant ($P<0.0001$) two-fold increase in the percentage of DN thymocytes and about a two-fold decrease in the percentage of DP thymocytes compared to the untreated control thymi (Fig. 2-2A). The CD4 to CD8 SP ratio was also disrupted in that the frequency of CD4 SP thymocytes was decreased and the frequency of the CD8 SP thymocytes was increased by an average 1.5 fold compared to untreated control (Fig. 2-2B, $P<0.05$). The CD45RB specific mAb, MB4B4, also induced a statistically significant ($P<0.05$) effect on the DN to DP (Fig. 2-2A) and the CD4 to CD8 (Fig. 2-2B) ratios by inducing a 1.5 fold increase and decrease in these ratios respectively. MB23G2 showed no significant difference compared to control mAb treatment (Fig. 2-2A & B). FTOC treatment with 14.8 mAb, specific for CD45RA containing isoforms, did not have any effect on T cell progression as expected as there is minimal staining for the RA isoform in thymocytes ((McNeill et al. 2004), and Appendix A2). The contribution of RC containing isoforms was not assessed as very limited expression of this isoform was found in fetal thymocytes (Appendix A2). As indicated above, addition of isotype control antibodies had no effect on T cell development (Figs. 2-2A & B).

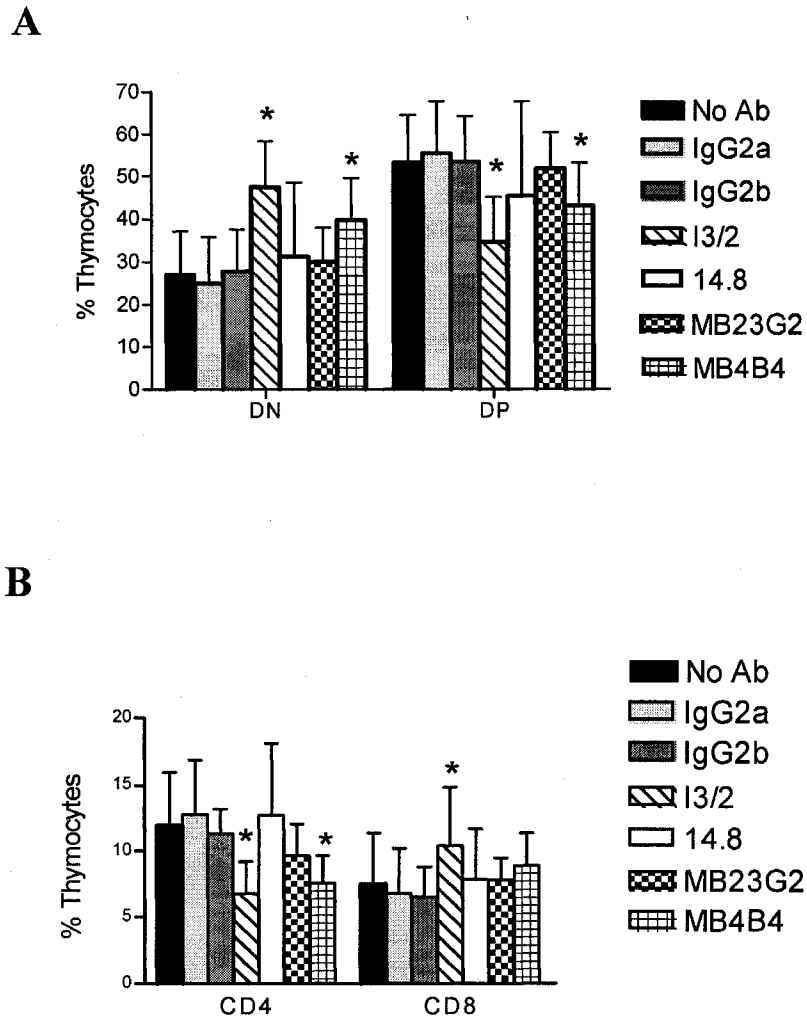

FIGURE 2-2. *Different CD45 antibodies have varying effects on T cell development in FTOC.* Thymic lobes derived from wildtype d14 fetal mice were pooled and cultured as described, in the presence or absence of different CD45-specific antibodies, or rat IgG2a or IgG2b isotype controls. I3/2 is pan-specific, 14.8 is specific for CD45RA containing isoforms, MB23G2 and MB4B4 are specific to CD45RB containing isoforms, Y13-238 was used as a rat IgG2a isotype control, and anti-DR5 was used as a rat IgG2b isotype control. The average percentage of thymocytes in each population, the DN and DP (A), and (B) the CD4 and CD8 SP populations are shown for each culture condition. Error bars represent SEM. * ($P<0.05$), n=7.

CD45 mAb treated FTOC and CD45-/- FTOC exhibit stage-specific blocks in thymocyte development.

As adult CD45 deficient mice are known to exhibit developmental defects from the DN to DP and the DP to SP progression (Byth et al. 1996), T cell development in CD45 mAb treated FTOCs derived from Wt mice and CD45 deficient mice was compared. The effect of I3/2 on thymocyte development in Wt FTOC was found to be similar in phenotype to thymocyte development in FTOCs derived from CD45-/- fetal mice (Fig. 2-3A). Both exhibit an approximately 2-fold decrease in DP thymocyte percentages and a corresponding 2-fold increase in the DN thymocyte percentages compared to untreated Wt control cultures (Fig. 2-3B, $P<0.0001$). I3/2 treated Wt FTOC, however, exhibited a dramatic decrease in the percentage of CD4 SP cells and an increase in the percentage of CD8 SP T cells, whereas FTOCs derived from CD45-/- mice, exhibited a small decrease in CD4 SP percentages and no major change in the CD8 SP percentages (Fig. 2-3B). The effect of I3/2 was specific to CD45, as I3/2 treatment of CD45-/- FTOC had no additive effect (Fig. 2-3B).

A block in thymocyte development during β chain selection, which occurs at the DN3 to DN4 stage has been observed in CD45-/- adult mice (Byth et al. 1996); therefore, harvested thymocytes from I3/2 treated Wt FTOC or CD45-/- FTOC were examined for developmental progression through the DN stages. CD45-/- FTOC exhibited over a 3-fold increase in the percentage of DN3 ($CD44^-CD25^+$) thymocytes and an over 2-fold decrease in the percentage of DN4 ($CD44^-CD25^-$) thymocytes compared to Wt control FTOC (Fig. 2-3C, $P<0.001$), which is consistent with previous studies in adult CD45 knockout mice (Byth et al. 1996). I3/2 treated Wt FTOC displayed only an a 1.6-fold

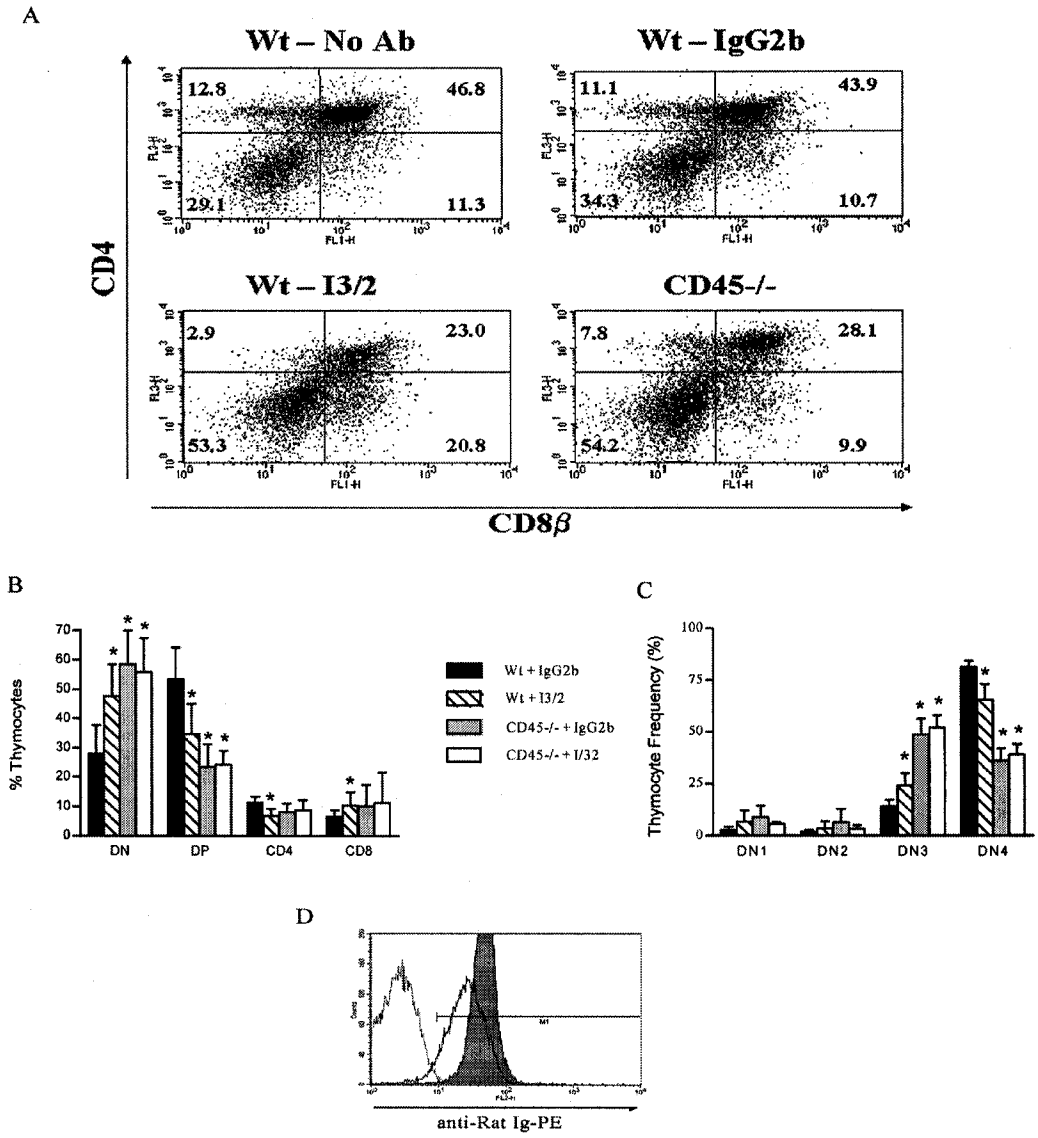

FIGURE 2-3. *CD45 mAb treated FTOC and CD45-/- FTOC exhibit stage-specific blocks in thymocyte development.* FTOC cultures derived from wt or CD45-/- fetal mice were treated with I3/2 or isotype control and Ab. A) Representative flow cytometry profiles of thymocytes isolated and stained for CD4 and CD8β. The average percentages of thymocytes at each developmental stage as determined by CD4 and CD8 staining (B), or with CD25 and CD44 (C) are shown graphically. Error bars represent SEM. * ($P<0.05$), n=8. D) Representative histograms of thymocytes harvested from isotype control (grey dashed line) or I3/2 treated (dark line) wt FTOC cultures stained directly with anti-rat IgG-PE. Thymocytes derived from I3/2 treated cultures were also stained with fresh I3/2 and then with secondary anti-rat IgG-PE (filled histogram).

increase in DN3 thymocyte percentages and a 1.2-fold decrease in DN4 thymocyte percentages, which was much lower in comparison to FTOC derived from CD45-/- mice, but still significantly different from untreated Wt control, ($P<0.05$). Thus, it appears that I3/2 treatment does not induce a DN3 to DN4 block to the same extent as that seen in CD45-/- thymi, even though they exhibit comparable percentages of total DN cells (Fig. 2-3 B & C).

To rule out the possibility that I3/2 causes a CD45 deficient phenotype by removing CD45 from the cell surface, CD45 expression on thymocytes derived from I3/2 treated Wt FTOC was indirectly confirmed by staining the harvested thymocytes with fluorescently conjugated anti-rat Ig or with staining with saturating I3/2 concentration followed by anti-rat Ig. The I3/2 antibody used during FTOC treatment was found to remain bound to the cell surface throughout the duration of the culture period as indicated by the high staining intensity that was only slightly reduced compared to thymocytes stained with fresh I3/2, indicating that CD45 remained on the cell surface (Fig 2-3D). Therefore, I3/2 is inducing an effect on thymocyte development by binding to the external domain of CD45 and remaining on the cell surface.

Ab engagement of CD45 does not affect upregulation of the selection markers CD5 or CD69 on developing thymocytes.

As the major block on thymocyte development caused by CD45 Ab treatment was occurring later at the DP and SP stages, the effects of CD45 Ab engagement and CD45 deficiency were examined on the upregulation of CD5 and CD69, markers of positive

selection (Swat et al. 1993; Tarakhovsky et al. 1995; Azzam et al. 1998). In CD45-/- mice, CD69 or CD5 are not up regulated as a result of defective positive and negative selection caused by CD45 deficiency (Mee et al. 1999). Examination of the DP populations in FTOC revealed that I3/2 Ab treatment did not affect the percentage of thymocytes that up regulated CD5 (Fig. 2-4A) or CD69 (Fig. 2-4B) and expression was found similar to Wt control. DP thymocytes derived from CD45-/- FTOCs, however, showed a drastic reduction in the basal expression of CD5 (Fig. 2-4A) as well as a much reduced percentage of $CD5^{hi}$ expressing cells, which is consistent with the literature (Mee et al. 1999). The expression of CD69 on CD45-/- fetal thymocytes, however, did not seem to be impaired at the DP stage, but was notably absent from $CD4^+$ SP thymocytes (Fig 2-4B). Upregulation of CD5 expression also occurred on a percentage of thymocytes reaching the CD4 SP stage in a similar manner on both the Wt and I3/2-treated FTOCs, whereas in the CD45-/- FTOCs, a very limited percentage of $CD4^+$ SP thymocytes up regulated CD5 expression (Fig. 2-4A). CD45 Ab treatment does not, therefore, affect the upregulation of selection markers unlike CD45 deficiency, which results in a reduction of their expression at specific stages of thymocyte development.

Thymic cellularity is decreased in I3/2 treated and CD45-/- FTOC.

Thymic cellularity was found to be significantly decreased after I3/2 treatment of Wt FTOC (Fig. 2-1B), therefore, total cellularity of FTOCs derived from CD45-/- fetal mice was also examined. As shown in Fig. 2-5A, the average I3/2 treated thymus contained about half the number of cells compared to that of untreated control thymi; $2.2x10^5$ cells compared to $1.0x10^5$ cells, respectively which was statistically significant

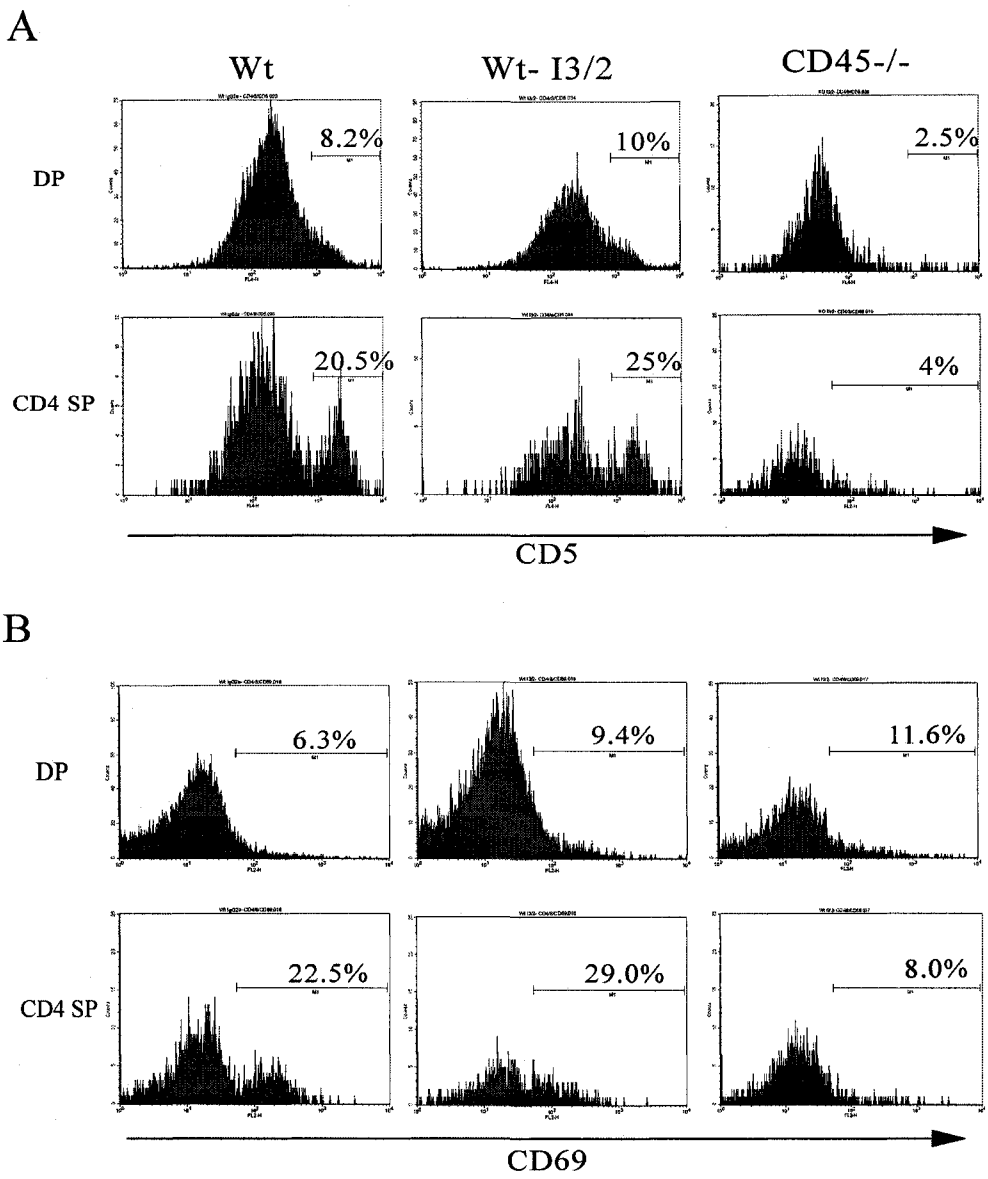

FIGURE 2-4. *CD45 mAb treatment does not prevent up regulation of the selection markers CD5 and CD69.* Representative histograms (n= 4) of A) CD5 or B) CD69 expression in gated DP (upper panels) or CD4 SP (lower panels) thymocytes derived from wt control (left) or I3/2 treated (middle), or CD45-/- (right) 6-day FTOC. Harvested thymocytes were stained with CD4-PE-Cy5, CD8β-FITC, and CD5-APC or CD69-PE and analyzed by 3 color flow cytometry. The percentage of high expressing cells is shown above the marker bounds.

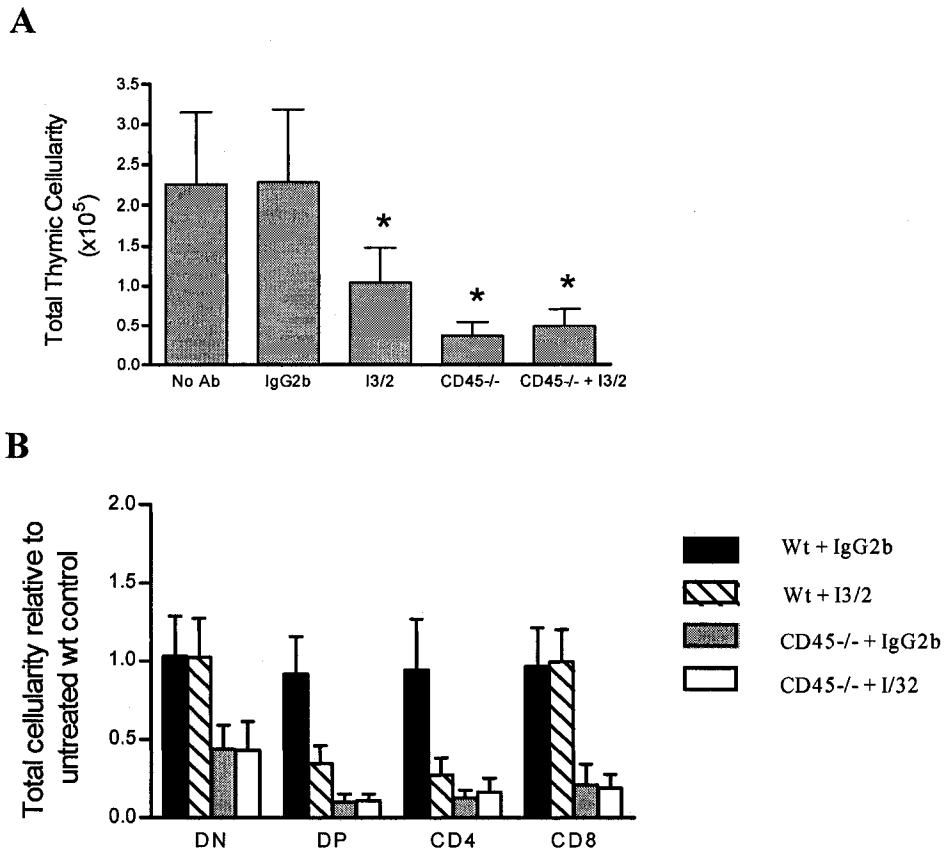

FIGURE 2-5. *Cellularity is reduced in I3/2 treated wildtype FTOC and CD45-/- FTOCs.* Wt or CD45-/- FTOC were treated with either isotype control or I3/2, A) total thymic cellularity was determined from pooled thymic cultures by hemocytometer counting and dividing by the number of thymic lobes per culture condition. B) The number of thymocytes at each developmental stage was determined from wt and CD45-/- FTOC cultures (untreated or treated with I3/2) by multiplying the total thymic cellularity by the population percentages and then displayed relative to the cellularity of untreated control FTOCs. Errors represent SEM. * ($P<0.05$), n=6.

($P<0.01$). CD45-/- thymi exhibited a more dramatic decrease in thymic cellularity, containing only a quarter of the number of thymocytes found in Wt control thymi, ($P<0.0001$). I3/2 treatment had no enhanced effect on CD45-/- FTOC cellularity. Further analysis of cellularity at each developmental stage, by taking into consideration the total thymic cellularity and the percentages of thymocytes at each developmental stage, revealed that FTOCs derived from CD45-/- mice exhibited decreased cell numbers at all stages of development relative to Wt control cultures (Fig. 2-5B). I3/2 treated Wt FTOCs, on the other hand, showed selective thymocyte loss mainly at the DP and CD4 single positive stages. The numbers of DN and $CD8^+$ SP cells in the I3/2 treated FTOCs was the same as untreated control, explaining why the frequencies of these populations were found to be higher (Fig. 2-5A). The loss of thymocyte numbers observed from harvested I3/2 treated, and CD45-/- FTOCs was not due to an enhanced or aberrant thymocyte release from the thymus as no detectable thymocytes were found in the culture media after 6 days of culture. Therefore, engagement of CD45 by I3/2 reduces thymocyte cellularity at selective thymocyte stages, whereas, CD45 deficiency leads to a global reduction in thymocyte numbers.

CD45 engagement or deficiency results in increased annexin V positive fetal thymocytes.

To determine if the decreased cellularity seen in I3/2 treated FTOC and CD45-/- FTOC was due to enhanced thymocyte apoptosis, harvested thymocytes were stained with annexin V to examine externalized phosphatidylserine that occurs upon early thymocyte apoptosis (Koopman et al. 1994; Vermes et al. 1995), and 7AAD, a vital dye that stains nucleic acids upon loss of cell membrane integrity indicating later apoptotic or

necrotic stages of cell death (Schmid et al. 1992). As shown in Figure 2-6, Wt FTOC treated with I3/2 or CD45-/- FTOC exhibit increased percentages of annexin V positive cells (Fig 2-6A) and exhibited about a two-fold increase in both annexinV^+/7AAD^- and annexinV^+/7AAD^+ thymocytes compared to control (Fig. 2-6B). Three color staining was used to examine which populations exhibited annexin V staining. In accordance with the cellularity data, I3/2 treatment induced increased annexin V positive thymocytes selectively at the DP and $CD4^+$ SP stages, up to 4-5 fold over control (Fig. 2-6C). Thymocytes derived from CD45-/- FTOC exhibited enhanced annexin V staining at all thymocyte stages, up to 6 fold higher than Wt control.

Induction of annexin V staining by CD45 mAb treatment or CD45 deficiency does not correlate with a loss of mitochondrial membrane potential.

The increased annexin V staining suggested apoptosis was induced by CD45 mAb treatment and deficiency; therefore, we examined other markers of apoptosis. The contribution of caspases could not be analyzed as treatment of FTOC with the pan-caspase inhibitor zVAD-fmk alone resulted in increased annexin V staining (Appendix A3). Investigation of whether the annexin V binding observed correlated with a loss of mitochondrial membrane potential, another well documented marker of apoptosis (Zamzami et al. 1995; Marchetti et al. 1996; Jayaraman 2005), was performed. Thymocytes were incubated with the cell permeable TMRE stain which only enters the mitochondria and fluoresces in healthy mitochondrial maintaining normal membrane potential. While I3/2 treatment and thymocytes from CD45-/- FTOC exhibited increased annexinV staining, there was no corresponding loss of mitochondrial membrane potential

A

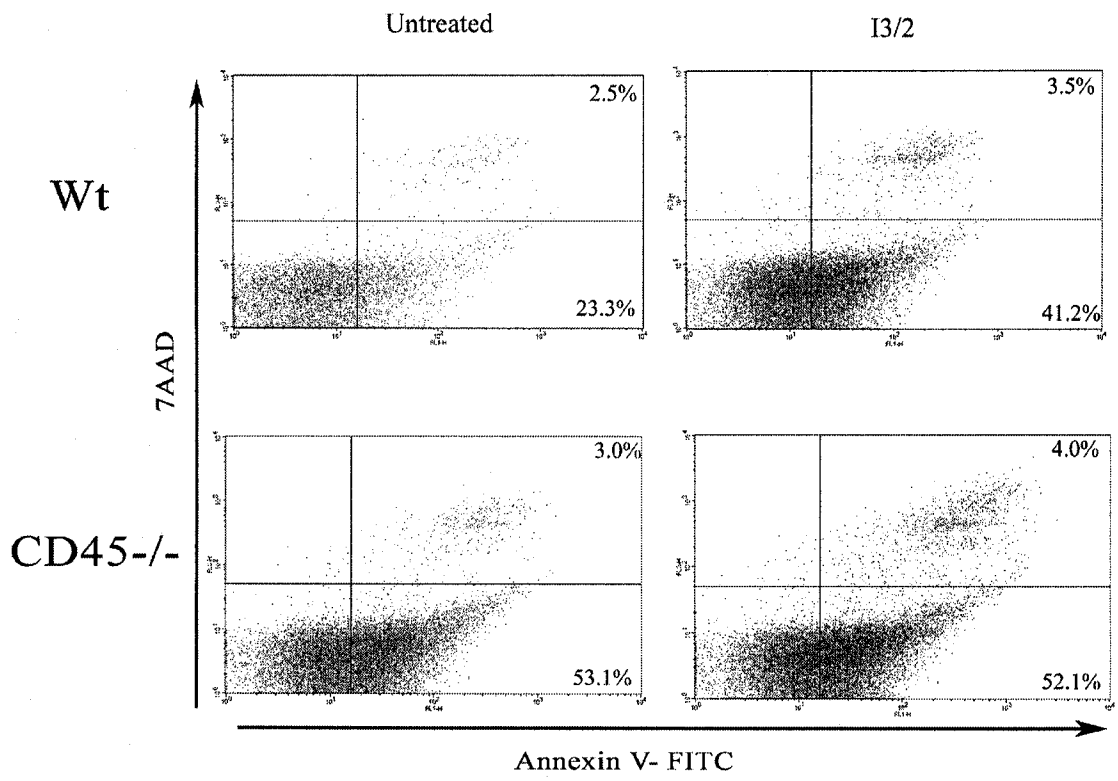

FIGURE 2-6 A. *CD45 mAb treatment and CD45 deficiency induces increased annexin V staining of thymocytes.* FTOC cultures derived from wt or CD45-/- fetal mice were cultured with isotype control or I3/2 mAb. After 6 days in culture, harvested thymocytes were stained with annexin V and 7AAD and the percentage of annexin V positive cells is represented by flow cytometry plots.

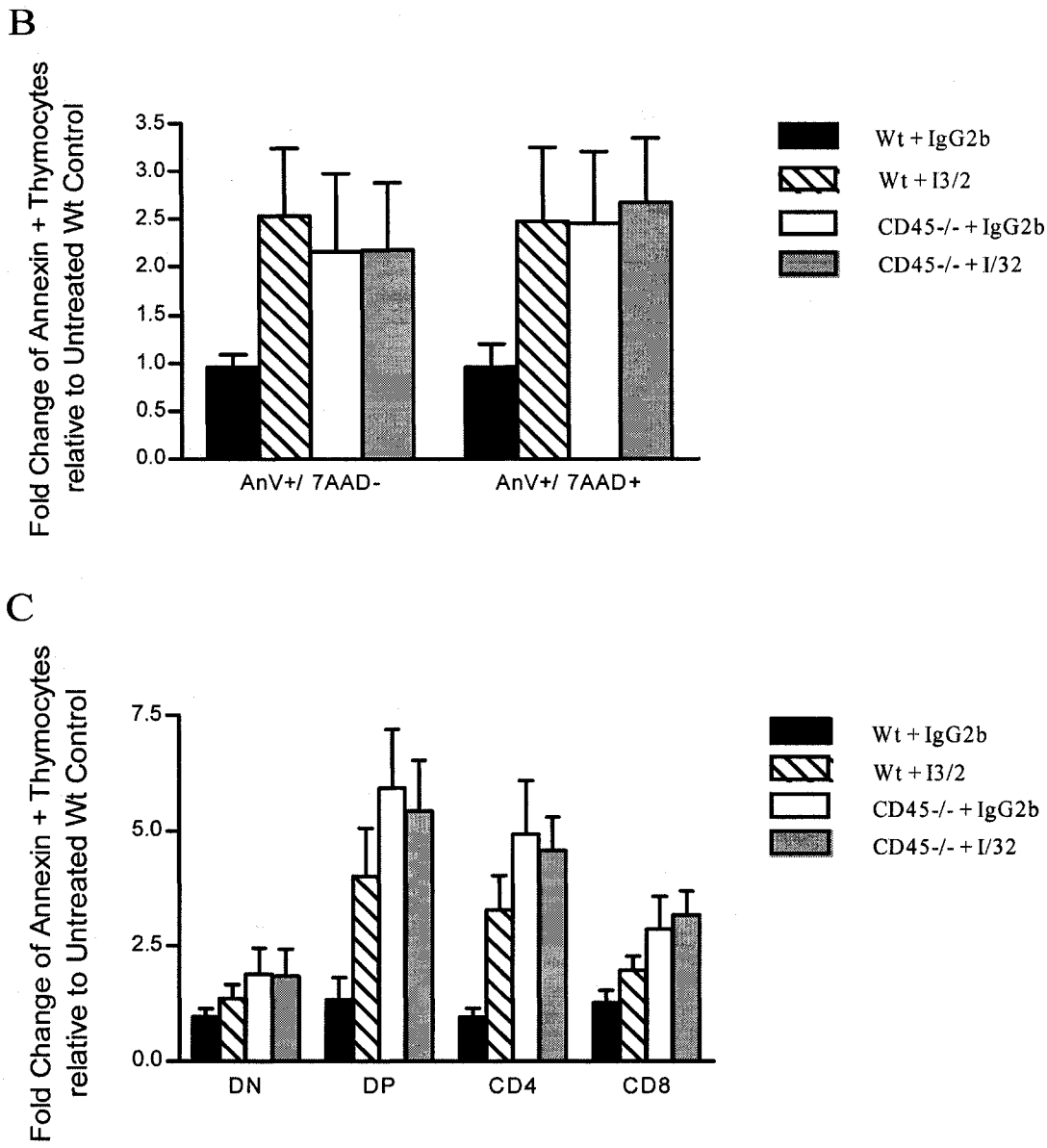

FIGURE 2-6 B&C. *CD45 mAb treatment and CD45 deficiency induces increased annexin V staining of thymocytes.* FTOC cultures derived from wt or CD45-/- fetal mice were cultured with isotype control or I3/2 mAb. After 6 days in culture, harvested thymocytes were stained with B) annexin V and 7AAD, or C) CD4, CD8β, and annexin V. The percentage of annexin V positive cells is represented relative to the percentages in wt untreated control cultures. Errors represent SEM, n=6.

(Fig 2-7A). As a positive control, adult thymocytes were treated with the apoptotic agent etoposide to show a loss of TMRE staining in conjunction with positive annexin V staining (Fig 2-7B). Thus, CD45 Ab treatment or CD45 deficiency causes an induction of annexin V positive thymocytes without a corresponding loss of mitochondrial potential, suggesting that these thymocytes are not reaching the later stages of apoptosis.

The percentage of thymocytes undergoing proliferation is not impeded by CD45 Ab engagement or deficiency.

The potential for CD45 Ab treatment or CD45 deficiency to affect the ability of developing thymocytes to proliferate, and thereby contribute to the loss in thymic cellularity observed, was also examined. To investigate this possibility BrdU was added to Wt, I3/2 treated Wt, or CD45-/- FTOC-6 cultures to examine the amount of BrdU incorporation as a measure of DNA synthesis and proliferation. Focusing on the DN and DP populations where thymocyte proliferation is expected to occur, the percentage of thymocytes that incorporated BrdU in I3/2 treated and CD45-/- FTOC was not found to be decreased but was actually slightly increased compared to untreated Wt cultures (Fig. 2-8A). Examination of phospho-STAT5, as an indicator of downstream IL-7 signaling, which is a regulator of thymocyte proliferation (Hare et al. 2000), also revealed increased phospho-STAT5 levels in thymocytes from I3/2 treated and CD45-/- FTOC compared to Wt FTOC (Fig 2-8B). Thus thymocyte proliferation and IL-7 signaling are not impeded but are actually increased in I3/2 treated and CD45-/- FTOC.

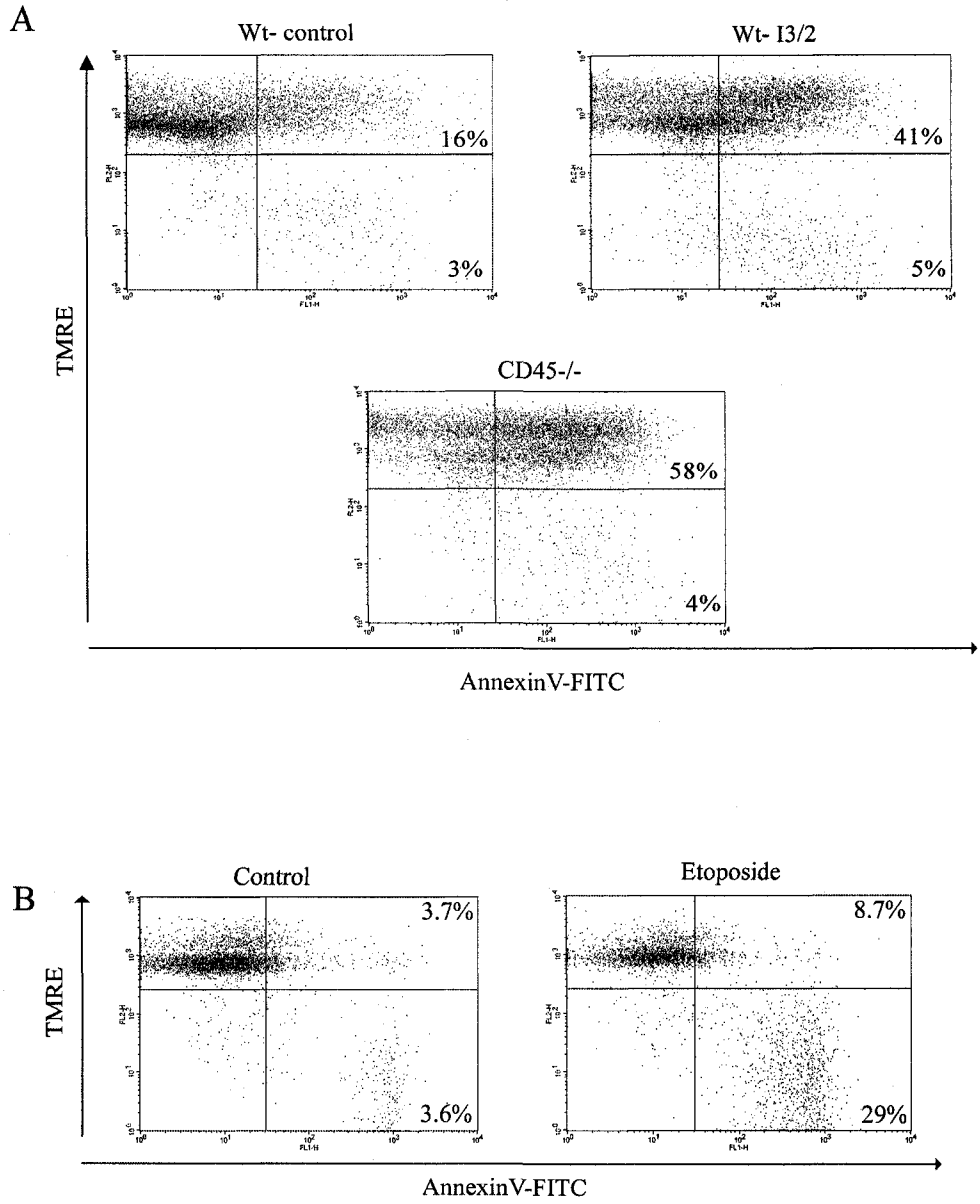

FIGURE 2-7. *Annexin V staining induced by CD45 mAb treatment or CD45 deficiency does not correlate with a loss of mitochondrial membrane potential.* A) Thymocytes harvested from wt, wt treated with I3/2, or CD45-/- FTOC-6 were stained with (TMRE) for 30 mins at 37°C, and then with annexin V-FITC and analyzed by two color flow cytometry. B) Adult thymocytes were left untreated, or treated with 20μM etoposide for 4hrs to induce apoptosis and then thymocytes were stained with TMRE and annexin V.

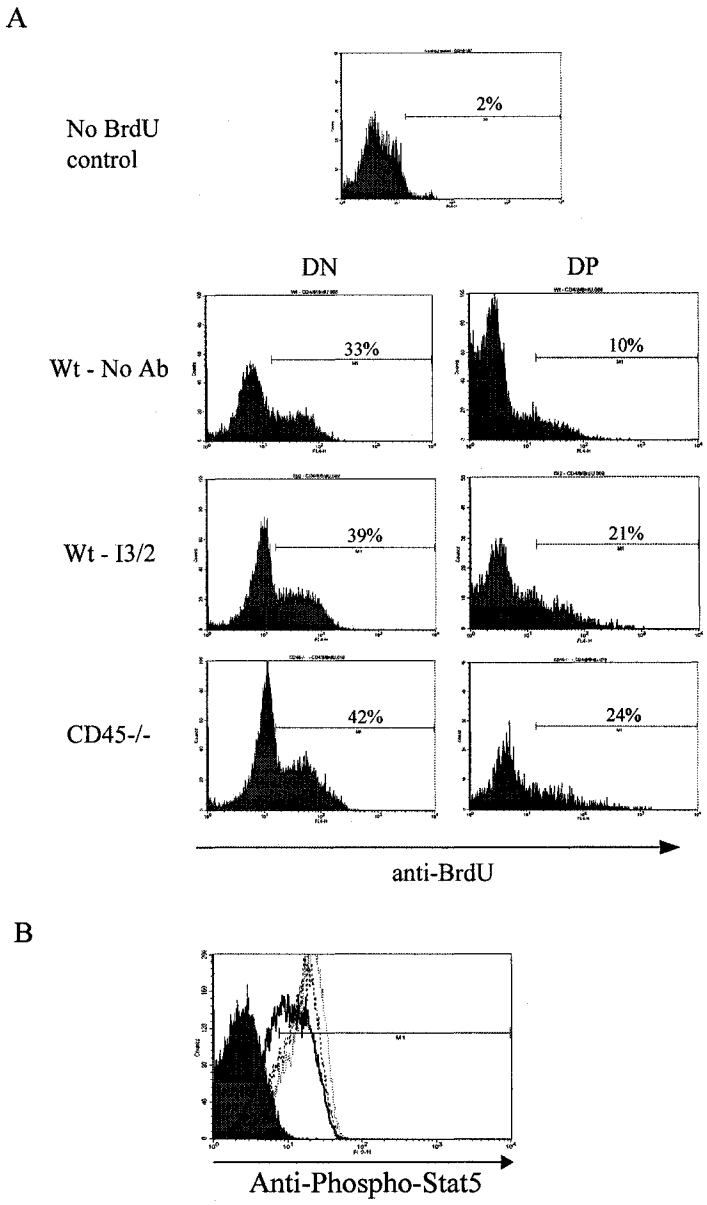

FIGURE 2-8. *Ab manipulation of CD45 or CD45 deficiency does not reduce the percentage of proliferating thymocytes.* A) BrdU was added for 45 min at 37°C to FTOC-6 cultures derived from wt or CD45-/- fetal mice cultured with or without I3/2 mAb. Thymocytes were then harvested, stained for CD4 and CD8β, and BrdU incorporation. Representative histograms of the percentage of BrdU positive cells at the DN or DP stage are shown relative to background in cultures where BrdU was not added. B) Thymocytes derived from wt (dark line histogram), I3/2 treated wt (dashed line histogram), or CD45-/- (grey dot histogram) FTOC were stained intracellularly with anti-phospho-STAT5, or left unstained (filled histogram).

Crosslinking antibodies do not enhance I3/2 mediated effects in FTOC.

As it has been shown by others that mAb crosslinking of CD45 can induce death in adult lymphocytes in culture (Klaus et al. 1996; Steff et al. 2003), I3/2 on its own was verified to not induce annexin V staining on adult thymocytes in culture (Fig 2-9A). Addition of secondary crosslinking mAb had no enhanced effect on I3/2 treated FTOC (Fig 2-9B), even though it did induce increased annexinV binding in cultured adult thymocytes after 4 hrs of crosslinking I3/2 treatment (Fig 2-9A) consistent with previous data (Steff et al. 2003). To address if epitope crosslinking was required for the effect of I3/2 on thymocyte development, Fab fragments of I3/2 were generated. Treatment of FTOC with I3/2 Fabs or I3/2 Fabs in addition with a secondary crosslinking antibody did affect development impairment (Fig 2-9B); however, the I3/2 Fabs exhibited a markedly reduced binding ability from that of the intact antibody (Fig 2-9C), therefore, it cannot be definitively concluded whether CD45 crosslinking is absolutely required. Nonetheless, this work suggests that soluble CD45 antibodies specifically disrupt interactions occurring during development in FTOC.

Global tyrosine phosphorylation is not affected by CD45 Ab treatment.

In an attempt to address the mechanism by which CD45 mAb treatment impairs development, signaling in developing thymocytes was examined by examining total protein tyrosine phosphorylation to determine if antibody treatment was perhaps mediating an effect on the phosphatase activity of CD45. Western blot analysis of lysates generated from FTOC-6 treated with various anti-CD45 antibodies revealed no major difference in protein tyrosine phosphorylation compared to isotype control treatment

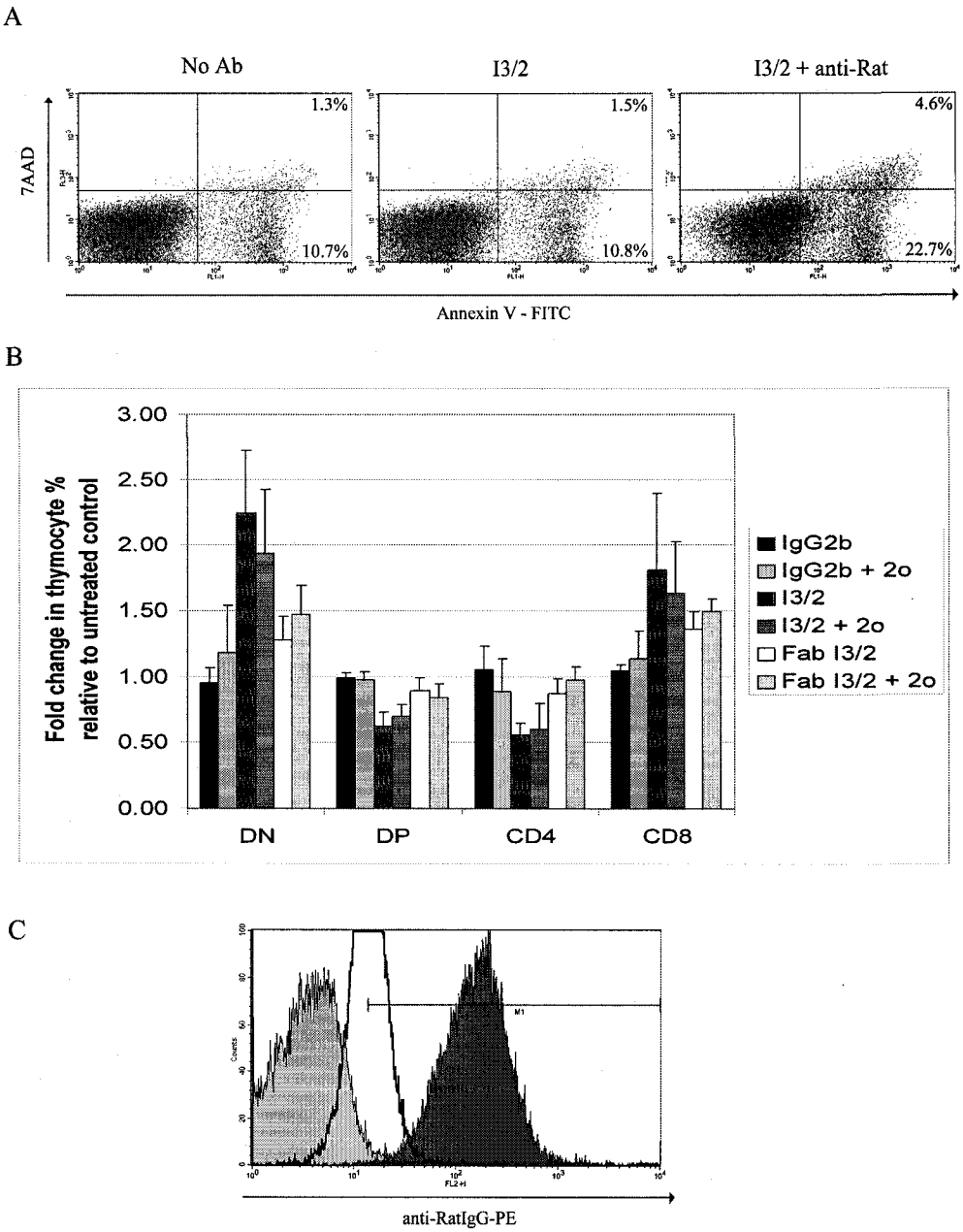

FIGURE 2-9. *Crosslinking antibodies do not enhance I3/2 mediated effects.* A) Adult thymocytes from B6 mice were cultured for 4 hrs at 37°C with IgG2b control or I3/2, with or without the addition of a secondary crosslinking anti-ratIg. Thymocytes were then stained with Annexin V-FITC and 7AAD. B) Wt FTOC were treated with 40ug/ml of IgG2b, I3/2, or I3/2 Fabs, with or without the addition of a secondary anti-ratIg. After 6 days, thymocytes were harvested and stained for CD4 and CD8β. C) Histogram showing binding of anti-ratIg-PE to thymocytes derived from FTOCs treated with IgG2b (grey filled), I3/2 (dark filled), or I3/2 Fab (dark line).

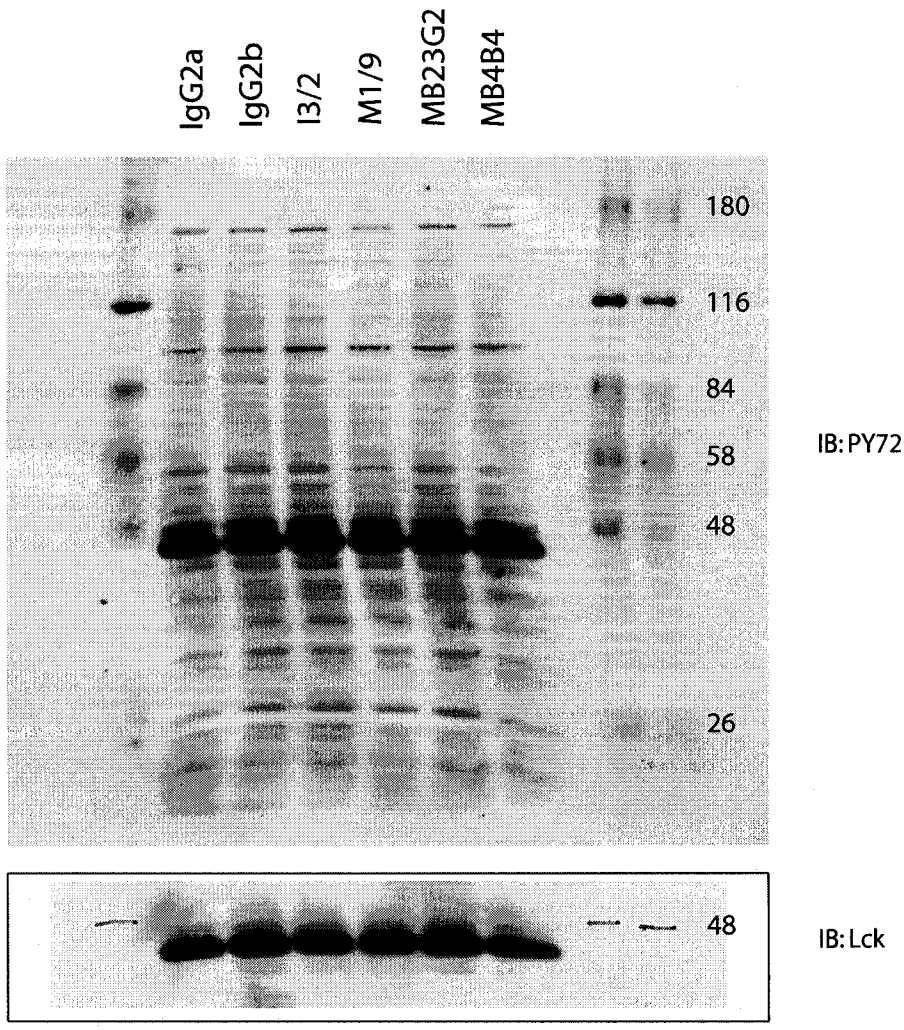

FIGURE 2-10. *Anti-CD45 Ab treatment has no effect on global protein tyrosine phosphorylation in developing thymocytes in FTOC.* Thymocytes from FTOC-6 treated with anti-CD45 Abs or isotype controls were lysed in 1% NP40 and run on a 12% SDS-PAGE and transferred to PVDF Immobilon membrane. The membrane was then immunoblotted (IB) with anti-phosphotyrosine, PY72, (upper panel) or anti-Lck (lower panel).

(Fig 2-10 upper). Furthermore, anti-CD45 treatment was not found to exhibit any noticeable effect on lck expression or mobility shift (Fig 2-10 lower). Hence CD45 Ab treatment did not induce a remarkable effect on protein phosphorylation in developing thymocytes in FTOC.

2.4. Discussion

This work demonstrates that engagement of the external domain of CD45 in an epitope specific manner can modulate specific stages of thymocyte development. Addition of certain CD45 specific antibodies to developing thymocytes in fetal thymic organ culture caused a disruption of normal thymocyte maturation resulting in increased ratios of DN to DP and CD8 to CD4 SP thymocytes. This disruption was found to be due to a decrease in thymocyte numbers at the DP and CD4 SP stages as an apparent result of apoptosis based on annexin V and 7AAD staining. The developmental impairment induced by mAb treatment was found to be similar to, yet distinct, from thymocyte development in FTOC derived from CD45 deficient fetal mice. CD45 mAb treatment of wildtype FTOC affected mostly the later stages of T cell development, resulting in depletion of the DP and CD4 SP thymocytes specifically, while not appearing to affect thymocyte selection. Thymocyte development in CD45-/- FTOC, however, displayed a more impaired phenotype, including disruption earlier at the DN3 stage of development, impairment of selection marker induction, and thymocyte depletion at all stages of thymocyte development. Thus, manipulation of the external domain of CD45 results in the impairment of thymocyte development distinct from that of CD45 deficiency,

implying a role for the external domain in regulating thymocyte survival and developmental progression.

It has been shown previously that anti-CD45 mAb treatment in mice could prevent thymocyte progression to the SP stages and was suggested to disrupt selection events in TCR-Tg mice (Benveniste et al. 1994). Here these studies were expanded to discover that the mechanism by which CD45 antibodies impair thymocyte development is through the specific depletion of DP and CD4 SP thymocytes. The loss of thymocytes was an apparent result of apoptosis induction exhibited by externalization of phosphatidylserine residues, based on annexinV and 7AAD staining; although a loss of mitochondrial potential using TMRE staining was not observed. Non-specific binding of TMRE to organelle membranes, however, cannot be ruled out as a mitochondrial potential uncoupler was not used to verify the membrane potential dependence of the TMRE dye within fetal thymocytes (Scaduto et al. 1999). Nonetheless, CD45 manipulation does induce the expression of an early apoptotic marker, phosphatidylserine, which can signal for uptake and clearance by macrophages (Fadok et al. 1992; Surh et al. 1994; Hoffmann et al. 2001; Williamson et al. 2002). The thymocyte depletion observed was not a result of proliferation impairment, as it was found that I3/2 treatment or CD45 deficiency resulted in increased percentages of proliferating thymocytes compared to Wt controls, as assessed by BrdU incorporation (Fig. 2-8A). CD45 deficiency has been shown previously to result in increased IL-7 signaling in B cells causing hyper-proliferation (Fleming et al. 2004), which were verified to be increased in CD45-/- FTOC and I3/2 treated FTOC by examining phospho-STAT5 staining (Fig 2-8B). This data supports the function of CD45 as a negative regulator of

the JAK/STAT pathway, and also suggests regulation of this pathway can be influenced by manipulation of the external domain of CD45.

The ability of different CD45 isoform-specific antibodies to block thymocyte development was also investigated in order to examine the potential involvement of different CD45 isoforms. Anti-CD45RA specific mAb (14.8) had no effect which is not surprising since thymocytes mainly express the R0 and RB isoforms of CD45 (McNeill et al. 2004)(Appendix A2). Currently there is no specific Ab available to the murine CD45R0 isoform, therefore, its specific contribution could not be evaluated, however, the effects of two CD45RB specific antibodies were examined. One of the CD45RB antibodies, MB4B4, did cause a partial disruption of thymocyte development, but not the other, MB23G2. This was surprising as MB23G2 prevents allograft rejection whereas MB4B4 can not; although the mechanism by which MB23G2 functions and the differences in biological function between the two antibodies is not very clear (Lazarovits et al. 1999). Both antibodies are capable of binding CD45 on thymocytes, and MB23G2 even exhibited higher staining intensity by FACS analysis than MB4B4 (Appendix A4), yet had no effect on thymocyte development (Fig. 2-2A & B). The epitopes for these antibodies have not yet been mapped, however, it is clear through FACS analysis that they bind non-identical epitopes ((Birkeland et al. 1988), and Hargrove and Ostergaard, unpublished observations). Interpretation of these data suggests that the mAb-induced impairment of thymocyte development is most likely not isoform dependent, but rather it depends on the nature of the specific Ab-epitope interaction. The CD45 specific monoclonal antibodies used in this study were isolated from hybridomas generated by immunizing rats with either T lymphoma cells in the case of I3/2 (Trowbridge 1978), or

with B cell lines in the case of 14.8 (Coffman 1982), and MB4B4 and MB23G2 (Birkeland et al. 1988). Antibody specificity to CD45 exon restricted epitopes was established by assessing the binding ability of the mAbs to cell lines retrovirally transduced with different exon containing CD45 constructs (Johnson et al. 1989). Although the pan CD45 specific antibodies recognized all the different CD45 transduced cell lines, the possibility exists that the mAbs may not be able to bind all possible specific exon containing isoforms due to glycosylation differences of some isoform subsets. Therefore, the differing ability of the CD45 specific antibodies to block thymocyte development may have to do with their overall ability to bind all possible forms of the different exon containing isoforms.

Other groups have documented previously the ability of CD45 antibodies to induce annexin V binding *in vitro* of adult thymocytes and mature T and B cells from mouse (Klaus et al. 1996; Lesage et al. 1997) and human (Gregori et al. 2005). Crosslinking of the CD45-specific mAb by either adding secondary antibodies, crosslinking agents, or by immobilizing the mAb on plates or beads was found to be required to induce annexin V staining of adult thymocytes *in vitro*; soluble anit-CD45 was not sufficient to induce annexin V staining (Klaus et al. 1996; Lesage et al. 1997). The I3/2 mAb alone was confirmed to not directly induce annexin V staining *in vitro* unless secondary mAb to induce crosslinking was added (Fig 2-9A). Addition of crosslinking anti-rat Ig to the I3/2 treated FTOC did not have any additional effect on development. To further examine the requirement of CD45 crosslinking, Fab fragments of I3/2 were also generated to examine their impact on thymocyte development in FTOC. The Fab fragments only exhibited minor effects on thymocyte development compared to

intact Ab, however, efficient binding of the Fab fragments to thymocytes could not be achieved, therefore, whether CD45 crosslinking is absolutely required cannot be definitively concluded. Nonetheless, this study suggests that soluble CD45 antibodies in the context of FTOC, specifically induce annexin V staining on specific thymocyte subsets.

The effects of soluble CD45 mAb in FTOC cultures did yield some similar effects to those observed by Hugo and colleagues in adult thymocytes *in vitro* with crosslinking Ab (Lesage et al. 1997). They found, similar to our findings, that the DP as well as the CD4 SP thymocytes were the most sensitive to anti-CD45 treatment, whereas, the DN and CD8 were more resistant as measured by annexin V binding. They also found that the apoptosis induced by crosslinking CD45 mAbs was caspase independent and did not result in DNA fragmentation (Lesage et al. 1997). An examination of the involvement of caspases using the caspase inhibitor z-VAD-fmk was attempted; however, the drug alone induced increased annexin V staining and therefore a role for caspases was not evaluated (Appendix A3). Other studies have shown that externalization of phosphatidylserine residues is not always accompanied by classical markers of apoptosis during death of T lymphocytes (Lesage et al. 1997; Doerfler et al. 2000; Bidere et al. 2001; Ferraro-Peyret et al. 2002; Jaattela et al. 2003). This is not surprising in light of the increasing evidence to suggest that thymocytes are efficiently cleared by phagocytes prior to detection of classical apoptosis markers (Lesage et al. 1997; Dias-Baruffi et al. 2003). A recent publication by Elliott et. al. (Elliott et al. 2005) has also reported a correlation between CD45 expression and phosphatidylserine (PS) exposure in *ex-vivo* peripheral lymphocytes that lacked any other signs of apoptosis. They have suggested that the

expression of CD45 is a negative regulator of PS translocation, however, the mechanism by which this occurs remained to be elucidated (Elliott et al. 2005). The data presented here are consistent with their hypothesis that CD45 may be regulating PS externalization without apoptosis induction. However, the function of the PS externalization in FTOC in relation to the developmental effects observed in this study would be hard to explain other than the ultimate fate of cell death considering the correlation to the loss of cellularity and annexin V staining of the same populations.

Manipulation of the CD45 external domain by mAb treatment in Wt FTOC resulted in a developmental phenotype as extensive as that observed in CD45-/- FTOC, however, the mechanisms by which the block occurs is distinct. Engagement of the external domain mainly affected specific stages of development, whereas, CD45 deficiency resulted in developmental impairment at all stages of development. CD45-/- adult mice show disruption of thymocyte development at both the DN stage during β-chain selection and later at the DP to SP progression and exhibit defects in both positive and negative selection (Byth et al. 1996; Conroy et al. 1996; Mee et al. 1999). In this study CD45 mAb treatment did not appear to affect thymocyte selection processes as CD69 or CD5 upregulation, a marker of cells undergoing positive selection (Tarakhovsky et al. 1995; Azzam et al. 1998), was not found to be changed by I3/2 treatment. Thymocytes from CD45-/- FTOCs, in contrast, were found to lack the upregulation of these selection markers, which is consistant with previous literature (Byth et al. 1996; Mee et al. 1999). Therefore, this study suggests that CD45 mAb treatment does not affect thymocyte selection processes directly, but more likely may be enhancing death by neglect of thymocytes as a result of failure to receive adequate survival signals during

development. This could also account for the differences observed between the sensitivity of the $CD4^+$ SP thymocytes compared to the $CD8^+$ SP thymocytes, as $CD8^+$ SP thymocytes have been documented to require lower activation thresholds for survival and maturation (Germain 2002). More specifically strong lck signaling is required for CD4+ SP development, whereas lower lck signaling is sufficient to promote CD8 SP development (Hernandez-Hoyos et al. 2000). The total lack of CD45 in CD45-/- thymocytes, on the other hand, results in impaired β-selection and both positive and negative selection, therefore, accounting for the more generalized defect observed on thymocytes at all stages of development.

The disruption of thymocyte development in CD45-/- animals has been previously attributed to the lack of CD45 phosphatase activity; however, reconstitution of CD45-/- mice with constitutively active lck effectively rescued thymocyte development only to the DP stage (Pingel et al. 1999). Efficient development to the SP stages was only achieved by active lck when a Tg TCR was also expressed (Seavitt et al. 1999) which may influence the normal progression of thymocyte development. Antibody engagement of CD45 *in vitro* has been reported to not effect phosphatase activity (Fortin et al. 2002) and we have found that engagement of the external domain with soluble I3/2 also does not affect global protein phosphorylation (Fig 2-10). Therefore, the effects on thymocyte development seen upon mAb engagement of CD45 can likely be attributed to the manipulation of the external domain, possibly by regulating its localization or distribution on the cell surface, rather than by altering the intrinsic phosphatase activity of the molecule. This could also account for why selection processes are not affected by I3/2 treatment, as phosphatase activity is still present, whereas in CD45 deficiency it is not.

These data reveal that the external domain of CD45 regulates thymocyte survival and development *in vivo* in a developmental stage specific manner. The exact mechanism behind the stage specific role is unknown, but it is likely that the differential expression of certain carbohydrate ligands on CD45 during development may be required at the DP and CD4 SP stage to enable survival signals *in vivo*. This study shows that engagement of the external domain of CD45 modulates thymocyte development by affecting the survival of developing thymocytes at these stages of development. This work suggests a role for the external domain of CD45 in regulating thymocyte survival, and that external interactions of CD45 can modulate specific stages of thymocyte development.

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CHAPTER 3:

CD45 DEFICIENCY ALTERS THE STRUCTURE OF LYMPHOID COMPARTMENTS AND ANNEXIN V STAINING OF LYMPHOCYTES.

3.1 Introduction

Thymocyte development occurs within distinct microenvironments of the complex three-dimensional organization of the thymus. The thymus consists of an outer region known as the cortex and an inner region known as the medulla, each containing their respective specialized epithelial cells, in addition to macrophage and dendritic cells (Anderson et al. 1996). Thymic precursors seed the thymus on d11 of gestation (in mice) and enter near the cortex-medullary junction (Anderson et al. 2001). During progression through the DN stages, the DN thymocytes migrate outwards towards the outer cortex and subcapsular region (Takahama 2006). Development to the DP stage and positive and negative selection on cortical epithelial cells expressing MHC occurs, inducing migration back towards the corticomedullary junction (Witt et al. 2005; Ladi et al. 2006). Development to the SP stage allows migration into the medulla, where SP thymocytes are further subjected to negative selection processes, and mature into functional T cells (Ladi et al. 2006)

Localization of thymocytes within the thymus is controlled by localized expression of chemokines and the regulated expression of chemokine receptors on thymocytes at distinct stages of development. Outward movement of DN cells is regulated by the expression of CXCR4, CCR7, and CCR9 (Takahama 2006). Positive selection then signals the down regulation of CCR9 (Uehara et al. 2006) and upregulation

of CCR7 on DPs and SPs allowing their migration towards the medulla which predominately expresses the CCR7 ligands, CCL19 and CCL21 (Ueno et al. 2002; Kwan et al. 2004; Ueno et al. 2004). Emigration of mature SP thymocytes then occurs via expression of the sphingosine-1-phosphate receptor 1 ($S1P_1$) (Matloubian et al. 2004) which allows mature SP cells to enter the blood.

Lympho-stromal interactions are crucial for the development and proper organization of the thymus. Proper maturation of the cortex depends on lineage committed T cells to develop to the DN3 stage. Thymocytes unable to reach this stage, as demonstrated in human CD3ε Tg mice, do not form mature differentiated and structured cortical epithelial cells (Hollander et al. 1995). Maturation to the DN3 stage results in proper cortex development, but lack of a mature medullary compartment, as revealed in RAG deficient mice (Shores et al. 1991; Shores et al. 1994). Proper expression of the TCR complex and thymocyte development to the SP stage is required for the full maturation of the thymic medulla (Shores et al. 1994; van Ewijk et al. 2000). Crosstalk between developing thymocytes and the developing thymic stroma is key for proper development. For example the upregulation of lymphotoxin β receptor (LTβR) ligand(s) (Boehm et al. 2003) and keratinocyte growth factor (Erickson et al. 2002) on SP thymocytes, is required for full differentiation of thymic medullary epithelial cells.

Interactions between lymphoid cells and other stromal cells are important for structural organization of peripheral lymph node and spleen. The spleen is organized into white pulp regions, consisting of lymphocytes, and the red pulp region, which contains aged erythrocytes. Within the white pulp, lymphocytes are, for the most part, segregated into T cell zones consisting mainly of T cells and dendritic cells, and B cell zones, also

know as B cell follicles, which contain B cells and antigen trapping follicular dendritic cells. Localization of lymphocytes to their respective regions in the spleen is also regulated by localized chemokine production. During immune activation, lymphocytes differentially express different chemokine receptors allowing T cell interaction with B cells to mediate their further activation and germinal center formation (Mebius et al. 2005).

Macrophages are also important constituents of the thymus and spleen. The large percentage of dying thymocytes during development is very rapidly and effectively cleared by thymic resident macrophages (Surh et al. 1994). Externalization of phosphatidylserine residues on apoptotic cells is a well characterized signal for clearance by macrophages (Fadok et al. 1992). Interestingly, lectin interactions have also been demonstrated to promote phagocytosis. Galectin-1 and galectin-3, which have been shown to bind to CD45 have also been implicated in phagocytosis. Addition of dimeric galectin-1 to neutrophils resulted in externalization of phosphatidylserine and enhanced phagocytosis by macrophages (Dias-Baruffi et al. 2003; Karmakar et al. 2005). Galectin-3, which is produced by macrophages and found internally in phagosomes, was found to be important for phagocytosis as revealed by galectin-3 deficient macrophages which were impaired in phagocytosis of apoptotic thymocytes *in vitro* and experimentally *in vivo* (Sano et al. 2003). MBL has also been shown to bind to apoptotic thymocytes preferentially (Nauta et al. 2003; Stuart et al. 2005) and mice deficient for MBL also exhibit defective clearance of apoptotic cells (Stuart et al. 2005). However the cell surface molecules these lectins bind to mediate phagocytosis of apoptotic cells is yet to be defined.

As is shown in chapter 2, CD45-/- thymocytes exhibit increased annexin V binding and reduced thymocyte cellularity. CD45-/- mice also exhibit impaired maturation of B lymphocytes in the spleen (Byth et al. 1996; Fleming et al. 2004), and impaired NK cell function (Huntington et al. 2005; Hesslein et al. 2006). Recently the generation of CD45-/- deficient mice with a defect in the B cell compartment only demonstrated the importance of CD45 for B lymphocyte survival and for maintenance of germinal centers (Huntington et al. 2006). These data suggest that lectin interactions perhaps mediated through CD45 may be important for thymocyte survival and clearance within the thymus. Therefore, in this chapter, the role of CD45 in lymphocyte survival and clearance was examined in primary and secondary lymphoid tissues of CD45-/- mice.

3.2. Materials and Methods

Mice, antibodies and reagents

Details regarding the mice used are documented in Chapter 2 materials and methods. The following antibodies and reagents were obtained from BD Pharmingen: CD4-PECy5 (RM4-5), CD8β-FITC (53-5-8), thy-1.2-PE (53-2.1), CD19-PE (1D3), CD45-APC (30-F11), and MTS10. CD11b-PE (M1/70) and F4/80-PE (BM8) were purchased from eBioscience (San Diego, CA). Rabbit antiserum H2, specific for GIIβ, was previously described (Arendt et al. 1997). Biotinylated mannose binding lectin was previously described (Baldwin et al. 2001). Anti-calreticulin (SP-600) was purchased from Stressgen (Victoria, BC). FITC labeled donkey anti-rabbit-Ig and DTAF-streptavidin were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Thymocyte staining and flow cytometry

Freshly isolated thymocytes were washed with PBS and then stained at a concentration of $1x10^6$ cells with 10μg/ml of the indicated Ab, annexin V-FITC or with biotinylated mannose binding lectin in PBS containing 1% defined calf serum for 30 mins on ice. For uncoupled antibody staining, cells were then washed and stained with isotype specific secondary antibodies or streptavidin-DTAF at a dilution of 1:100 for 30 mins on ice. Cells were washed, fixed with 4% formaldehyde and analyzed by flow cytometry using a BD FACSCalibur (BD Biosciences, San Jose, CA). Both cell populations were visualized on the FSC/SSC plot and gated individually using the following approximate settings then fined tuned using single cell suspension controls: FSC voltage E(0), 1.00 amp gain, SSC 400(lin), FL1 500(log), FL2 450(log), FL3 500(log). If required during multi-color acquisition, compensation was set at: FL1 0.4% FL2, FL2 30-40% FL1, FL2 0.2% FL3, FL3 20-30% FL2. Data was collected and analyzed using Cell Quest Pro software (Santa Rose, CA).

DNA isolation and PCR

DNA was isolated from tail biopsies of F2 litters using a DNeasy Tissue Kit (Qiagen, Mississauga, ON). Standard PCR was then performed using HotStarTaq DNAPolymerase (Qiagen) and primers specific to exon 9 of CD45 (5'-GTA ATC AGA CTT TTA AGG CAG ACC TC-3' and 5'-GCT GGA GCA CAT GAG TCA TTA GAC AC-3'). The PCR was run for 35 cycles in a Mastercycler (Eppendorf, Mississauga, ON). PCR products were run out on a 1% agarose gel and examined for the appearance

of a small product (~150 bp) in Wt mice, a large product containing the neomycin cassette (~1500bp) in the CD45-/- mice, or both products in heterozygous mice.

Histology

For gross morphology examination, freshly isolated thymi and spleen were rinsed in PBS and then immediately fixed with Z-fix (Anatech Ltd., Battle Creek, MI), embedded in paraffin, sectioned and stained with hematoxylin and eosin. Frozen sections for immunofluorescence were generated by embedding freshly isolated thymus samples in Tissue Tek O.C.T. compound (Fischer Scientific), snap frozen in liquid nitrogen, and kept frozen at -80°C.

Immunofluorescent staining of frozen sections.

Frozen sections were fixed with acetone at -20°C for 3 minutes and then dried at room temperature. Slides were then blocked with 2% FCS for 30 mins, stained with primary antibody (MTS10, 1:25) for 1 hr, and then washed with blocking buffer (2% FCS). Secondary Ab was added (anti-mouse-FITC, 1:100) in blocking buffer and incubated for 30 mins. Slides were washed with PBS and mounted with VectaShield mounting media (Vector labs, Burlington, ON). Slides were then examined using Leitz Dialux 22 fluorescence (440-480nm) microscope (Leica Microsystems, Richmond Hill, ON) and Retiga 1300 mono 12 bit camera (QImaging, Burnaby, BC) and analyzed using OpenLab3 software (Improvision Inc, Lexington, MA).

Chemotaxis Assay

Thymocytes were put in the top well of a 5 μm polycarbonate membrane transwell dish (Costar) at a concentration of $5x10^6$ cells in 500 μl. The bottom chamber contained 500 μl of media alone, or media containing 250 ng/ml of chemokines CCL19 or CCL21 (PeproTech, Ottawa, ON). Thymocytes were incubated in the transwell for 3 hrs at 37°C and then were isolated from the top and bottom chambers separately, subjected to centrifugation, counted and then stained for CD4 and CD8β.

FTOC reconstitution experiments.

Day 14 fetal thymi were cultured for 5 days in the presence of 1.35 mM 2'-deoxyguanosine (Sigma) to deplete endogenous thymocytes. Depletion of thymocytes was confirmed by FACS analysis. Thymi were then extensively washed by multiple passing in fresh media prior to reconstitution. Thymocytes used for reconstitution were harvested from d14 fetal thymi and then added to Terasaki dishes (Fischer) at a ratio of 3 thymi equivalents of thymocytes per reconstitution of one depleted thymus. Reconstituted thymi were left overnight using the hanging drop method by inverting the Terasaki dish, and then placed onto membranes and cultured in FTOC conditions (Chapter 2 Materials and Methods) for 8 days.

Phagocytosis Assay

Macrophages were derived from bone marrow (BM) isolated from the hind femurs and tibia of C57BL/6 or CD45-/- mice (refer to Chapter 2 material and methods regarding mice). BM was cultured at $2x10^5$ cells/ml in 10% FCS complete RPMI

supplemented with 5% GM-CSF supernatant derived from transfected CHO cells for 6 days. Macrophages were then removed from tissue culture plates using cold versene (1:5000, Gibco) and gentle scraping, washed and placed in 5 ml tubes at $1x10^6$ macrophages per tube. Thymocytes were derived from Wt or CD45-/- litter mates, stained with 0.2 μg/ml thy-1.2-PE, and then incubated with or without 10 ug/ml of recombinant protein A/G (Pierce). Thymocytes were then added ($2x10^6$) to the macrophages on ice and then left on ice or put at 37°C for indicated time points. At the experimental end point, tubes were vortexed for 10 seconds and then fixed immediately with 4% formaldehyde. Alternatively samples were treated with 5 μM EDTA for 5 min, vortexed, and then fixed. Macrophages were then analyzed by flow cytometry and gated based on forward and side scatter properties and green autofluorescence.

3.3 Results

Adult CD45 deficient thymocytes exhibit increased annexin V staining

Annexin V staining is found to be increased upon CD45-/- fetal thymocytes, as shown in the previous chapter; therefore, annexin V staining was examined in CD45 deficient adult mice. In order to accurately compare genetic and aged matched mice, the CD45-/- mice were crossed with C57BL/6 mice to generate heterozygous progeny. These CD45+/- mice were then crossed to produce a mixed F2 generation. F2 littermates of 8-10 wks of age were first genotyped by PCR using primers specific to the exon 9 of CD45 that flanked the region of insertion of the neomycin cassette in the CD45-/- mice (Appendix A5), and CD45 protein expression was confirmed by FACS analysis (Fig. 3-1C). Thymi were then extracted and thymocytes assessed for CD4 and CD8β staining

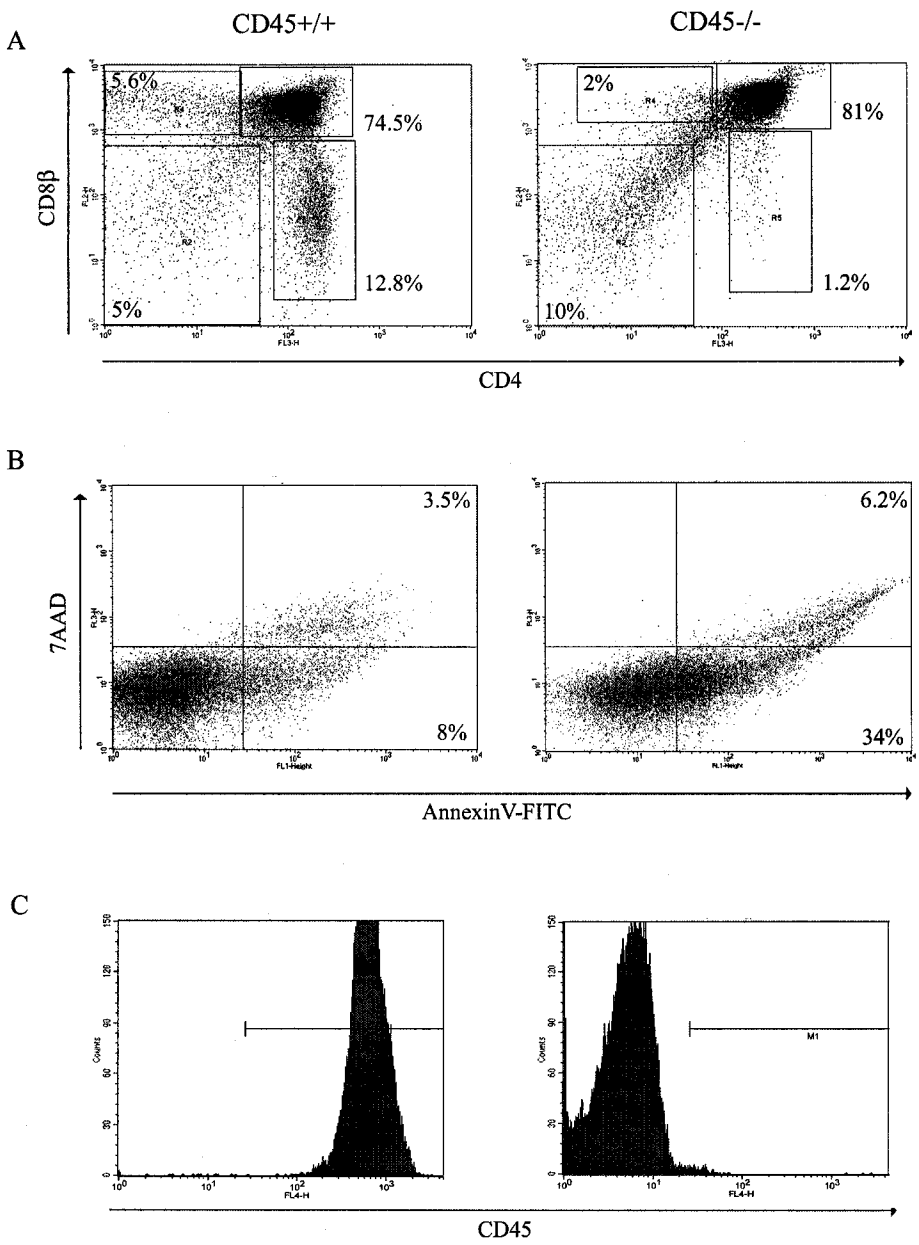


FIGURE 3-1. *Adult CD45-/- thymocytes exhibit higher annexin V staining compared to their Wt littermates.* Thymocytes were harvested from CD45+/+ or CD45-/- litter mates and then stained with A) CD4 and CD8β or B) annexin V and 7AAD and analyzed by flow cytometry. C) CD45 expression on thymocytes from CD45 +/+ or CD45-/- littermates by staining with 30-F11 Ab.

(Fig. 3-1A), and annexin V and 7AAD staining (Fig. 3-1B). Thymocyte ratios were as expected for Wt and CD45-/- mice; the CD45-/- mice exhibited an increase in the percentage of DN thymocytes and a dramatic loss of the SP population. From Fig. 3-1B it is clear that thymocytes derived from CD45-/- mice exhibit increased annexin V binding (34% AnV+/7AAD-) compared to their Wt littermates (8% AnV+/7AAD-). Thus even in adult mice, there is an accumulation of annexin V positive thymocytes within the thymus.

CD45-/- thymi show disrupted thymic morphology

To examine if this increased population of annexin V^+ cells was affecting the normal morphology of the thymus, thymi from F2 littermates were fixed, embedded in paraffin and cross sections cut and stained with hematoxylin and eosin (H&E) to examine basic structural organization. The darkly stained outer cortex region was easily distinguishable from the lighter stained inner medullary regions in thymi from CD45+/+ and CD45+/- mice (Fig. 3-2). In striking contrast thymi from the CD45-/- littermates exhibited a more uniform dark staining, indicating that thymocyte dense regions extended throughout the thymus with no identifiable border between cortex and medullary regions (Fig. 3-2).

Since it appeared that there was a lack of distinguishable medullary regions in CD45-/- thymi, thymic sections were examined for the presence of properly differentiated medullary epithelial cells. Frozen thymic sections derived from CD45+/+ and CD45-/- littermates were stained for the medullary epithelial cell marker using the MTS10 antibody. As expected Wt thymi exhibited medullary epithelial cell staining near the

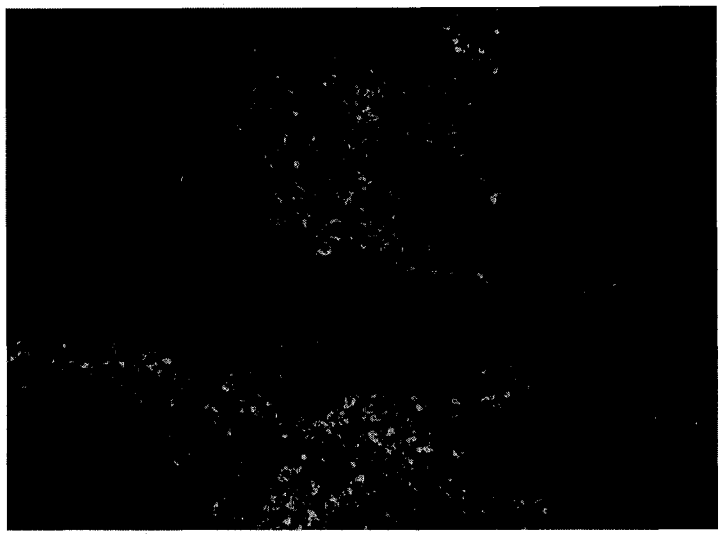
FIGURE 3-2. *Thymic architecture is disrupted in CD45-/- mice.* A) Histological sections of thymi isolated from 8 week old CD45+/+ (left), CD45+/- (middle), or CD45-/- (right) littermates, stained with hematoxylin & eosin. (25X magnification)

central regions of the section, and staining appeared in large clusters of localized cells (Fig 3-3A). In CD45-/- thymi, less positive stained medullary epithelial cells were present and they appeared more scattered and did not form the large clusters of positively stained cells as seen in Wt thymi (Fig. 3-3B). Thus there is an obvious defect in thymic organization and structure in the CD45-/- mice.

CD45-/- thymocytes do not exhibit increased response to CCR7 ligands.

The CCR7 ligand, CCL19 has been shown to be important for directing developing thymocytes into the thymic medulla, where it is expressed (Ueno et al. 2002). In mice made transgenic to overexpress CCR7, these thymocytes prematurely migrated and overwhelmed the thymic medulla. Thymic histology of thymic sections from these mice revealed markedly similar dark H&E staining throughout the thymic section (Kwan et al. 2004). Given the similarities in phenotype, CD45-/- thymocytes were examined for potential premature migration into the medulla and hence accounting for the lack of a distinguishable lightly H&E stained medulla in these mice. Thymocytes from Wt and CD45-/- littermates were assessed for their ability to migrate to CCR7 ligands in a transwell migration assay (Fig. 3-4). A significant percentage of CD4 and CD8 SP thymocytes of Wt mice migrated to CCL19, and to a lower extent to CCL21, compared to media alone. Of the CD45-/- thymocytes, only the CD4 SP population and not the CD8 SP thymocytes migrated in response to the CCR7 ligands; albeit to a lower extent than Wt thymocytes. Thus, CD45-/- cells were not hyper-responsive to CCR7 ligands, but were in fact hypo-responsive, indicating that they were not prematurely chemotaxing into the thymic medulla.

+/+



-/-

FIGURE 3-3. Thymic *medullary epithelial cells do not form large clusters in CD45-/- mice.* Thymic frozen sections generated from CD45 +/+ (upper) or CD45-/- (lower) thymi were stained with the medullary epithelial marker MTS10 Ab and anti-mouse-Ig-FITC and visualized by fluorescent microscopy (100X magnification).

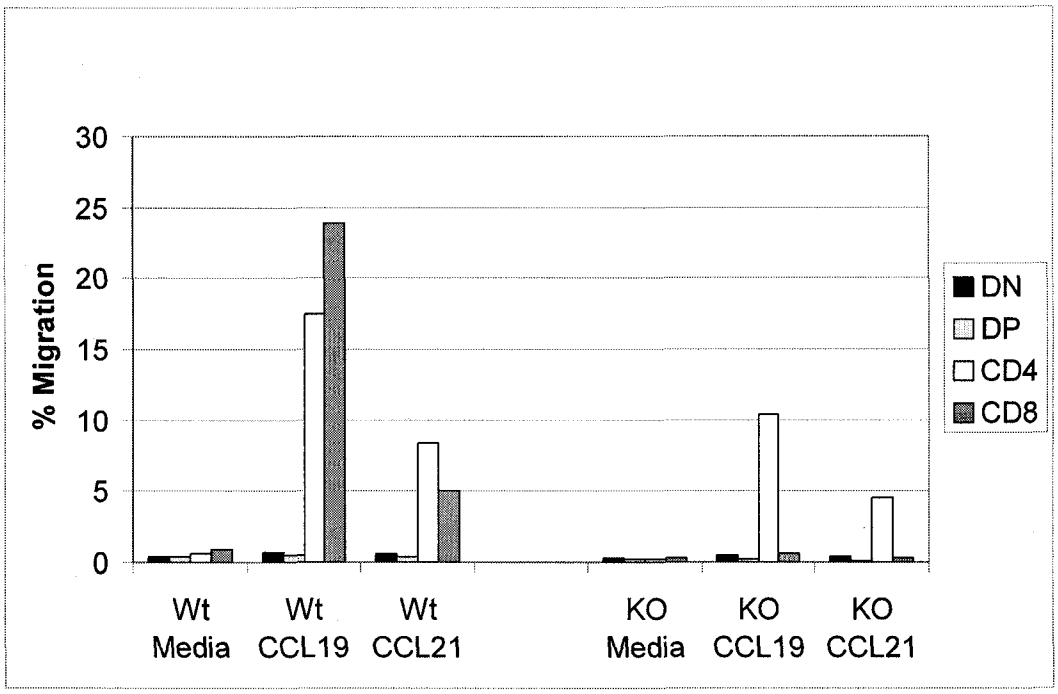


FIGURE 3-4. *CD45-/- thymocytes do not exhibit increased response to CCR7 ligands.* Wt (CD45+/+) or KO (CD45-/-) thymocytes were placed in the upper chamber of transwell plates and assessed for their ability migrate to the bottom chamber containing either media alone, 250 ng/ml of CCL19 or CCL21 for 3 hrs at 37°C. Thymocytes were then harvested from both the upper and lower chambers, counted, and then stained for CD4 and CD8β. The percentage of thymocytes that migrated was determined by calculating the number of thymocytes at each developmental stage in the bottom chamber divided by the total amount of thymocytes put into the top chamber.

Thymocyte development of CD45-/- thymocytes is not rescued by development in a Wt environment.

In order to examine the contribution of CD45 expression on thymocytes compared to its expression on the surrounding thymic environment, FTOC reconstitution experiments were performed. Thymocytes from Wt or CD45-/- deficient d14 fetal thymi were used to reconstitute Wt or CD45-/- fetal thymi depleted of thymocytes by deoxyguanosine treatment. The percentage of thymocytes at each stage of development after reconstitution and 8 days of culture is depicted in Figure 3-5A. Wt thymocytes exhibited similar thymocyte ratios (Fig. 3-5A) and annexin V staining (Fig. 3-5B) when developing in Wt or CD45-/- thymi. This was found to be similar to development in unmanipulated Wt FTOC (Chapter 2: Fig. 2-3). CD45-/- thymocyte reconstitution of Wt thymi resulted in thymocyte development that remained impaired (Fig. 3-5A). The percentage of CD45-/- annexin V+ thymocytes was also found to be increased compared to Wt thymocytes, when used to reconstitute either, Wt or CD45-/- thymi (Fig. 3-5B). These data demonstrate that the developmental defects and the increased annexin V binding is intrinsic to the CD45 deficient thymocytes and is not a result of a CD45 deficient thymic environment.

CD45-/- mice also exhibit abnormal spleen morphology.

Upon dissection of CD45-/- mice it was observed that they exhibited a much larger spleen than that of their Wt counterparts (Fig. 3-6A). Wt spleens were smaller and pinker in color compared to CD45-/- spleens, which were darker red/purple, much larger, and much tougher in texture. Histological investigation revealed abnormal H&E staining

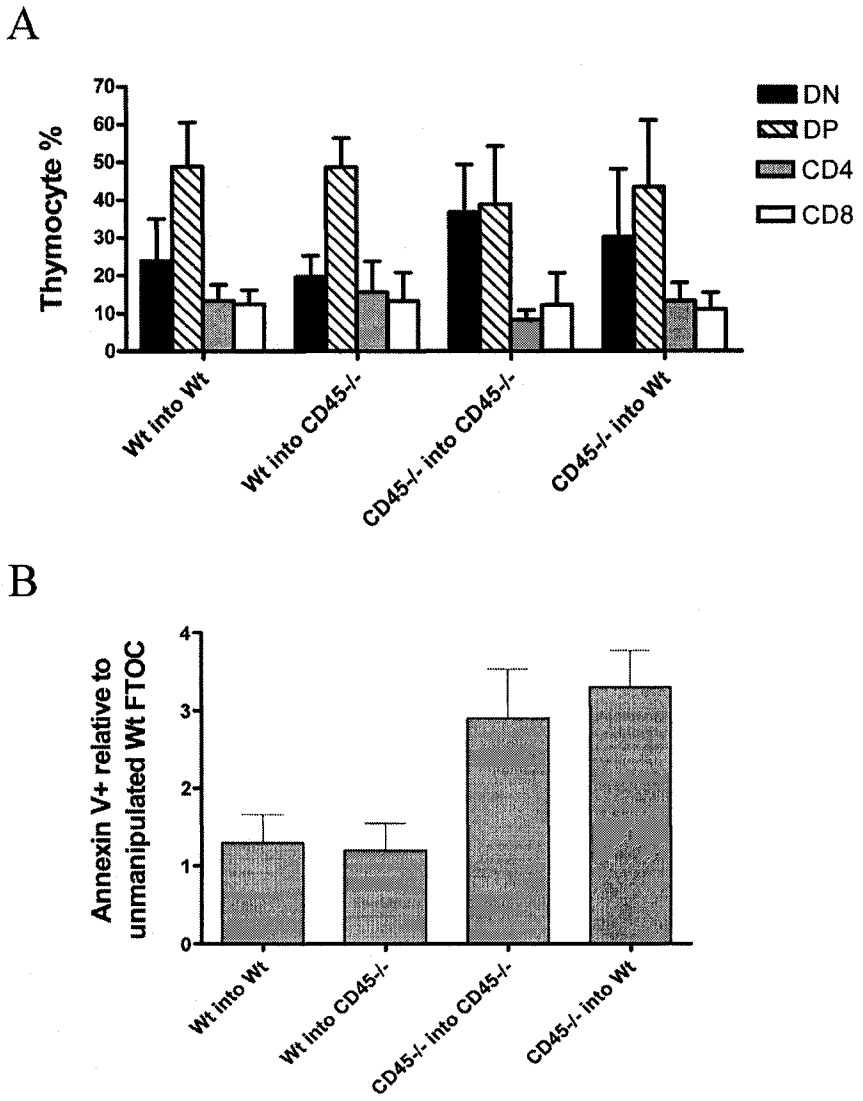


FIGURE 3-5. *CD45-/- deficient thymocyte development is not rescued by reconstitution of wt thymi.* Thymi from Wt or CD45-/- d14 fetal mice were depleted of thymocytes by deoxyguanosine treatment for 6 days and then reconstituted with Wt or CD45-/- thymocytes. Reconstituted thymi were cultured for 8 days and then thymocytes were harvested and stained for CD4 and CD8β, or annexin V. A) The percentage of thymocytes at each developmental stage is shown per reconstituted condition. B) The percentage of annexin V+ thymocytes in reconstituted thymi is shown relative to the percentage of annexin V+ thymocytes in unmanipulated (non-depleted) Wt FTOC-8 cultures. Error bars represent S.E.M.

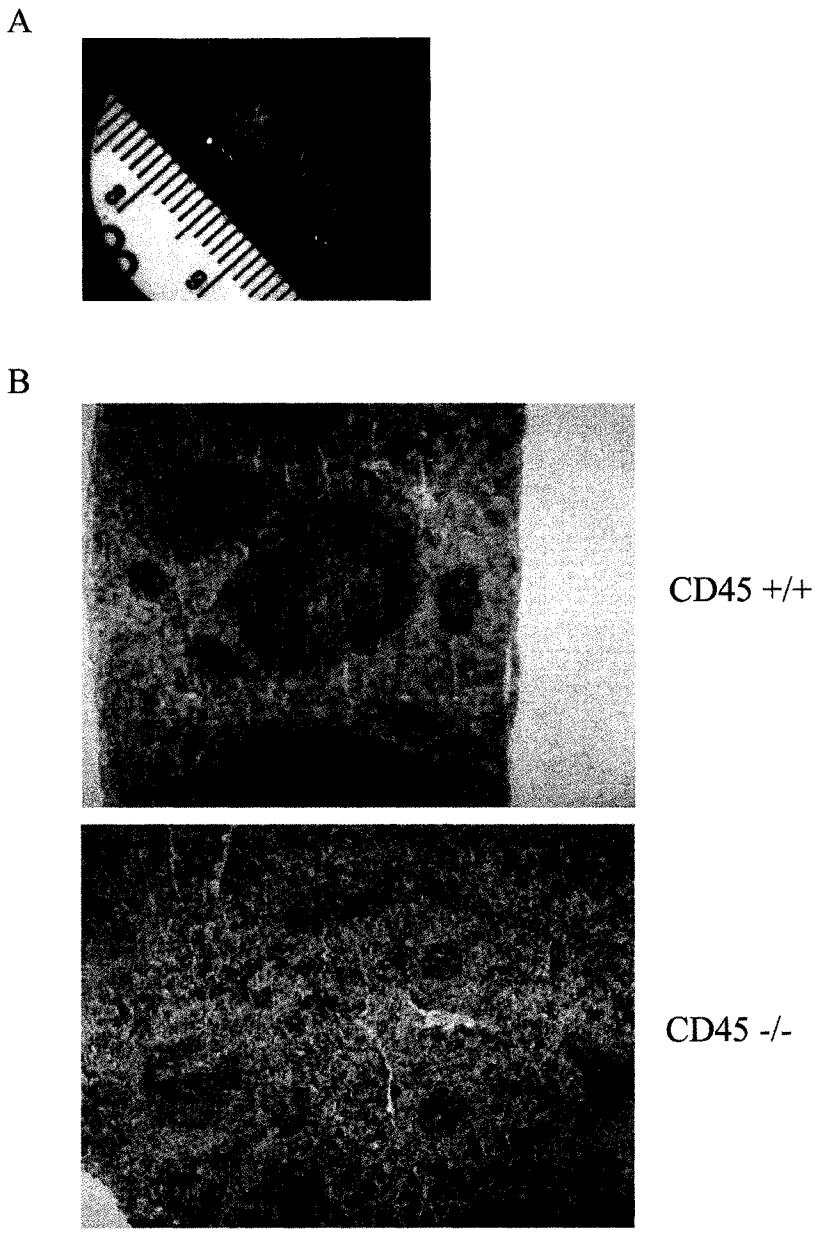


FIGURE 3-6. *CD45-/- mice exhibit abnormal spleen morphology.* A) Spleen were isolated from Wt (right) or CD45-/- (left) littermates and then compared for gross morphology under a dissecting microscope. B) Longitudinal sections of spleen isolated from Wt (top) or CD45 -/- (bottom) mice were stained with H&E and examined under light microscope (25X magnification).

in longitudinal sections of spleen from the CD45-/- mice (Fig. 3-6B). The lymphocyte dense white pulp regions were not as well formed and were found to be much smaller and scattered throughout the spleen of CD45-/- animals. Thus, CD45 deficiency affects the morphology of both thymus and spleen.

Lymphocytes in the periphery of CD45-/- mice also exhibit higher annexin V staining compared to their Wt littermates.

Due to the increased annexin V staining of CD45-/- thymocytes, and to the morphological differences in CD45-/- spleen, annexin V staining of lymphocytes in the peripheral lymphoid organs was examined. Freshly isolated splenocytes from Wt and CD45-/- littermates were stained with annexin V and T (TCRβ) or B (CD19) cell markers. The small percentage of T cells within the CD45-/- spleen were found to be highly annexin V positive (Fig. 3-7A). B lymphocytes from CD45-/- mice also exhibited much higher annexin V staining compared to Wt B cells (Fig. 3-7B).

Total lymphocytes isolated from the lymph nodes of CD45-/- littermates exhibited dramatically increased annexin V staining compared to their Wt littermates, with only a small percentage of healthy cells remaining (Fig. 3-8A). T lymphocytes found within the lymph nodes of CD45-/- also exhibited higher percentage of annexin V staining (Fig. 3-8B). The majority of the lymphocytes were B cells and they were also highly annexin V positive (Fig. 3-8B). Thus, in the periphery of CD45-/- mice, the lymphoid compartments also exhibit an increased population of annexin V positive lymphocytes.

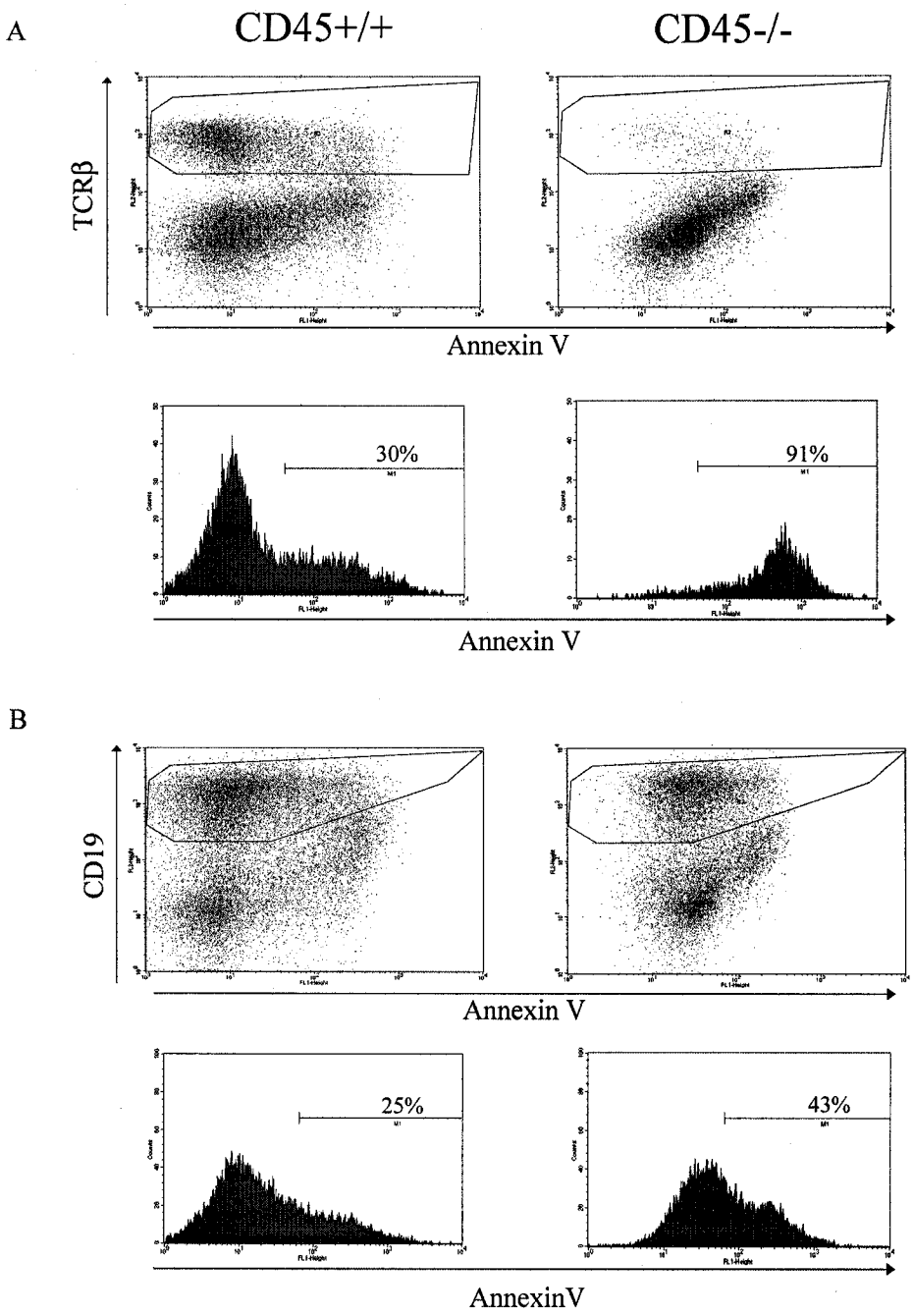


FIGURE 3-7. *Splenocytes from CD45-/- mice also exhibit higher annexin V staining compared to their Wt littermates.* Splenocytes from Wt or CD45-/- mice were harvested from spleen and profiles of TCRβ (A) or CD19 (B) in conjunction with annexinV are shown in the upper panels. Histograms (bottom) show annexin V staining of TCRβ positive gated splenocytes (A), or CD19 positive gated splenocytes (B).

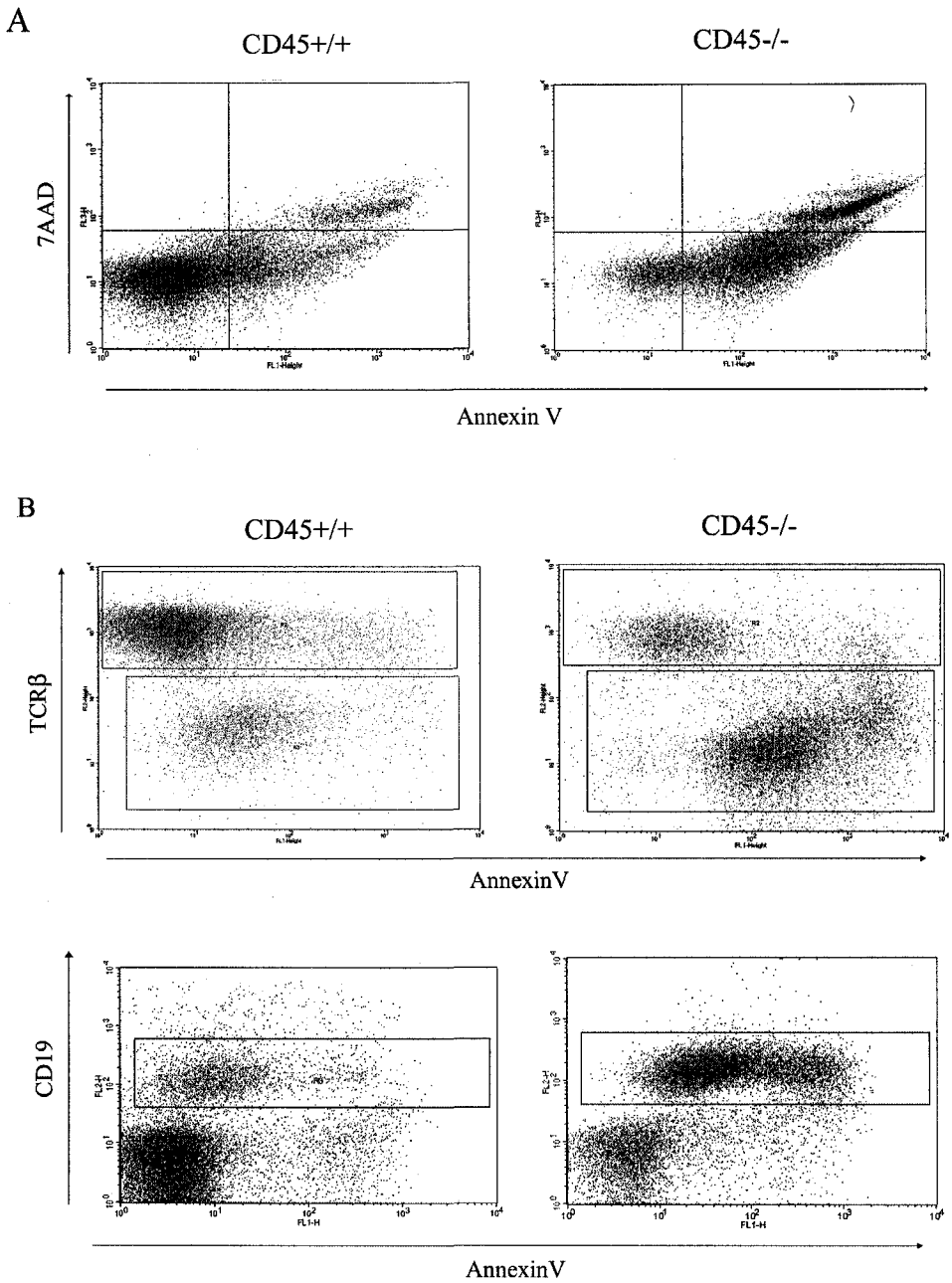


FIGURE 3-8. *Lymph node cells exhibit very high annexin V staining in CD45-/- mice.* Lymphocytes were isolated from the auxillary, inguinal, and popliteal lymph nodes of Wt (left panels) or CD45-/- (right panels) littermates. A) Total LN cells were stained with annexin V and 7AAD. B) Flow cytometric profiles of lymph node cells stained with annexin V and TCRβ (upper) or CD19 (lower).

Phagocytic clearance of apoptotic cells is not impaired in CD45-/- mice

Cell surface externalization of phosphatidylserine is thought to be a marker for clearance by phagocytes (Fadok et al. 1992). As CD45-/- mice exhibit increased percentages of annexin V positive cells in both the primary and secondary lymphoid organs, it was hypothesized that these cells were not being properly phagocytosed and that perhaps CD45 was involved in the clearance of apoptotic lymphocytes. As CD45 binds to many molecules through lectin interactions, the binding of lectins known to bind CD45 and that may be involved in phagocytosis, were examined. Thymocytes isolated from Wt and CD45-/- littermates were compared for their ability to bind mannose binding lectin (MBL), which has been shown previously in our lab (Baldwin et al. 2001) to bind to CD45 on DP thymocytes, and is also is known to promote phagocytosis by macrophages (Ogden et al. 2001; Stuart et al. 2005). DP thymocytes from Wt and CD45-/- mice were found to bind MBL equally, as examined by FACS analysis (Fig. 3-9A). GIIβ, an ER protein which has also has been shown previously by our lab to bind to CD45 on the cell surface (Baldwin et al. 2001) was also not found to differ in its expression on the cell surface of Wt and CD45-/- thymocytes (Fig. 3-9B). Another ER protein, calreticulin has been shown to promote phagocytosis *in trans* through its cell surface expression on apoptotic cells (Gardai et al. 2005). Although it has not been shown to interact with CD45 on the cell surface, it does interact with proteins through lectin interactions; therefore, its cell surface expression was also examined. Calreticulin cell surface expression also did not differ between Wt and CD45-/- thymocytes (Fig. 3-9C). These data show that the overall cell surface binding of lectins to thymocytes is not affected by CD45 deficiency.

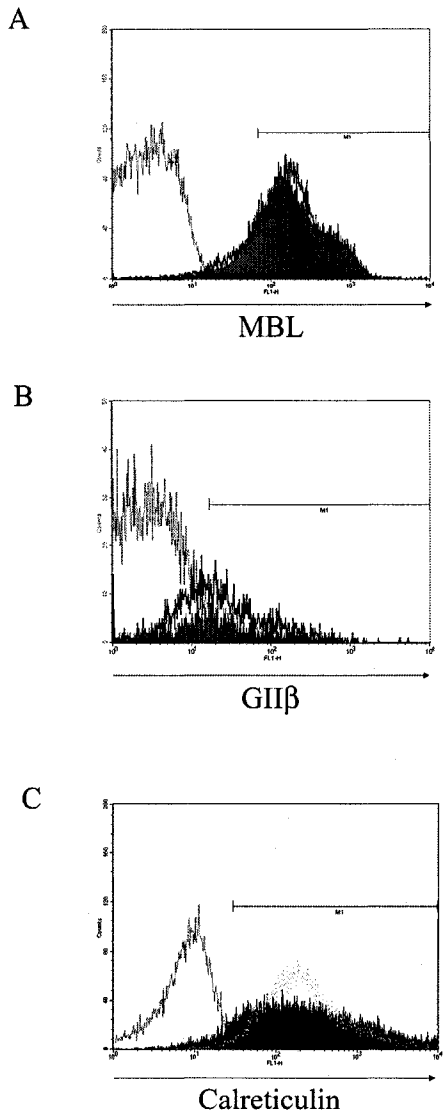


FIGURE 3-9. *Wt and CD45-/- thymocytes show similar staining patterns of lectins involved in phagocytosis.* Thymocytes from Wt (filled histogram) or CD45-/- (dark black lined histogram) littermates were stained for CD4 and CD8β and with antibodies to lectins. Histogram plots are shown of thymocytes stained with 10ug/ml of anti-GIIβ in gated DN thymocytes (top), biotinylated mannose binding lectin on gated DP thymocytes (middle), or anti-calreticulin on gated DP thymocytes (bottom). Unstained samples are shown in light grey lines.

As there are many redundant pathways and receptors involved in phagocytosis (Savill et al. 2002), the phagocytosis of thymocytes isolated from Wt and CD45-/- mice by bone marrow derived macrophages was investigated directly. Bone marrow derived macrophages were generated from both Wt and CD45-/- mice to examine the additional possibility of an intrinsic defect in macrophage function in CD45-/- mice. Macrophages generated from bone marrow of Wt and CD45-/- mice, appeared to differentiate similarly and expressed similar levels of the macrophage markers CD11b (Fig. 3-10A) and F4/80 (Fig. 3-10B). To examine the phagocytic ability of these macrophages, thymocytes were isolated from Wt and CD45-/- mice, stained with fluorescent Thy1.2.-PE, and combined with the macrophages for various lengths of time. The macrophages were then examined by flow cytometry for the uptake of PE fluorescence. The percentage of Wt macrophages (Fig. 3-10C, circles) that phagocytosed PE labeled Wt thymocytes was similar to that of macrophages that phagocytosed CD45-/- thymocytes. In fact, CD45-/- thymocytes were phagocytosed to a slightly greater extent at later time points. Macrophages derived from CD45-/- bone marrow (Fig. 3-10C, squares), were found to be more phagocytic compared to their Wt counterparts, as assessed by the increased PE positive macrophages at all time points observed. Treatment of samples with EDTA at the experimental end point to remove adhered thymocytes, resulted in similar percentages of positive stained macrophages to untreated samples (Appendix A6). Prior blocking of the Fc portion of the Thy1 antibody with protein A/G did not block phagocytosis, suggesting that the phagocytosis observed was not FcR mediated (Appendix A7).

Thus, it appears that phagocytosis of apoptotic thymocytes is intact, and somewhat enhanced in the CD45-/- mice.

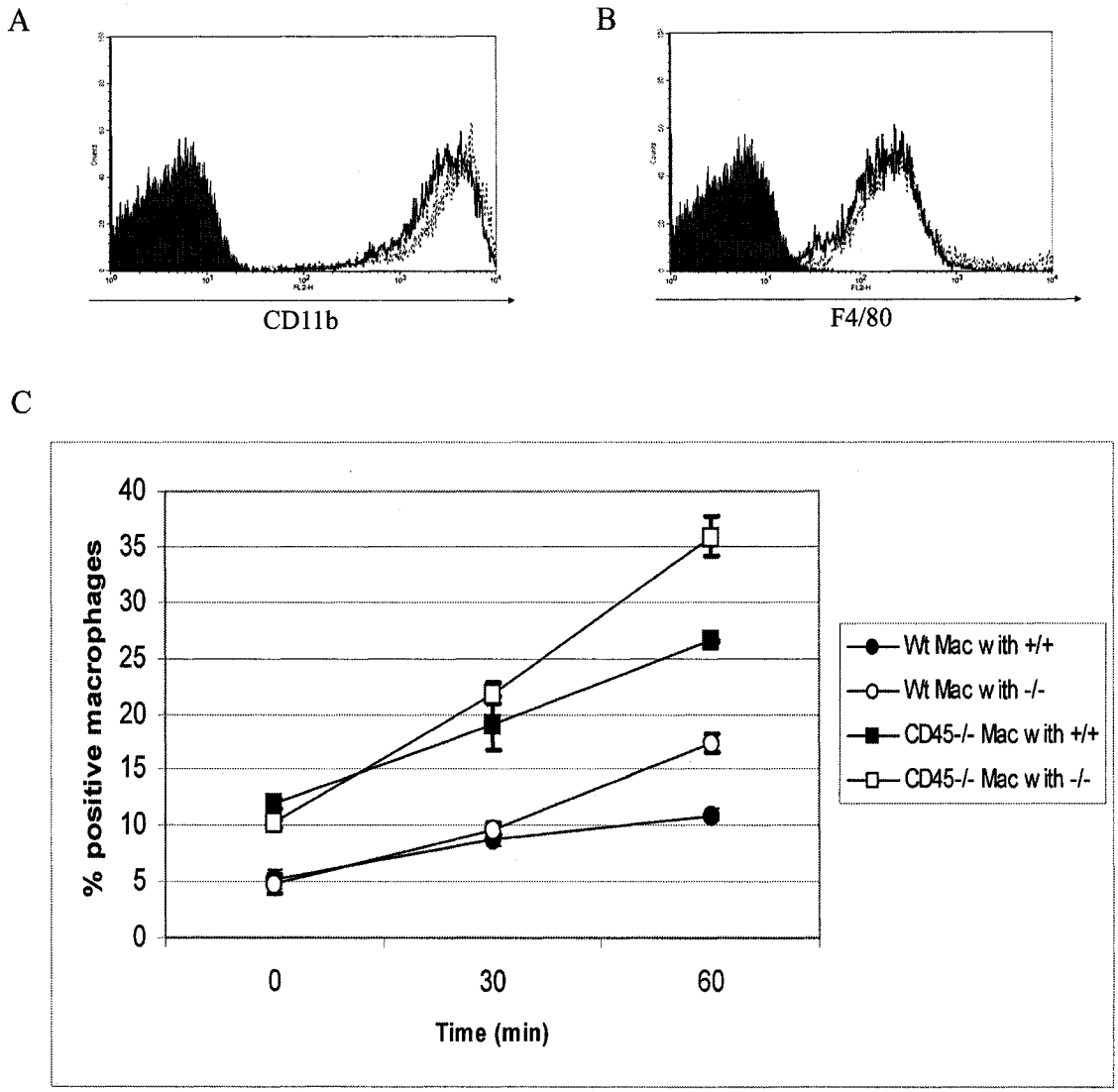


FIGURE 3-10. *Phagocytosis of thymocyte is not impaired in CD45-/- mice.* A) Histogram profiles of BM derived macrophages from Wt (dark line) or CD45-/- mice (light dashed line) stained with anti-CD11b or F4/80, or left unstained (filled). B) Phagocytosis of Thy-1-PE stained thymocytes from Wt or CD45-/- mice incubated at 37°C for 0, 30, and 60 min, vortexed, and then fixed and analyzed by flow cytometry.

3.4 Discussion

These studies demonstrate that lymphocytes within CD45-/- mice exhibit increased phosphatidylserine externalization compared to their Wt littermates, as assessed by annexin V binding. Histological investigation of the thymus and spleen of CD45-/- mice revealed disrupted morphology. Thymocytes were not localized to discernable cortex and medullary areas in the thymus of CD45-/- mice, but rather were distributed throughout the thymus. Medullary thymic epithelial cells in the CD45-/- mice were not found to be organized into large clusters as compared to that seen in Wt thymi. Spleens of CD45-/- mice were also enlarged and exhibited disrupted organization; the white pulp regions were not as highly formed and organized compared to Wt littermates. Despite an observed disruption in the lymphoid compartments and the appearance of annexin V positive lymphocyte accumulation in the CD45-/- mice, phagocytic clearance of these cells was not impaired in *in vitro* phagocytosis assays.

The morphological differences observed in the thymus and spleen are most likely the consequence of impaired development of lymphocytes in CD45-/- mice that results in the increased annexin V+ binding observed, and not caused by an accumulation of annexin V+ thymocytes due to defective clearance. In CD45-/- mice, thymocyte development is impaired as demonstrated in chapter 2, and published by others (Kishihara et al. 1993; Byth et al. 1996; Mee et al. 1999), hence the defective generation of SP thymocytes in these mice might simply account for the underdeveloped medullary epithelium, since thymocyte interactions are required for proper thymic structural development as seen in RAG-/- mice (Shores et al. 1991; Shores et al. 1994; van Ewijk et al. 2000). Therefore, the H&E staining observed in CD45-/- mice might better be

explained by a detainment and accumulation of thymocytes in the cortex due to their lack of positive selection, thereby not enabling thymocyte migration into the medulla.

While the expression of CCR7 on thymocytes was not assessed due to the unavailability of a specific antibody for the receptor, the notion of a lack of thymocyte migration into the medulla was supported by the chemotaxis data. CD45-/- thymocytes exhibited decreased responsiveness to CCR7 ligands and hence one would expect decreased movement into the medullary region. Interestingly, it was found that only the CD4 SP, CD45-/- thymocytes exhibited notable chemotaxis towards the CCR7 ligands; although, to a lesser degree than Wt thymocytes. The lack of responsiveness of CD45-/- CD8 SP thymocytes is unknown, but suggests that the CD8 SP population in the CD45-/- mice may be more functionally immature. Localization of CD4+ and CD8+ thymocytes within the thymic sections was not successfully performed; however, it would have been interesting to note the thymic localizations of these different populations in comparison to each other, and to Wt thymocyte localization. Because of the effects of CD45 on thymocyte development, impaired chemotaxis may be the result of reduced CCR7 receptor expression. CD45 has also been documented to positively influence chemokine responsiveness (Mitchell et al. 1999; Fernandis et al. 2003; Okabe et al. 2006), thus impaired chemotaxis signaling may also account for the observed reduced CD45-/- thymocyte chemotaxis.

This study also demonstrated that thymocyte developmental defects associated with CD45 deficiency are due to its lack of expression on the thymocytes themselves, and not due defects of the thymic environment. Development of Wt thymocytes was not found to be impaired when allowed to develop in a CD45 deficient environment. Also

CD45-/- thymocyte reconstitution of Wt or CD45-/- thymi resulted in an equally impaired developmental phenotype and increased annexin V staining, suggesting that a CD45 sufficient environment cannot rescue the developmental defect of CD45-/- thymocytes. These data indicate that the effects of CD45 deficiency on thymocyte development and the accumulation of annexin V positive thymocytes is not the result of defective thymic stroma. These data also suggest that the effects of I3/2 treatment in FTOC shown in Chapter 2 are in fact due to direct binding of the antibody on the thymocytes and not due to effects on other cell types.

The observed defects in the periphery of CD45-/- mice are most likely a combination of developmental defects of both T and B lymphocyte populations (Byth et al. 1996; Mee et al. 1999; Huntington et al. 2006). The lack of mature T cells generated from the thymus would result in defective lymphocyte interactions in the spleen and lymph nodes. The lack of T cell help would exacerbate the already developmentally compromised B cells from proliferating and surviving hence attributing to the abnormal and small white pulp regions of the spleen and the increased annexin V staining in both spleen and lymph node.

The observed increased percentage of cells exhibiting annexin V staining on CD45-/- lymphocytes in both primary and secondary lymphoid compartments was intriguing since resident macrophages and other phagocytes are highly efficient at removing apoptotic cells expressing phosphatidylserine (Fadok et al. 1992; Surh et al. 1994). Therefore the possibility of defective clearance of thymocytes in the CD45-/- mice was considered since that could account for the increased percentage of annexin V positive lymphocytes observed both in the thymus and peripheral lymphoid organs. The

possibility that CD45 might be involved in phagocytosis was further supported by findings in the literature showing the importance of lectins in phagocytosis, which are known to interact with CD45 (Dias-Baruffi et al. 2003; Sano et al. 2003; Karmakar et al. 2005). However, cell surface staining of Wt and CD45-/- thymocytes revealed no difference in lectin binding. Although these lectins have other binding partners on the cell surface of thymocytes, it does not preclude the importance of the interactions of these lectins with CD45, which is yet to be determined.

To directly analyze the possibility that CD45 was required on thymocytes for their efficient phagocytosis by macrophages, *in vitro* phagocytosis assays were performed; however, CD45-/- thymocytes were found to be phagocytosed as efficiently if not more so than Wt thymocytes. The slightly enhanced phagocytosis of CD45-/- thymocytes correlates with their increased annexin V staining, and suggests that the externalized phosphatidylserine on these thymocytes promotes phagocytosis. The alternative possibility that macrophages in CD45-/- mice were impaired in phagocytosis ability was also examined; although the thymic reconstitution experiments suggested that this was not the case. It was found that macrophages derived from CD45-/- bone marrow actually exhibited greater phagocytic capacity than Wt derived macrophages. Others have also reported an inverse correlation of CD45 expression and phagocytic capability (Bohm 2004). The difference in phagocytic ability was not investigated further, but may be due to a lack of negative regulation of src kinases by a loss of CD45 phosphatase activity, since src kinases are involved in mediating phagocytosis (Berton et al. 2005).

Thus, a high percentage of annexin V+ lymphocytes are present within the primary and secondary lymphoid organs of CD45-/- mice and this is not due to defective clearance of apoptotic cells by phagocytosis. Rather, this work suggests a lymphocyte intrinsic role for CD45 in mediating survival during development and also in the periphery for maintaining healthy lymphocyte populations.

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CHAPTER 4: FINAL DISCUSSION

4.1 Thesis Summary

CD45 is a crucial molecule for proper thymocyte development. These studies demonstrate a role for the external domain in regulating thymocyte development at specific stages, and implicate a role for CD45 in mediating thymocyte survival. Ab manipulation of the external domain of CD45 impaired thymocyte development by causing a loss of thymocytes, specifically the DP and CD4+ SP stages, which correlated with phosphatidylserine externalization, as determined by annexin V staining. Manipulation of the external domain of CD45 resulted in distinct developmental consequences compared to the lack of the whole molecule, as shown using CD45-/- FTOC. CD45 deficiency resulted in dramatically reduced thymocyte cellularity and increased annexin V staining at all stages of development, and also impaired selection based on upregulation of selection markers CD5 and CD69. Manipulation of the external domain of CD45, however, only resulted in a loss of thymocytes specifically at the DP and CD4+ SP stages with no apparent effect on the upregulation of selection markers. Therefore, these data suggest that the external domain has a distinct function in regulating thymocyte survival during specific stages of development, whereas, the entire molecule is required for mediating thymocyte selection and survival at all stages.

The lack of positive selection of thymocytes and progression to the SP stage in CD45-/- mice, resulted in disrupted thymic morphology. The thymic environment, however, was not the cause of the annexin V+ thymocytes observed in CD45-/- FTOC and adult mice, because reconstitution of CD45-/- thymocytes into wildtype thymi did

not rescue their development or annexin V+ phenotype. The few peripheral T cells present, as well as B cells in the lymph node and spleen, also exhibited annexin V+ staining. The presence of increased annexin V+ lymphocytes however, was determined not to be due to defective phagocytosis of these cells based on *in vitro* phagocytosis assays. Collectively these studies demonstrate a lymphocyte intrinsic role for CD45 in maintaining thymocyte survival and healthy lymphocyte populations.

4.2 CD45 External Interactions

Although a specific ligand to CD45 has not been identified, many different molecules have been shown to interact with CD45, including CD22, galectin-1, GII, and MBL (Baum et al. 1995; Sgroi et al. 1995; Uemura et al. 1996; Baldwin et al. 2001). Notably, all do so through carbohydrate-lectin interactions. The most well characterized interaction is with galectin-1, a lectin expressed in lymphoid tissues including the thymus, which has been found to bind a number of T cell surface glycoproteins including CD45, CD44, CD7, and CD2 (Perillo et al. 1995). Treatment of thymocytes with galectin-1 results in apoptosis, which is regulated by CD45 (Perillo et al. 1995; Nguyen et al. 2001). Death mediated through galectin-1 also requires the regulation of glycosyltransferase expression (Nguyen et al. 2001) that occurs at the DP stage of development and therefore, DP thymocytes are most sensitive to galectin-1 mediated death. Upon galectin-1 engagement CD45 is relocalized along with CD3 away from other molecules such as CD44 and CD7 into distinct punctate cell surface microdomains (Pace et al. 1999; Nguyen et al. 2001). Therefore, the apoptosis induced by galectin-1 appears to require segregation of CD45 away from other signaling molecules which then

allows a death signal to proceed. This suggests the possibility that the expression of certain carbohydrate ligands on CD45 during development may cause thymocytes to become susceptible at the DP stage to mAb-mediated death *in vivo*. This is highly plausible as it was observed in these studies that the mAb mediated effects occurred in a stage specific manner; CD45 mAb treatment only affected the later stages of development, even though CD45 specific antibodies were added upon initiation of the cultures. The antibodies remained bound to the cell surface of all thymocytes throughout the culture period, but only mediated specific loss of the DP and CD4+ SP populations. The exact mechanism behind this stage specific depletion is unknown, but it is likely that different signals and genes upregulated at the DP and CD4+ SP stages may be regulating the susceptibility of the thymocytes to engagement of CD45, which may modulate survival or death signals *in vivo*.

The data presented in this thesis demonstrate that the external domain of CD45 can regulate thymocyte survival and development *in vivo*, in a developmental stage specific manner. CD45 may be engaging ligands, such as galectin-1, in the thymus leading to re-localization of cell surface CD45, which can then allow for death signaling to occur. In mAb-treated FTOC, the mAb may cause premature death of the thymocytes perhaps by mimicking galectin-1 binding and forcing re-localization of CD45 on the cell surface. Alternatively the antibodies could be disrupting survival signals mediated through CD45 that are selectively required by DP and CD4 SP cells, thereby blocking survival at these certain stages of thymocyte development. CD45 mAb treatment in culture has also been shown to uncouple signals through the TCR upon TCR engagement (Shivnan et al. 1996) and perhaps this is also occurring in our FTOC cultures resulting in

premature death instead of survival signals, however, this remains to be tested. I showed that engagement of the external domain of CD45 modulates thymocyte development by affecting the survival of developing thymocytes at specific stages of development. This work suggests a role for the external domain of CD45 in regulating thymocyte survival, and that external interactions of CD45 can modulate specific stages of thymocyte development.

4.3 CD45 and lymphocyte survival

Clearly CD45 is important during thymocyte development for thymocyte survival since deficiency or antibody manipulation of CD45 results in a reduction of thymocyte cellularity. The loss of thymocytes appears to be mediated by an apoptotic mechanism based on correlating increased annexin V staining of those specific populations. However, latter stages of apoptosis including loss of mitochondrial membrane potential were not detected, however the specificity of the TMRE was not verified. Alternatively, this may be a result of efficient clearance of annexin V+ cells by resident macrophages before later stage apoptosis can be detected (Nakamura et al. 1997). Large scale apoptosis of thymocytes even without MHC selecting ligands (in MHC deficient mice) was not detected due to the highly efficient engulfment by macrophages (Surh et al. 1994). In support of this, human dimeric galectin-1 mediated externalization of phosphatidylserine on neutrophils and some T cell lines did not result in DNA fragmentation, yet still enhanced their phagocytosis by macrophages (Dias-Baruffi et al. 2003; Karmakar et al. 2005). Macrophages have also been shown to phagocytose lymphocytes after apoptosis induction, even before the detection of phosphatidylserine

externalization (Kurosaka et al. 2003), illustrating the sensitivity and efficiency of macrophage clearance mechanisms.

As presented in the Introduction (Chapter 1), the mechanisms of cell death during thymocyte development and selection are not well defined. A role for caspases is limiting, while members of the Bcl-2 family have been implicated in regulating thymocyte apoptosis. However, the signaling mechanisms involved and final outcomes of apoptotic signaling during thymocyte development *in vivo* have not been well characterized (Marsden et al. 2003; Palmer 2003; Starr et al. 2003). A recent paper has shown that CD45-/- B cells lack upregulation of anti-apoptotic Bcl-2 proteins following BCR stimulation, thus affecting survival (Huntington et al. 2006). Further delineation of the survival mechanisms involved during thymocyte development will allow further characterization of the mechanism behind CD45 regulated thymocyte survival.

The mechanism of phosphatidylserine externalization is not well defined, but involves the downregulation of the ATP-dependent aminophospholipid translocase and increased activity of a lipid scramblase (Verhoven et al. 1995; Williamson et al. 2002). The aminophospholipid translocase is responsible for translocating the randomly flipped aminophospholipids (mainly phosphatidylserine) from the outer leaflet to the inner leaflet of the outer plasma, while the scramblase randomly flips phospholipids across the membrane leaflets. How these lipid translocators are regulated is unknown. The role of caspases in mediating phosphatidylserine exposure also depends on the cell type and apoptosis agent inducing agent used (Williamson et al. 2002). There are a number of studies that have demonstrated caspase-independent phosphatidylserine externalization in cell lines (Verhoven et al. 1999; Egger et al. 2003) and primary T cells and thymocytes

(Bidere et al. 2001; Ferraro-Peyret et al. 2002; Egger et al. 2003; Jung et al. 2004). Thus it is plausible that increased annexin V binding observed due to CD45-/- in these studies does not need to correlate with other apoptotic features. Galectin-1 mediated exposure of phosphatidylserine on neutrophils has also been shown to involve src kinase activity, as src kinase inhibitors reduced phosphatidylserine mobilization (Karmakar et al. 2005). Therefore, CD45 may be able to regulate phosphatidylserine exposure through src kinase activity.

The phosphatidylserine exposure detected on thymocytes during development in CD45-/- correlated with reduced cellularity and, therefore, it was rationalized that the outcome is cell death and clearance. However, the presence of high percentages of annexin V+ lymphocytes in the periphery of CD45-/- mice may suggest an alternative outcome. Viable B cells have been shown to bind annexin V during selection events in the BM and in the spleen (Dillon et al. 2000; Dillon et al. 2001); however, the annexin V binding exhibited in CD45-/- mice was found to surpass that of normal basal annexin V binding exhibited by Wt B cells. Despite this, both the high annexin V+ B and T cells remained within the lymph node and spleen, and their presence at such large percentages indicates that they are not being phagocytosed, even though phagocytotic clearance mechanisms appear to function in CD45-/- mice. Perhaps the large percentage of annexin V+ lymphocytes within the periphery are overwhelming the phagocytic cells and hence leading to their accumulation. Alternatively these cells may not exhibit proper phagocytotic signals despite externalized PS, as other signals including glycosylation status and protein expression can influence phagocytosis (Williamson et al. 2002; Guzik et al. 2006).

Non apoptotic externalization of phosphatidylserine has also been described recently for T cells by Elliott et. al (Elliott et al. 2005). These authors found phosphatidylserine exposure on T cells exhibiting $CD45RB^{lo}$ phenotype that did not correlate with cell death as determined by propidium iodide exclusion, and healthy mitochondrial activity. They demonstrated that stimulation of the $P2X_7$ receptor, an ATP-dependent cationic receptor channel results in phosphatidylserine exposure, which in turn, regulates the channel opening and results in CD62L shedding. The authors suggest that externalized phosphatidylserine may enhance migration of lymphocytes into peripheral tissues, and that CD45 acts as a negative regulator of phosphatidylserine translocation (Elliott et al. 2005). However, the evidence for CD45 involvement was merely correlative and no mechanism was delineated. Thus the role of phosphatidylserine exposure on lymphocytes within the lymph node and spleen and of thymocytes within the thymus of CD45 -/- mice observed remains unknown, but may also reflect the consequences of disregulated signaling pathways involving non-apoptotic phosphatidylserine externalization as a result of CD45 deficiency.

The nature of the regulatory capacity of CD45 is diverse and complex. These studies demonstrate that CD45 positively regulates survival of developing thymocytes in addition to its role in mediating thymocyte positive selection signals. The external domain of CD45 contributes to thymocyte survival at specific thymocyte developmental stages, as demonstrated by antibody manipulation which results in reduced cellularity and annexin V staining of DP and CD4 SP thymocytes. These studies also support a negative regulatory role of CD45 in the JAK/STAT pathway as CD45-/- thymocytes exhibit

enhanced IL-7 signaling and proliferation and suggest a role for the external domain in regulating this pathway as well, since mAb manipulation also exhibited enhanced phosphorylation of STAT5 and BrdU incorporation. Survival signals regulated by CD45 therefore are most likely mediated by regulating TCR signaling threshold or other unidentified survival signal pathways rather than IL-7 mediated survival. Yet other pathways not fully defined may also be regulated by CD45 accounting for the increase of phosphatidylserine externalization on peripheral lymphocytes.

The phenotype observed in CD45-/- mice is extremely complex due to the number of pathways regulated by CD45 in essentially all hematopoietic cell types. Dissecting the role for CD45 in distinct pathways, therefore, will remain complex, and further delineation of a function for the external domain of CD45 even more complicated due to its dynamic nature. Nonetheless these studies have demonstrated a role for CD45 and its external domain in intrinsically regulating thymocyte survival during development.

4.4 Model

These data suggest that CD45 and the external domain regulate survival signals during thymocyte development. Interactions of the external domain of CD45 may occur in *trans* with molecules expressed by thymic stromal cells, or alternatively by interacting with molecules in *cis* on the cell surface of the thymocyte. In either case, interactions are most likely mediated through lectin interactions either directly with glycoproteins or soluble lectins, such as the galectins, via complex networks of lectin-protein interactions. Differential expression of CD45 isoforms and glycoylation status would in turn regulate lectin interactions and modulate the outcome of the interaction. Lectin interactions may

mediate localization of CD45 and hence alter the functional outcome of CD45. Translocation of CD45 away from the TCR would result in reduced signaling and, in turn, survival signals. Alternatively CD45 may be regulating another unknown pathway involved in mediating survival signals to developing thymocytes.

4.5 Future Directions

Further examination of the mechanism(s) behind CD45 mediated survival during thymocyte development is still required. The downregulation of Bcl-2 anti-apoptotic proteins and upregulation of pro-apoptotic members should be examined in CD45-/- thymocytes to further define the mechanisms involved in CD45 positive regulation of survival. Further dissection of the role of the complex external domain will provide more knowledge as to how thymocyte survival signals are regulated. If feasible, expression of distinct CD45 isoforms in CD45-/- thymocytes within FTOC would allow for further understanding of the role of individual isoforms in response to Ab mediated reduced survival. Also to dissect the function of the external domain from the intracellular domain, truncated and phosphatase dead mutants could be expressed to address the mechanism of CD45 mAb manipulation and its effects on survival and phosphatidylserine exposure; however the limitations of expression of such a large molecule such as CD45 may preclude such studies.

As the external domain specifically influences DP and CD4+ SP thymocyte survival it would be interesting to determine if this effect is restricted to those thymocytes committed to the CD4+ lineage. The use of FTOC derived from TCR transgenic models in which thymocytes either develop into the CD4 or CD8 linage would allow for

examination of the effect of mAb treatment on the DP population of CD4 or CD8 lineage committed thymocytes. This would also provide more supportive evidence for the requirement of CD45 in mediating CD4-lck signals. Further delineation of the differences between CD4 and CD8 thymocyte development will also be important for determination of the distinct pathways that CD45 may be involved in to differentially influence survival.

As knowledge regarding *cis* and *trans* splicing factors involved in mediating CD45 splicing as well as epigenetic control of the CD45 locus continues to be brought forward, this will also provide new tools in which to examine the CD45 external domain. Further understanding of the glycosylation patterns and functional consequences of the diversity of the external domain, will also be important in determining the function of the external domain in lymphocyte development and function.

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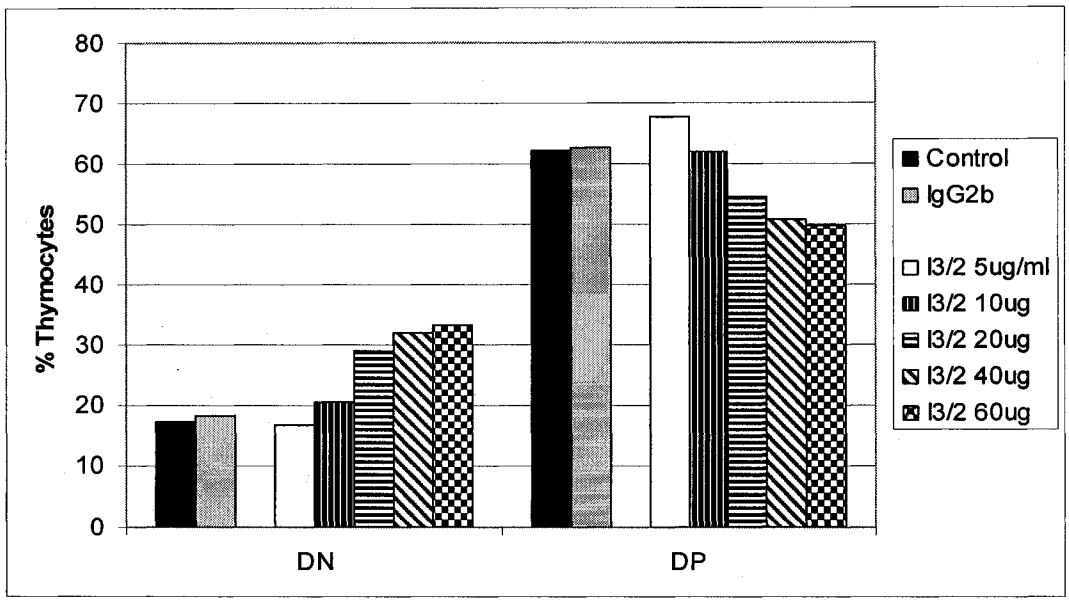
APPENDIX:

Supporting Results

A



B

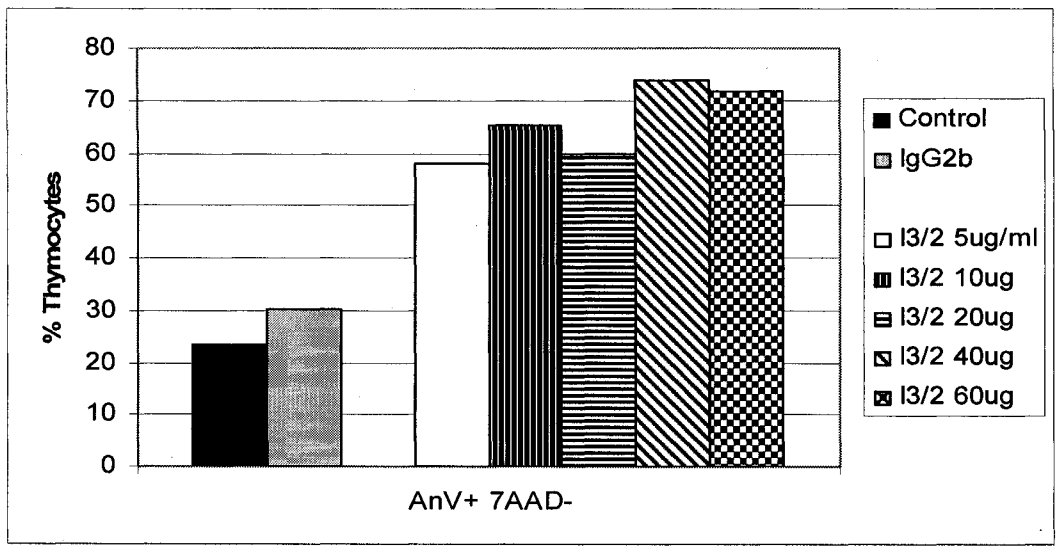


FIGURE A1. *I3/2 Ab titration*

FTOC were left untreated, or treated with isotype control or with differing concentrations of I3/2 Ab Harvested thymocytes after 6 days of culture were then stained for CD4 and CD8 according to the Chapter 2 Materials and Methods and percentages of thymocytes at the DN and DP stages of development are shown in (A) or with annexin V-FITC and 7AAD and the percentage of thymocytes that were AnV+, 7AAD- is shown in (B).

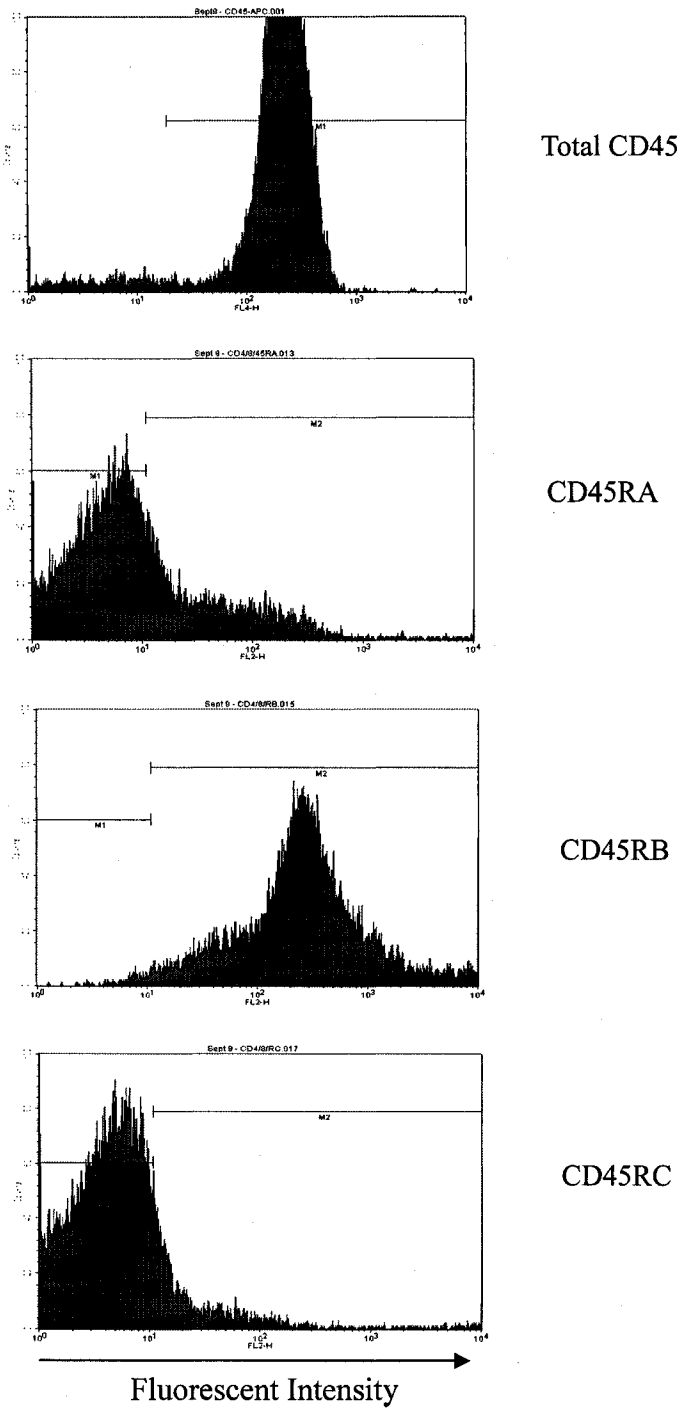


FIGURE A2. *CD45 isoform expression in FTOC-6 fetal thymocytes*
Total thymocytes isolated from FTOC-6 cultures were stained for total CD45 (I3/2), or CD45RA (14.8), CD45RB (16A), and CD45RC (DNL.9) and analyzed by flow cytometry according to the Chapter 2 Materials and Methods.

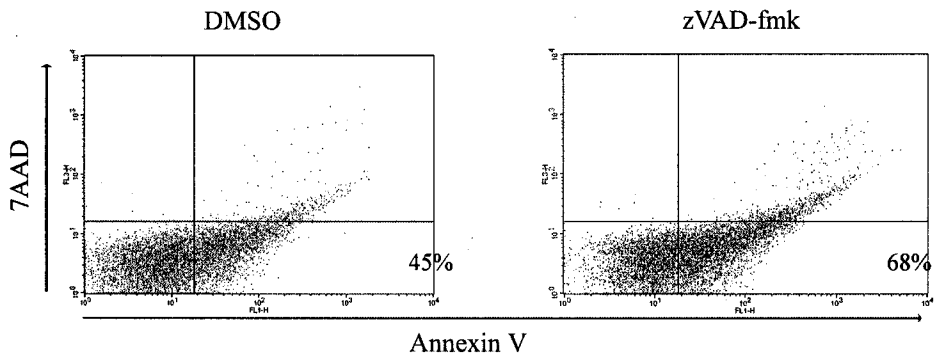


FIGURE A3. *Caspase inhibitor zVAD-fmk is toxic to thymocytes in FTOC.*
FTOC were treated with 100 μM zVAD-fmk or equal volume of DMSO alone, then after 6 days thymocytes were harvested and stained with annexin V-FITC and 7AAD to assess viability, according to Chapter 2 Materials and Methods.

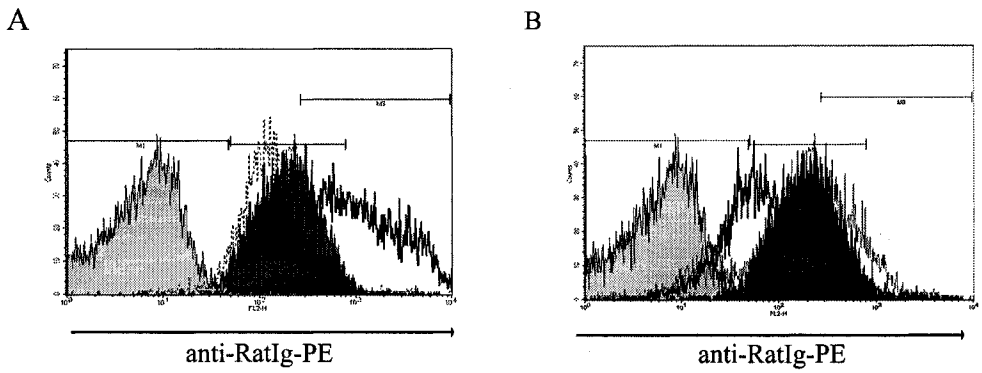


FIGURE A4. *Cell surface binding of CD45 isoform specific mAbs in FTOC.* FTOC were treated with the indicated isoform specific mAbs, harvested at day 6 as described in Chapter 2 Materials and Methods and stained with directly coupled PE-anti-RatIg to detect cell surface Ab binding. A) Cultures were treated with isotype control (grey filled histograms), I3/2 (black filled histograms), M1/9 (black line), or M1/8.9 (dashed line). B) Cultures were treated with isotype control (grey), I3/2 (black histograms), MB4B4 (black line), or MB23G2 (dashed line).

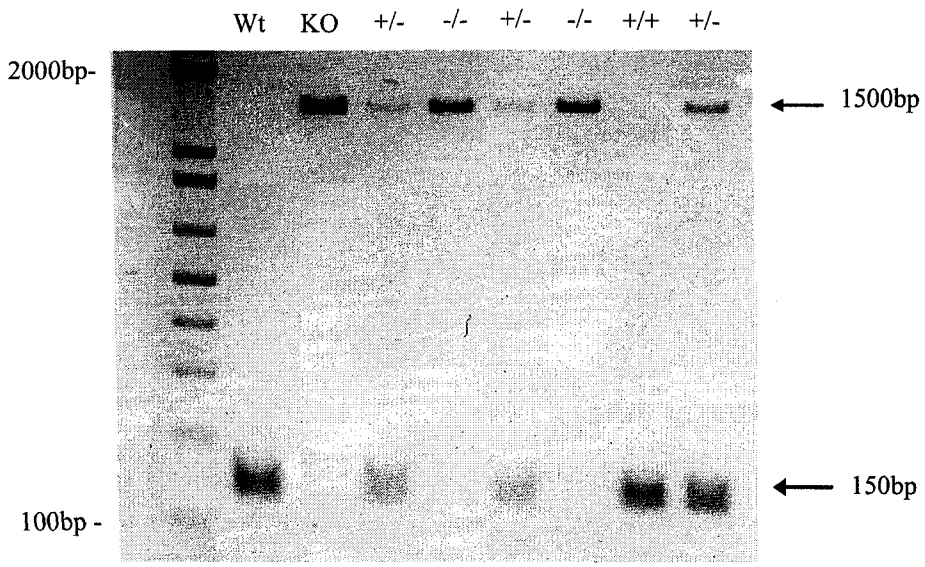


FIGURE A5. *PCR of CD45 exon 9 from F2 littermate mice.*
DNA was isolated from litters from CD45 heterozygous breeding and PCR was performed using primers specific to exon 9 of CD45 according to Chapter 3 Materials and Methods. PCR of samples from F2 litters were run along side known Wt and CD45-/- (KO) DNA to determine proper product band locations.

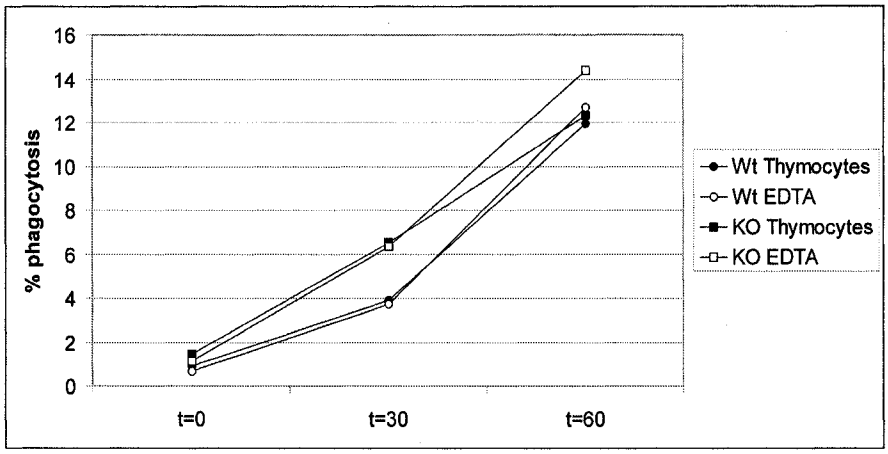
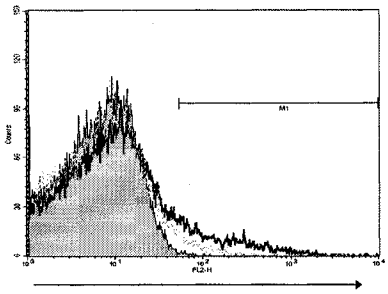


FIGURE A6. *EDTA treatment does not effect % phagocytosis.* Macrophages were allowed to phagocytosis labeled thymocytes as described in Chapter 3 Materials and Methods, for varying time points and then samples were vortexed and treated with 5mM EDTA or left untreated on ice for 15 min, vortexed, fixed and analyzed by flow cytometry. % phagocytosis, indicates the percentage of macrophages staining positive for fluorescent labeled thymocytes.

A



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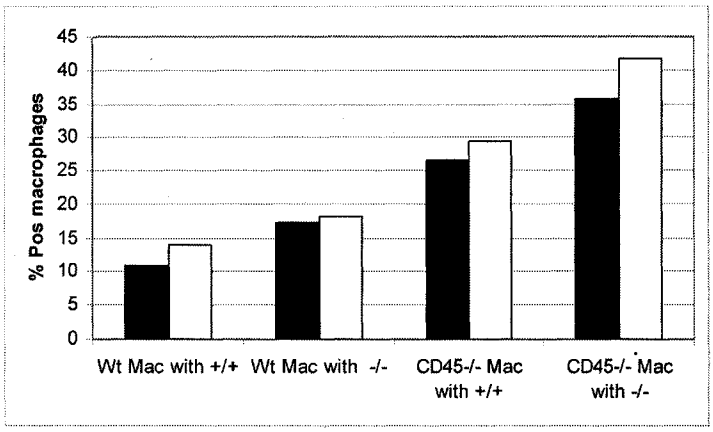

FIGURE A7. *Phagocytosis is not FcR mediated.*

A) Histogram depicting bone marrow derived macrophages (as described in Chapter 3 Materials and Methods), stained directly with thy-1 (dark line), or thy-1 incubated prior with recombinant Protein A/G (grey dashed line), or unstained (filled). B) Comparison of phagocytosis at the 60 min time point of thymocytes stained with Thy-1 alone (dark bars) or Thy-1 pre-incubated with Protein A/G.