University of Alberta

Examining the origin of peripheral self-reactive T cells and the contribution of Gadd45β to T cell selection events in the HY^{cd4} TCR transgenic mouse model

by

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Abstract

Thymic negative selection is important for preventing self-reactive T cells from entering the circulation. However, some self-reactive T cell clones can escape negative selection and induce autoimmunity. The molecular pathways that regulate negative selection are currently unclear, but PD-1 and Gadd45β have been implicated. Using the HY^{cd4} mouse model, we found an absence of self-reactive CD8SP thymocytes, but the presence of self-reactive T cells in the periphery in adult mice. Ontogeny studies demonstrated the presence of self-specific DP and CD8+ T cells in the periphery at Day 3 post-birth that expressed the co-inhibitory receptor PD-1. The presence of self-reactive T cells was not dependent on negative selection occurring in a neonatal thymus. By studying Gadd45β deficient mice, no evidence was found to support a role for Gadd45β in negative selection. Collectively, these data shed light on the source of self-reactive peripheral T cells and the molecular mechanism underlying negative selection.

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List of Abbreviations

AGM – aorta-gonad-mesonephros

AIRE – autoimmune regulator

B6 – C57BL/6 wildtype mice

Bim – Bcl-2-interacting mediator of cell death

BM – bone marrow

BSO – bothionine-sulfoximine

CAR – constitutive active/androstane receptor

CD4SP – CD4 single positive T cell

CD8SP – CD8 single positive T cell

CD62L – L-selectin

CFSE – carboxyfluorescein diacetate succinimidyl ester

ChIP – chromatin immunoprecipitation assay

c-Kit – tyrosine kinase receptor c-Kit

CLP – common lymphoid progenitor

CMJ – cortico-medullary junction

cTECs – cortical thymic epithelial cells

CTLA-4 – cytotoxic T-lymphocyte antigen-4

DAG - diacylglycerol

DN – double negative

DP – double positive

ED – embryonic day

ELP – early lymphocyte progenitors

ERK – extracellular signal-regulated kinase

ETP – early thymic progenitors

FSC – fetal calf serum

Flt3 – fms-tyrosine kinase 3 receptor

Gadd45β – Growth arrest and DNA-damage-inducible 45 β

GR – glucocorticoid receptor

HA – influenza viral protein hemagglutinin

HBSS – Hank's Balanced Salt Solution

HSC – hematopoietic stem cells

IEL – intestinal epithelial lymphocyte

IL-7Rα – IL-7 receptor alpha (α) chain

INS-HA TCR-HA – double transgenic mice expressing the hemagglutinin epitope (HA110-120) of A/PR/8/34 influenza virus under control of the rat insulin promoter and generating CD4+ T cells specific for the hemagglutinin epitope

IP_3 - inositol triphosphate

ISP – immature single positive thymocytes

ITIM – immunoreceptor tyrosine-based inhibitory motif

ITSM – immunoreceptor tyrosine-based switch motif

JNK – Jun N-terminal kinase

KO – knock-out

LAT – linker for the activation of T cells

Lck – leukocyte-specific tyrosine kinase

Lin – lineage

LMPP – lymphoid multi-potent progenitors

LSK – lineage negative stem-cell antigen-1 positive tyrosine kinase receptor c-Kit high

MAPK – mitogen-activated protein kinase

MEKK4 – mitogen activated protein kinase kinase kinase 4, regulator of p38

MHC – Major histocompatibility complex

MKK7 – mitogen activated protein kinase kinase 7, regulator of JNK

MPP – multi-potent progenitors

mTEC – medullary thymic epithelial cells

MyD118 – myeloid differentiation immediate early gene 118 (Gadd45β)

NAC – N-acetyl-L-cysteine

NIH – National Institutes of Health

NK cell – natural killer cell

NP – nucleocapsid peptide of influenza, cognate TCR antigen for F5 TCR

transgenic mice

Nur77-FL – Nur77 full length

OVA – chicken ovalbumin protein

PBS – phosphate buffered saline

PD-1 – programmed death-1

PD-L1 – programmed death-1 ligand 1

PD-L2 – programmed death-1 ligand 2

PIP_2 – phosphatidylinositol biphosphate

PLCγ – phospholipase C γ

pMHC – peptide MHC complexes

preTCR – pre T cell receptor

P-Sp – para-aortic splanchnopleura

PSGL-1 – P-selectin glycoprotein ligand-1

RAG – recombination activating genes protein

Rb – retinoblastoma tumor suppressor

RIP-mOVA – mouse strain expressing membrane bound OVA under control of the rat insulin promoter

ROS – reactive oxygen species

RP10 – RPMI-1640 media with fetal calf serum (FCS) and supplementary completum (SC)

RTE – recent thymic emigrants

S1P – sphingosine-1-phosphate

$S1P_1$ – sphingosine-1-phosphate receptor 1

SC – supplementary completum

SCZ – subcapular zone

SDF-1 – stromal-cell-derived factor 1

SP – single positive

T3.70 – monoclonal antibody recognizing HY TCR+ T cells

TCR – T cell receptor

TGF-β – transforming growth factor beta (β)

ThPOK – T-helper inducing POZ-Kruppel factor

Treg – T regulatory cell

TUNEL – Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling

UVC – ultra violet radiation chemotherapy

VP-16 – etoposide

WT – wildtype

Chapter 1: Introduction

Contribution of the T cell compartment to the Adaptive Immune Response

A host's immune system is composed of three branches of defense: the first involves the epithelial and mucosal barriers to the outside environment, the second is the innate immune response and the third is the adaptive immune response. Cooperation between the innate and adaptive immune responses ensures pathogen clearance. More specifically, the adaptive immune system functions to eliminate bacterial and viral infections and target cancerous cells for death in an antigen-specific manner. This is accomplished through the concerted effort of the adaptive immune cells, B and T cells. Its sophisticated development has allowed for the recognition of various antigens from different pathogens. However, deregulation of the immune system contributes to pathologies including primary immunodeficiencies and autoimmune conditions.

The T cell compartment is crucial to the function of the adaptive immune system and has a direct role influencing the outcome of acute and chronic infections, cancerous cell growth and autoimmune diseases. It is composed of many different cell types including, but not restricted to, CD8+ cytotoxic T cells, CD4+ T helper 1 cells and CD4+ T helper 2 cells. Following interaction between the T cell receptor (TCR) with peptide-bound major histocompatibility complex (MHC) class I or class II expressed on antigen presenting cells, T cells become activated through a complex signal transduction pathway downstream of the TCR. Once activated, T cells can elicit inflammatory and cytotoxic effector responses to clear pathogen-infected cells. However, these responses are generated to specific antigens, therefore it is crucial that T cells can discern between self antigens and foreign antigens. Discrimination between self and nonself is paramount to overall health in mammals (1).

The ability of T cells to distinguish self antigen from foreign antigen is initially

established in the thymus. This has been shown in autoimmune regulator (Aire) deficient mice, which develop peripheral autoimmune disease. Autoreactive T cells did not encounter cognate antigen, whose expression is regulated by Aire, therefore allowing cells to escape central tolerance (2). Numerous mechanisms cooperate to induce this "central tolerance." One of the most recognized mechanisms of central tolerance is negative selection. Negative selection events remove high affinity T cell clones from the T cell repertoire by clonal deletion, anergy or receptor editing. Some argue that clonal deletion is the most important process in generating a tolerant T cell repertoire (3). However, clonal deletion is not perfect at removing all potential high affinity T cells as self-reactive T cell clones have been found within human diabetic patients (4, 5). Therefore, the immune system has also developed fail-safe checkpoints within the periphery that function to prevent the activation of these self-reactive clones. Such peripheral tolerance mechanisms include clonal anergy, clonal deletion and dominant suppression (6). Failure of either central or peripheral tolerance can result in catastrophic developments of autoimmune diseases.

An extensive amount of research is focused on understanding the development of autoimmune disorders by deregulation of T cell development. The logic behind this approach is if our understanding of T cell development demonstrates how autoreactive T cell clones are escaping central tolerance, then one day we could prevent autoimmunity before it starts. Thymic development of T cells involves the migration of precursors to the thymus, whereby they undergo various cellular changes by altered gene expression, resulting in the generation of a tolerant T cell repertoire. The processes mediating thymic development are detailed below.

Hematopoietic stem cell development in murine and human neonates and adults

The generation of the first hematopoietic stem cells occurs at similar stages of embryonic development in mice and humans. Hematopoietic stem cells (HSCs)

first appear within the yolk sac at murine embryonic day (ED) 8.0-8.5 (human ED 18-24) (**Fig. 1-1 A**). However, HSC development soon transfers to an intra-embryonic compartment called the para-aortic splanchnopleura (P-Sp) at murine ED 8.5-9.0. The P-SP further develops into the aorta-gonad-mesonephros (AGM) compartment and at murine ED 10.5-11.5 (human ED 30-40) HSC development ceases in the P-Sp. The AGM-derived hematopoietic precursor cells then colonize the fetal liver and thymic rudiment and at murine ED 12 (human ED unknown), AGM-driven HSC generation ceases and the fetal liver provides the majority of immune cell precursors until birth (7). Bone marrow developmental support of HSCs begins at ED 17 and is sustained for the remaining life of the mouse. Fetal liver contribution to the immune progenitor populations ceases in the few days after birth (8).

At birth, the thymus of neonatal mice contains T cell precursors that originate from both the fetal liver and bone marrow (9). However, to qualify as a T cell progenitor, the cell must be able to traffic from the bone marrow to the thymus and undergo further T cell development upon arrival to the thymic tissue (10). Differences between the fetal liver and bone marrow derived progenitor populations are currently unclear *in vivo*, however, fetal thymic organ culture has shown that fetal liver derived cells generate T cells at a much faster rate than bone marrow derived cells (11). Additionally, unbiased gene cluster analysis suggests that fetal liver derived CD4SP thymocytes have a vastly different gene expression signature compared to adult bone marrow derived cells (12). When thymocyte development within fetal and adult thymic tissue is compared, the adult thymus can generate 100-times as many thymocytes compared to the fetal thymus possibly due to thymocyte capacity to proliferate prior to TCR-beta chain rearrangement (13). However the fetal thymic microenvironment and hematopoietic precursors are capable of giving rise to specific T cell clones that are absent within adult mice. The first evidence to suggest that HSC potential changes during ontogeny was demonstrated when either fetal liver-derived or adult bone marrow-derived HSCs were treated in a hanging drop culture and

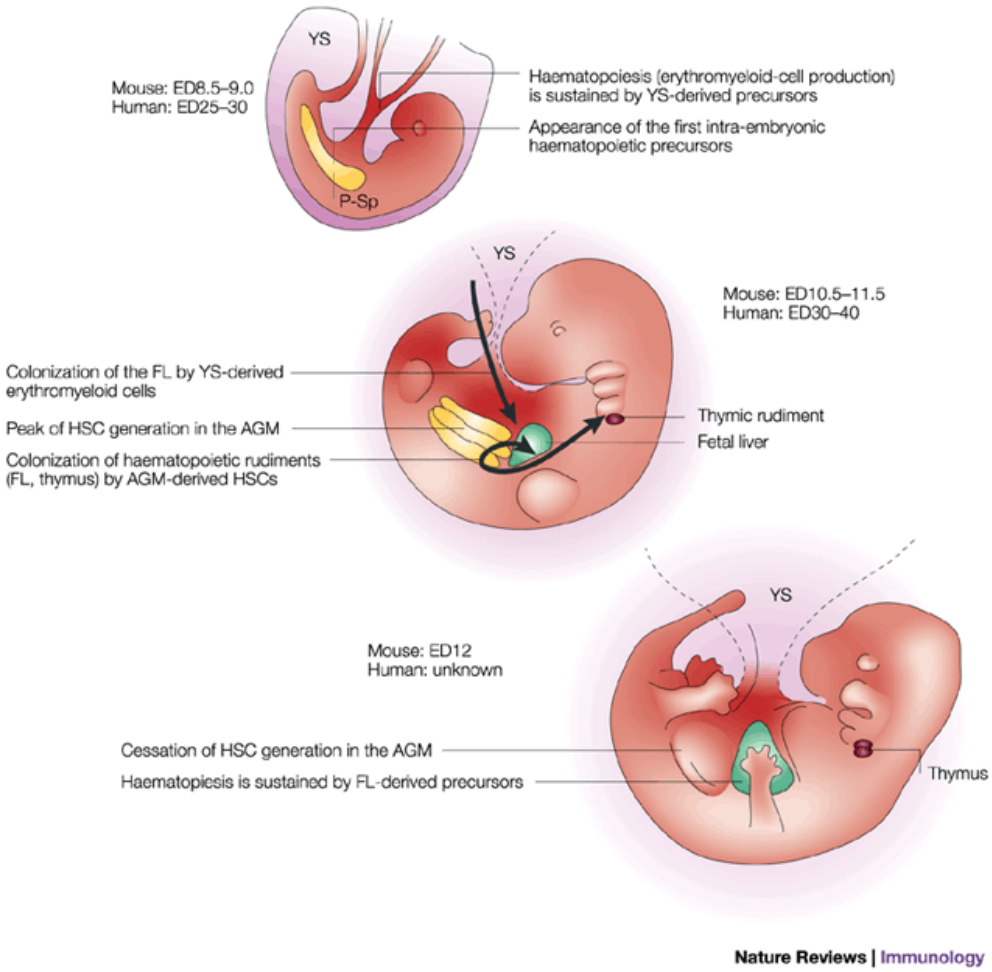


Fig. 1-1. Hematopoietic stem cell development during embryonic development of mice and humans. **A.** Detailed outline of the embryonic development of the hematopoietic stem cell (HSC) compartment within mice and humans. Hematopoietic precursors first appear within the para-aortic splanchnopleura (P-Sp). The P-Sp then develops into the aorta-gonad-mesonephros (AGM) and HSCs from the AGM begin to colonize the fetal liver and thymic rudiment at mouse embryonic day 10.5-11.5. At mouse embryonic day 12, HSC development within the AGM ceases and is entirely dependent on the fetal liver until birth. Figure from the following publication: Godin and Cumano (2002) The hare and the tortoise: an embryonic hematopoietic race. Nature Reviews Immunology **2** (8): 593-604, Figure 1.

grown *in vitro* with fetal thymic lobes. Only fetal liver-derived precursors developed into Vγ3 TCR expressing T cells within the fetal thymic organ culture, suggesting the adult hematopoietic precursors were unable to generate into the Vγ3 T cell subset (14). Furthermore, it has been shown that the Vγ5 TCR is preferentially expressed on fetal thymocytes whereas the Vγ4 TCR is abundantly expressed on adult thymocytes. Transplantation of the fetal liver or adult bone marrow progenitors into irradiated recipients demonstrated equal emergence of thymocytes following the procedure; however, only fetal liver receiving adult chimeras displayed Vγ5 TCR expressing thymocytes (15). Therefore, fetal liver derived T cell progenitors appear to have a predominance to generate specific T cell subsets compared to adult bone marrow derived progenitors. Despite these differences, hematopoietic precursors develop similarly within the fetal liver and bone marrow and T cell progenitors from these distinct sites undergo mostly the same developmental processes within the thymus.

Hematopoeisis is the process responsible for the generation of erythrocytes, cells of myeloid origin and lymphoid origin. The initial hemotopoietic cell population is called the LSK fraction, Lineage negative (Lin^-) stem-cell antigen-1 positive ($Sca\text{-}1^+$) tyrosine kinase receptor C-Kit high (Kit^{High}) and the long-term HSCs responsible for sustaining the LSK population accounts for only a small proportion of the total LSK cells (**Fig. 1-2 A**). The long-term HSCs are classified as lacking fms-tyrosine kinase 3 receptor (Flt3), lacking CD34 and expressing the Slam family receptor CD150 (10). Progenitor cells downstream of HSCs are multi-potent progenitors (MPPs) that have lost self-renewal capacity but maintain the potential for developing into various lineages (16). Cells that have lymphoid and myelo-erythroid potential are classified as LSK $Flt3^{low/+}$ $CCR9^-$ $VCAM\text{-}1^+$ (10). The cellular change to $Flt3^{high}$ expression classifies cells as lymphoid multi-potent progenitors (LMPPs) that maintain B and T cell potential (**Fig. 1-2 A**), as shown following *in vitro* culture on OP9-DL1 stromal cells (17). Much debate exists within the scientific literature as to the existence of the LMPP population, as LSK $Flt3^{high}$ cells have been shown to exhibit megakaryocyte and erythroid

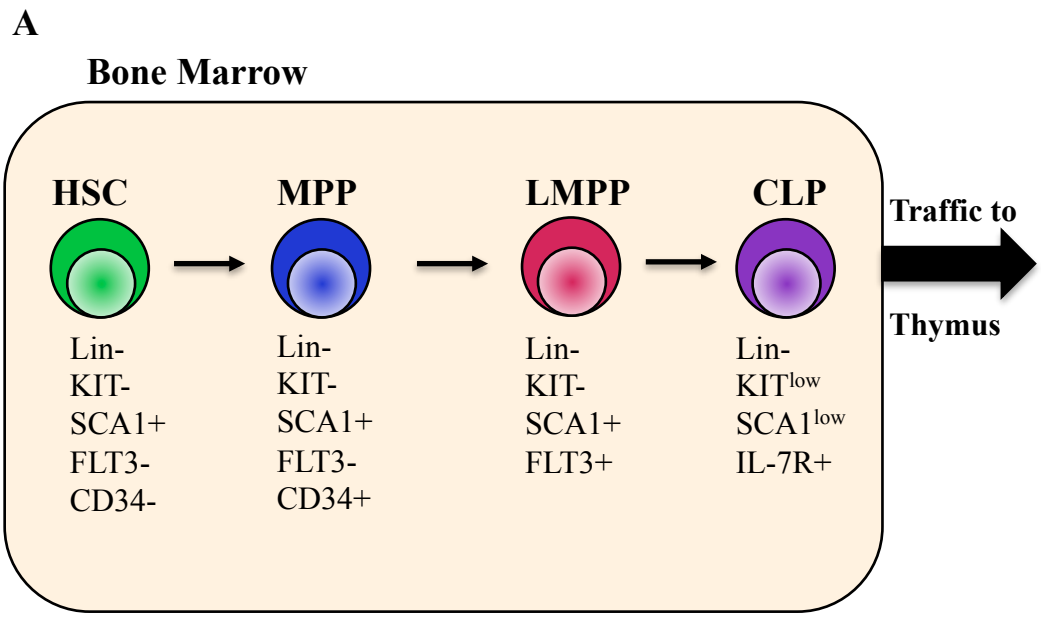


Fig. 1-2. Model of hematopoietic stem cell development of T cell precursors within the bone marrow. **A.** Bone marrow development of hematopoetic precursors and receptors associated with the specific hematopoietic subsets. Hematopoietic stem cells (HSC) develop into multiple potent-progenitors (MPP), then lymphoid multi-potent progenitors (LMPP) and then finally develop into common lymphoid progenitors (CLP). The CLP subset are the precursor cells to thymocytes and are responsible for leaving the bone marrow and trafficking to the thymus. This model was adapted from King, A.G. *et. al.* (2002) Lineage infidelity in myeloid cells with TCR gene rearrangement: a latent developmental potential of pro T cells revealed by ectopic cytokine receptor signaling. PNAS **99** (7): 4508-13, Figure 1 and Nagasawa, T. (2006) Microenvironment niches in the bone marrow required for B-cell development. Nature Reviews Immunology **6**: 107-116, Figure 2.

potential (17). However, the Jacobsen group demonstrated through quantitative PCR that LSK Flt3^{high} cells (LMPPs) lack expression of *GATA-1* and *EpoR*, genes required for megakaryocyte and erythroid potential, suggesting that LMPPs have undergone an irreversible lineage commitment to lymphoid potential. Up regulation of RAG-1 expression in the LMPP fraction expressing LSK Flt3^{high} results in the transition of LMPPs into early lymphocyte progenitors (ELPs) (18). Finally, cells progress from ELPs to common lymphoid progenitors (CLPs) with the up regulation of IL-7 receptor (IL-7R) (19, 20). CLPs require and maintain Flt3 expression to regulate and sustain the population as Flt3 ligand deficient mice show a drastic reduction in number of CLPs but not HSCs (21). Two receptors crucial for T cell progenitor homing to the thymus that are expressed on CLPs are the chemokine receptor CCR9 and P-selectin glycoprotein ligand-1 (PSGL-1) (10). Finally, the last definite developmental stage that divides the CLPs into T- or B-cell progenitors is the surface expression of Ly6d. B-cell progenitor CLPs express Ly6d+ whereas Ly6d- CLPs are defined as T-cell progenitors (22).

Trafficking of early thymic progenitors to the thymus

Mobilization from the bone marrow

Progenitors are required to traffic from the bone marrow to the thymic tissue to initiate T cell development. Ly6d- CLPs lack expression of VCAM-1 that reduces the tethering and adhesion of the cells to the bone (10, 23). However, CLPs require the adhesion molecule P-selectin and the chemoattractant shingosine-1-phosphate (S1P) to promote thymic recruitment. Intravenous injection of PSGL-1 heterozygous bone marrow (BM) and wildtype BM into adult mice demonstrated a two-fold reduction in chimerism of early thymic progenitors compared to wildtype chimeras. This demonstrates that PSGL-1 association with P-selectin is functionally required for CLP trafficking to the thymus (24). Furthermore, S1P is required for progenitor egress from the bone marrow to the thymus. The S1P gradient is higher within the blood circulation

compared to bone marrow therefore CLPs expressing sphingosine-1-phosphate receptor 1 ($S1P_1$) are drawn to the circulatory system. Additionally, S1P expression induces production of the potent HSC chemoattractant stromal-cell-derived-factor-1 (SDF-1 or CXCL12) via a reactive oxygen species (ROS) signaling mechanism, further enhancing progenitor egress from the bone marrow to the circulation. Treatment of total bone marrow cells with the ROS inducer buthionine-sulfoximine (BSO) induced SDF-1 secretion. Furthermore, while S1P treatment of total bone marrow cells induced SDF-1 secretion, this was blocked by co-treatment with S1P and the ROS inhibitor NAC (N-acetyl-L-cysteine)(25). Since high ROS levels have been associated with HSC mobilization (26), the authors attributed SDF-1 production to S1P and ROS expression (25).

Entry into the thymus

Thymic seeding of T cell progenitors is dependent upon chemokine signaling and gradients within fetal and post-natal T cell development. Within the fetal thymus, the chemokines CCL21 and CCL25 and their receptors CCR7 and CCR9 are required for T cell progenitor seeding. Treatment of fetal thymic lobes with neutralizing antibodies for the chemokines CCL21 and CCL25 reduced colonization of the thymic tissue by T cell progenitors; however, no change in colonization was observed when CXCL12 was blocked (27). Furthermore, CXCL12 is not required for thymic homing in embryonic day 12 fetal mice, as T cell progenitors within the thymic rudiment lacked expression of its receptor CXCR4 (28). However, the dependence on chemokine signaling for thymic seeding is not restricted to the fetal stage. CCR7 and CCR9 double-deficient mice show drastically reduced numbers within the adult thymus. This phenotype for CCR7 deficiency was not previously known due to the compensatory effects of CCR9 (29). Furthermore, adult thymus seeding is assisted by thymic epithelial expression of P-selectin and ligand recognition by PSGL-1 expressed on progenitor T cells (30). Finally, antibody-blocking studies have also identified CD44 surface expression as a requirement for T cell progenitor homing to the

thymus (31). Therefore, T cell precursors employ many different mechanisms to ensure proper trafficking from the bone marrow to the thymus. Despite the differences in origin of the hematopoietic precursor cells in fetal and adult mice, they require similar chemokine and signaling pathways to effectively traffic to the thymus and undergo T cell development.

Thymocyte development and the educational processes of selection

The thymus is the location where T cell progenitors complete their development and the thymic microenvironments of the cortex and medulla separate specific thymocyte populations. The outer portion of the thymus is referred to as the cortex, containing immature thymocytes, whereas the inner medulla portion houses mature single positive (SP) thymocytes prior to their egress (32). Early thymic progenitors (ETPs) enter the thymus at the cortico-medullary junction (CMJ) and undergo differentiation into the initial CD4- CD8- (double negative; DN) thymocyte stage, DN1 (CD44+ CD25-) (**Fig. 1-3 A**). From the DN1 stage, there is a sequential developmental pattern into the other DN thymocyte subsets: DN2 (CD44+ CD25+), DN3 (CD44- CD25+) and DN4 (CD44- CD25-) (33). Microscopic analysis has found that DN1 thymocytes appear to localize at the CMJ whereas the DN2 and DN3 thymocyte populations are found scattered from the CMJ to the subcapular zone (SCZ), based on immunofluorescence microscopy (34). At the DN3 thymocyte stage, expression of the chemokine receptor CCR9 is induced by pre-TCR signaling (35). Its expression is required for the localization of DN3 thymocytes to the SCZ as CCR9-deficient mice display heterogeneous dispersement of DN3 thymocytes throughout the cortex. Similar Vβ TCR specificities of CD4+ splenocytes within wildtype and CCR9-deficient mice suggests that T cell selection is not altered in the absence of CCR9 and intra-thymic localization is not required for thymocyte progression (36).

At DN2 and DN3 thymocyte stages, defined gene rearrangement of the *tcrb*, *tcrg* and *tcrd* loci by recombination activating genes proteins (RAGs) is initiated.

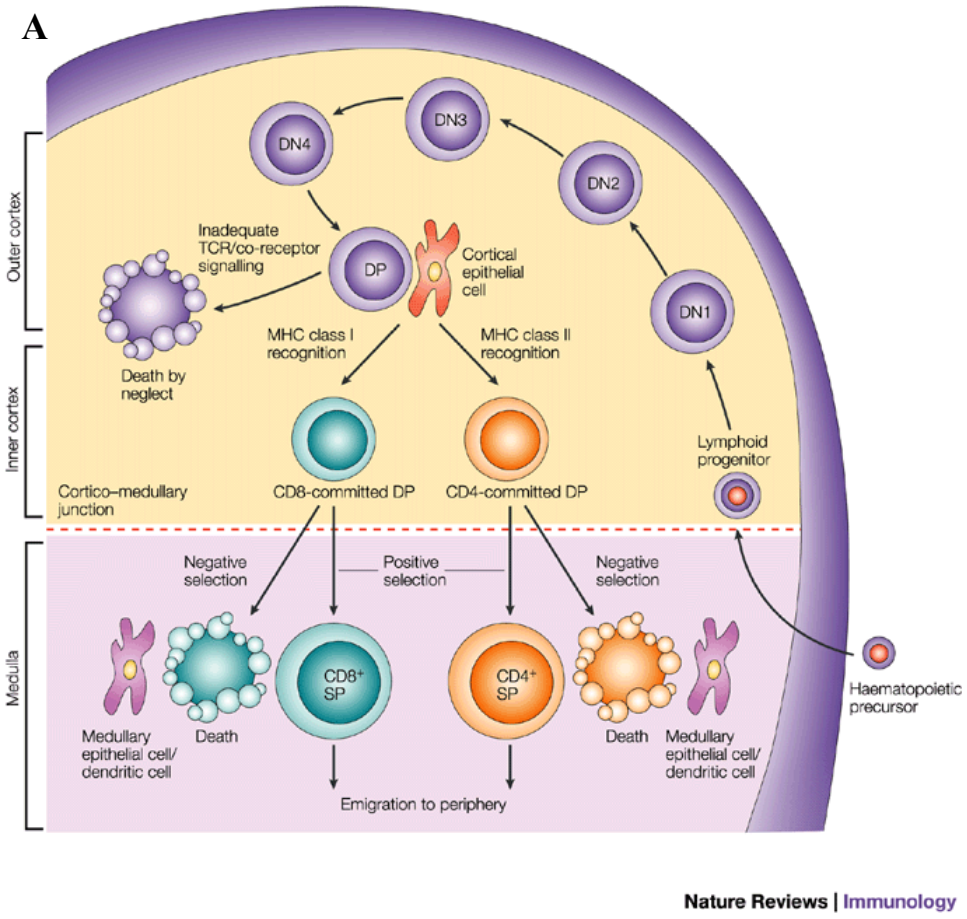


Fig. 1-3. Model of T cell development where hematopoietic stem cell precursors enter the thymus, progress through various developmental stages and undergo selection, generating a tolerant T cell repertoire. **A.** Detailed outline of thymocyte development. Lymphoid progenitors enter the thymus at the cortico-medullary junction and traffic through the cortex, progressing through the double negative (DN) 1-4 stages. At the double positive (DP) stage, thymocytes associate with cortical thymic epithelial cells (cTECs) and undergo positive or negative selection. DP thymocytes that survive positive selection within the cortex become CD8SP or CD4SP committed, traffic to the medulla and undergo another round of selection events to tissue-restricted antigens expressed on medullary thymic epithelial cells (mTECs) or dendritic cells. Positively selecting CD8SP or CD4SP thymocytes then migrate into the periphery. From Germain, R.N. (2002) T-cell development and the CD4-CD8 lineage decision. Nature Reviews Immunology **2**: 309-322, Figure 1.

Productive rearrangements at these loci results in expression of either a γδ TCR or pre-TCR. The pre-TCR consists of a rearranged β chain in association with the pre-Tα chain (37). Signaling through the pre-TCR promotes survival, αβ lineage commitment, proliferation and differentiation of DN3 thymocytes to the DN4 stage, where TCRα gene locus rearrangement begins (38, 39). Lineage commitment of T cells to either the γδ T cell or αβ T cell fate is thought to be regulated by differential signal transduction downstream of the TCR (40, 41). The DN4 stage is short-lived and all DN4 thymocytes become immature single-positive (ISP) thymocytes due to the expression of CD8 coreceptor. The exact role of ISPs is currently unclear (42) but cells do not remain in this stage for long, acquiring CD4 coreceptor expression and progressing to the CD4+ CD8+ (DP) thymocyte stage of development. At this stage, DPs complete TCRα gene rearrangement, resulting in the stable expression of a functional αβ TCR (38) .

Thymocyte selection: Death by neglect

DP thymocytes are the T cell progenitor populations that undergo the educational processes of positive and negative selection to generate a self-tolerant T cell repertoire following thymic stroma nurturing. Within the cortex, DP thymocytes interact with cortical thymic epithelial cells (cTECs) and dendritic cells via TCR-peptide MHC complexes (43). It was previously shown that approximately 90% of DP thymocytes that express TCR do not recognize peptide-MHC complexes and do not receive cytokine-mediated survival signals would undergo death by neglect (44, 45). However, a recent study that quantified the number of cells undergoing selection demonstrated that of the 0.5% of total thymocytes expressing active caspase 3, approximately 50% of them were identified as $CD5^{lo}$ $CD69^{lo}$ and presumably undergoing death by neglect (46). Therefore, it is likely the frequency of thymocytes undergoing death by neglect is much lower than previously considered.

The role of TEC-derived glucocorticoids has been implicated in death by neglect

with an *in vitro* assay using a glucocorticoid resistant PD1.6 Dex(-) cell subline (47). However, this is controversial as thymocyte development proceeded normally in glucocorticoid receptor (GR)-deficient mice (48). Furthermore, the T cell surface receptors CD8 (49), CD24 (50), CD45 (51) and the mouse homolog of CD99 have been implicated in death by neglect; however, their roles have not been definitively shown within an *in vivo* model (44).

Thymocyte selection: Positive selection

DP thymocytes undergo positive selection when the TCR associates with peptide-MHC (pMHC) complexes with low to moderate affinity, a concept first demonstrated with survival of ovalbumin protein variant E1 peptide treated OT-1 β2M-/- thymocytes in fetal thymic organ culture (52). DP thymocytes expressing TCRs specific for MHC Class I complexes develop into CD8SP thymocytes and those specific for MHC Class II complexes develop into CD4SP thymocytes. Recent evidence suggests that glucocorticoids are required for effective T cell selection events within a glucocorticoid receptor (GR) deficient mouse model, $GR^{lck\text{-}Cre}$. In the absence of GR, mice were severely immunocompromised and T cell affinity assays demonstrated that cells normally positively selected had undergone negative selection, suggesting that GR was required for gauging TCR responsiveness to pMHC interactions (53). Microarray analysis of Class I and II-selecting TCR transgenic mice (discussed later) found very similar gene expression profiles between the different positively selecting thymocyte populations (54). Therefore, positive selection signaling between MHC Class I and II-restricted thymocytes appears to be the same.

Interaction between the TCR and its positively selecting pMHC complex initiates a complex signaling cascade downstream of the TCR. This cascade can be divided into 3 distinct signaling pathways: (a) proximal signaling; (b) calcium-mediated signaling; and (c) GTPase Ras-mitogen activated protein kinase (MAPK) signaling pathway (55). The TCR signaling cascade begins with

localization and activation of various molecules proximal to the TCR. Upon ligand recognition, the src-family kinase leukocyte-specific tyrosine kinase (Lck) induces the phosphorylation and activation of CD3 (**Fig. 1-4 A**). This event promotes the localization of ZAP70 to the cytoplasmic side of the plasma membrane, promoting its phosphorylation and subsequent activation. ZAP70 activation then causes the activation of linker for the activation of T cells, LAT, followed by the localization and subsequent activation of SLP76 and phospholipase C γ (PLCγ) (55). The calcium-mediated and the GTPase Ras-MAPK signaling pathways are initially activated following the hydrolysis of phosphatidylinositol biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (56). Signaling through the MAPK extracellular signal-regulated kinase, ERK, is required for positive selection (57). DP thymocytes employ TCR signaling downstream of ERK to up regulate expression of Bcl-2 and Mcl-1, thereby promoting survival and further development (58). IL-7R expression is later restored following positive selection (59). Finally, positive selection induces the expression of the chemokine receptor CCR7 and signaling through CCR7 promotes trafficking to the medulla (60, 61). At this site, thymocytes undergo another round of selection events to tissue-restricted antigens.

Lineage commitment

There is much debate about the differentiating signals involved in the development of CD4SP and CD8SP thymocytes. Evidence supports the kinetic signaling model, which suggests that following TCR signaling, DP thymocytes down regulate CD8 coreceptor but not CD4 coreceptor to form the $CD4^+CD8^{lo}$ thymocyte population. If the MHC Class II-restricted TCR is signaled then $CD4^+CD8^{lo}$ thymocytes continue to develop into the CD4SP lineage. If the thymocytes do not signal through a MHC Class II-restricted TCR, then the signaling stops. These thymocytes then become responsive to IL-7 cytokine signaling to promote the lineage commitment to CD8SP thymocytes (62).

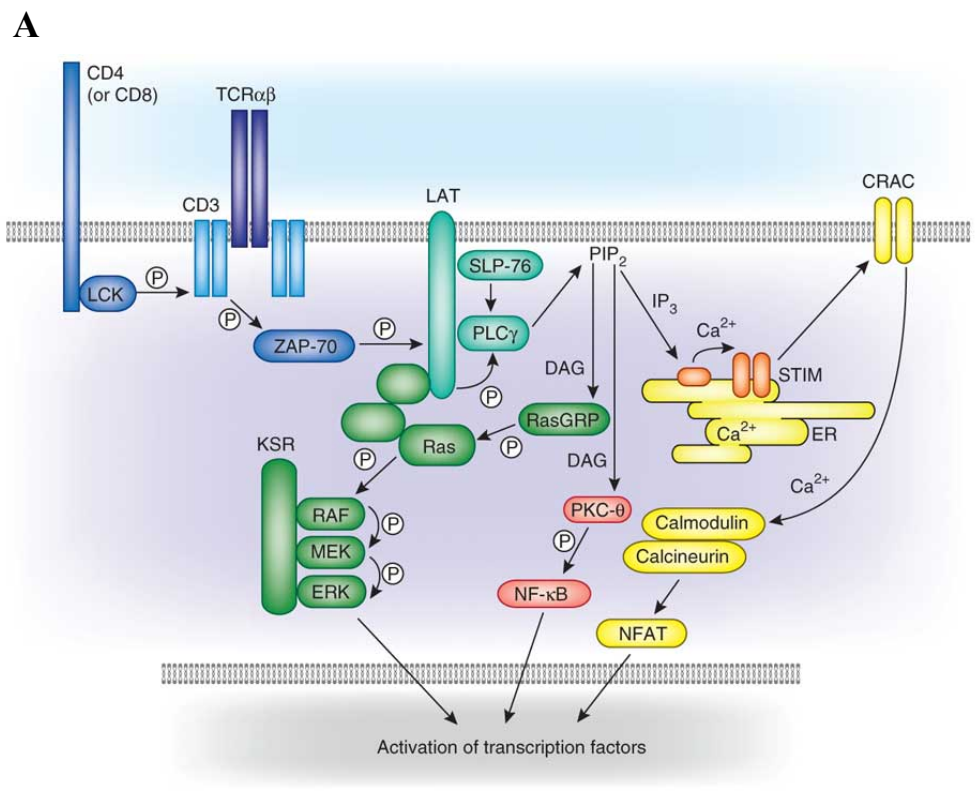


Fig. 1-4. Model of the signaling cascade downstream of the T cell receptor following engagement with peptide-MHC complexes. **A.** A detailed outline of the signaling cascade downstream of the T cell receptor. Following TCR engagement, Lck molecules sequestered at the CD4/CD8 coreceptors activate CD3 molecules. In turn, ZAP-70 molecules are localized to the cell surface and become phosphorylated. This activity promotes the activation of LAT, which then promotes the downstream activation of the Ras pathway and the calcineurin pathway via SLP-76 and PLCγ. Activation of the Ras pathway promotes the activation of the MAPK ERK, which has been shown to be required for positive selection. Downstream activation of these various molecules promotes the expression of various transcription factors important for positive selection. From Morris, GP. and PM. Allen. (2012) How the TCR balances sensitivity and specificity for the recognition of self and pathogens. Nature Immunology **13**: 121-128, Figure 2.

Specific transcription factors have been shown to promote commitment toward the CD4+ T-helper T cell lineage or the CD8+ cytotoxic T cell lineage. DP thymocyte commitment to the CD4+ T helper lineage is associated with the expression of transcription factors Gata-3 and T-helper inducing POZ-Kruppel factor (ThPOK). Gata-3 is a transcription factor known to be involved throughout T cell development. It is down regulated during the DN to DP thymocyte stage transition and is up regulated following positive selection (63). Furthermore, Gata-3 is required for directing thymocytes to the CD4+ T cell lineage but is not sufficient on its own (64). Therefore, Gata-3 induces the expression of the transcription factor directly involved in the CD4+ T cell lineage decision, ThPOK (63). It is known that ThPOK is the crucial factor for the CD4+ T cell fate as deletion of ThPOK redirected MHC Class II-restricted TCR expressing thymocytes to the CD8+ T cell lineage (65, 66). In comparison, Gata-3 deficiency causes a lack of ThPOK expressing pre-selection DP thymocytes (66). It has also been shown that Gata-3 directly binds with the transcription factor loci associated with the CD8+ T cell lineage fate, repressing its expression prior to the induction of ThPOK (67).

The expression of another family of transcription factors, the Runx factors, has been shown to be required for the CD8+ T cell lineage program (64). Runx proteins mediate their activity by associating as heterodimers and directly bind to specific DNA sites (63). A chromatin immunoprecipitation (ChIP) assay showed that Runx complexes directly associate with the silencer element on the *ThPOK* locus (68). Therefore, Runx complexes function to directly inhibit CD4+ T cell lineage-associated transcription factor activity. Runx activity toward the CD8+ T cell lineage decision was demonstrated when overexpression of the Runx3 transcription factor causes an increase in mature CD8+ T cells, partially due to redirection of MHC Class-II restricted T cells (69, 70). The opposite was also shown, when reduced Runx factor expression redirected CD8+ T cells to the CD4+ T cell lineage and therefore reduced CD8+ T cell numbers (71-73).

Thymocyte selection: Negative selection

Negative selection involves the removal of high-affinity TCR clones from the T cell repertoire. DP thymocytes that bind TCR peptide-MHC complexes with high affinity undergo one of the three mechanisms of negative selection: clonal deletion, anergy and receptor editing (74). Negative selection events of DP thymocytes are localized to the thymic cortex (75) however negative selection again occurs within the medulla. The spatial separation of antigen presentation within the thymus allow for DP thymocyte selection to ubiquitous antigen expressed by cTECs in the cortex and SP thymocyte selection to tissue-restricted antigens by medullary thymic epithelial cells (mTECs) and dendritic cells in the medulla (76). Clonal deletion involves the apoptotic death of self-reactive thymocytes and while the activation of caspase 3 can be measured as an early event during apoptosis of thymocytes, active caspase 3 is not required for negative selection (77, 78). Meanwhile, anergy and receptor editing are thought to play a lesser role in negative selection (74).

Several signal transduction pathways are involved in mediating clonal deletion following high-affinity signaling through the TCR. The mitogen-activated protein kinases (MAPKs) p38 and Jun N-terminal kinase (JNK) are activated following negative selection-inducing signals and have been shown to be required for mediating clonal deletion (79, 80). The contribution of p38 to thymocyte negative selection has been difficult to assess due to the embryonic lethality of the *p38α* knock-out mice (81, 82). However, treatment of thymocytes in a fetal thymic organ culture, from HY transgenic H-$2^{b/d}$ male fetuses, with the p38 inhibitor SB203580 showed an increase in the frequency of DP thymocytes and the appearance of a CD8SP thymocyte population. This CD8SP thymocyte population was absent within the control *in vitro* culture and demonstrated that p38 was directly involved in negative selection of the antigen-specific HY TCR expressing male thymocytes (79). Evidence of the contribution of JNK to clonal deletion was demonstrated when immature DP thymocytes from JNK-2 deficient

mice were resistant to apoptosis following *in vivo* injection of anti-CD3 antibody (83). In contrast to positive selection, ERK1/2 is not required for negative selection. This was concluded from a fetal thymic organ culture experiment performed with thymic lobes from fetal OT-1 wildtype (84) and OT-1 ERK1/2 single and double knock-out mice. Following a 24-hour incubation with OVA peptide, the absolute number of DP thymocytes within the thymic lobes did not differ in the presence or absence of ERK1/2, providing definitive evidence that ERK1/2 is not required for negative selection following antigen encounter (85).

Negative selection effector molecules

During clonal deletion, high affinity antigen encounter and signaling through the TCR initiates the mitochondrial apoptotic pathway, regulated by the Bcl-2 family of proteins. A pro-apoptotic member of the Bcl-2 family, Bim (Bcl-2-interacting mediator of cell death) has been implicated in negative selection (86) although its exact role has been controversial until recently. Studies with the TCR transgenic mouse models OT-1, a H-2K^{b} restricted TCR transgenic model specific for the chicken ovalbumin (OVA) protein (52), and HY, a H-2D^{b} restricted TCR transgenic model specific for the ubiquitous HY male antigen (87), have examined the contribution of Bim in negative selection. Initial evidence with Bim deficient mice on wildtype and TCR transgenic OT-1 and HY male backgrounds showed an increase in CD4+ CD8+ (DP) thymocyte number compared to heterozygous controls (88). This suggested that Bim was required for clonal deletion of thymocytes. However, analysis of physiological HY^{cd4} transgenic mice showed no alteration in DP thymocyte proportion and number in the presence or absence of Bim, suggesting that Bim is not required for thymocyte clonal deletion to ubiquitous antigen (89). Elegantly designed experiments using OT-1 Bim sufficient and deficient bone marrow into RIP-mOVA recipients, expressing OVA under control of the rat insulin promoter, examined the contribution of Bim to thymocyte negative selection against tissue restricted antigens. In the absence of Bim, antigen-specific OT-1 CD8SP thymocytes failed

clonal deletion to the tissue restricted antigen OVA, exited the thymus and persisted within the periphery. This evidence demonstrated that Bim is required for clonal deletion to tissue-restricted antigens (90).

In addition to Bim, the orphan nuclear steroid receptor Nur77 has also been implicated in negative selection. A rapid induction of Nur77 expression was observed following *in vitro* antibody stimulation of the TCR, CD2 and CD28 of B6 thymocytes, demonstrating a link between Nur77 expression and apoptosis (91, 92). Within a transgenic model system, AND transgenic mice on the negatively selecting H-$2^{b/s}$ background had a higher frequency of Nur77 expressing Vα11 thymocytes compared to AND H-$2^{b/b}$ mice undergoing positive selection (92). This provided further evidence that Nur77 was being expressed during thymocyte clonal deletion *in vivo*.

The contribution of Nur77 to clonal deletion is not entirely understood and different models have been generated to define Nur77 activity within developing thymocytes. Transgenic Nur77-FL (full length) mice that constitutively express Nur77 within thymocytes were generated to directly examine if Nur77 induces apoptosis. Constitutive expression of Nur77 caused a reduction in cell number of thymocytes and lymph node T cells and increased apoptosis of thymocyte determined by TUNEL staining, suggesting that Nur77 expression induced apoptosis (93). A dominant-negative Nur77 model went on to further show that Nur77 was implicated in negative selection, when F5 TCR transgenic mice lacking Nur77 expression were defective in clonal deletion (93). F5 TCR transgenic thymocytes recognize an influenza virus nucleocapsid peptide (amino acids 366-374, NP) in the context of H-$2D^b$ and are depleted following intraperitoneal injection of the nucleocapsid peptide (94).

It is not well understood how Nur77 activity contributes to the apoptotic pathway. Unlike other orphan nuclear steroid receptors, Nur77 activity does not require ligand binding. In fact, deletion of the C-terminal ligand-binding domain appears to enhance its activity (95). The role of Nur77 as a transcription factor has also

been described as a method for apoptosis induction. Nur77 contains a DNA binding domain and is a transcription factor that may directly regulate the expression of Bim in stimulated DP thymocytes (96). Therefore, Nur77 may induce apoptosis via transcriptional regulation of pro-apoptotic proteins within thymocytes. However, Nur77-mediated apoptosis induction has also been described by its activity at the mitochondrial membrane. Following intraperitoneal injection of NP peptide (366-374) into F5 TCR transgenic mice to induce negative selection of DP thymocytes, Nur77 translocated from the nucleus to the mitochondria to associate with Bcl-2 and thereby exposed its pro-apoptotic BH3 domain. Therefore, Nur77 may also function at the mitochondrial level to induce apoptosis during negative selection (97).

Peripheral T cell activity and tolerance mechanisms

Peripheral T cell subsets

Upon completion of T cell development within the thymus, mature CD4SP and CD8SP thymocytes are exported and enter the periphery. Egress from the thymus and entry into the blood stream requires T cell recognition of S1P via the S1P receptor 1 ($S1P_1$). $S1P_1$ knock-out fetal livers transplanted into B6 wildtype mice lacked peripheral T cells but had normal numbers of DP thymocytes and increased numbers of SP thymocytes, suggesting that the S1P-$S1P_1$ pathway is important for T cell export from the thymus (98).

Mature thymocytes that have entered the secondary lymphoid organs and have not been present for a long period of time are referred to as recent thymic emigrants (RTEs). Initial studies suggested that recent thymic emigrants were similar to mature peripheral T cells (99). However, it is now known that the RTE subset of T cells express high levels of CD24 and is functionally distinct from mature T cells (100, 101). Furthermore, the RTE subset undergoes further maturation within the periphery; this was shown when RTEs were activated with

concanavalin A and assayed for cytotoxicity against tumor cell targets. Within the mature peripheral T cell population, 2/3 of the total cells displayed cytotoxicity and were considered cytotoxic CD8+ T cell precursors whereas less than half of the RTE subset contained cytotoxic precursors (102). At later time points the surface expression of CD24, the marker for RTEs, is down regulated. Other markers of T cell maturation are initially expressed within the thymus, but may subtly change in the periphery. These markers include CD62L (L-selectin), IL-7 receptor α chain (IL-7Rα) and CD28 (102).

Egress from the thymus and homeostatic proliferation maintain the naïve T cell populations within the periphery. Naïve T cells are classified by the CD62L+ CD44- surface expression profile and are sustained within the periphery by homeostatic proliferation (103). Survival of the naïve T cell compartment requires a balance between TCR-peptide:MHC interactions and IL-7 mediated signaling (104, 105).

Following TCR encounter with foreign antigen and downstream activation signaling, naïve T cells differentiate into the effector T cell subsets, comprised of T helper 1 cells, T helper 2 cells or cytotoxic T cells. The T helper 1 and cytotoxic T cell subsets contribute to the cellular immune response whereas T helper 2 cells participate in the humoral immune response. Following a productive immune response and contraction of the effector T cell population, a few T cells survive to form the memory T cell population (106). The most commonly used markers of T cell memory populations used within mice are CD44 and CD62L. Therefore, central memory T cells are classified as CD62L+ CD44+ and effector memory T cells are classified as CD62L- CD44+ (107).

Mechanisms of peripheral tolerance

The enforcement of central tolerance by negative selection largely prevents self-antigen specific T cell clones from entering the periphery. However, this process

is not perfect and autoreactive T cell clones can leave the thymus and persist within the periphery. Therefore, peripheral tolerance mechanisms have been developed as 'fail-safe' measures to prevent T cell activation and responses of these T cell subsets.

Peripheral tolerance mechanisms can be categorized as extrinsic mechanisms or intrinsic mechanisms. Extrinsic mechanisms involve the dominant suppression of antigen-specific T cells in a cell-mediated fashion by regulatory T cells or dendritic cells (108). It is known that the regulatory T cell population, identified as CD4+ CD25+ Foxp3+, is crucial to T cell suppression and the prevention of autoimmunity. Genetic mutations in the Foxp3 gene result in the spontaneous development of autoimmune disease in the scurfy mouse (109). Dendritic cells have also been implicated in peripheral tolerance, either by inducing the regulatory T cell population or by antigen presentation in steady-state conditions. In a dendritic cell antigen-delivery model system using an antibody against the dendritic cell specific endocytic receptor DEC205, the initial T cell response following antigen encounter resulted in T cell proliferation. However, within 7 days, the number of antigen-specific T cells was drastically reduced and the remaining T cells were unresponsive to stimulation (110). Tolerance had been induced within this steady-state *in vivo* model system using immature dendritic cells. Evidence within the literature suggests that immature dendritic cell populations alone are capable of inducing tolerance whereas mature, activated dendritic cells induce effector functions within T cells (111). However, one study demonstrated that following activation by LPS, mature dendritic cells co-cultured with naïve CD4+ T cells induced an effector phenotype and expression of Foxp3. Furthermore, the CD4+ T cells exerted maximal suppressive effects on CD4+ T cells previously activated in an allogeneic mixed leukocyte reaction (112). This evidence suggested that mature dendritic cells also contribute to T cell suppression in a contact dependent manner.

Intrinsic mechanisms of peripheral tolerance also contribute to the maintenance of

autoreactive T cell clones. Clonal deletion and clonal anergy are two methods of intrinsic tolerance and involve TCR-pMHC interactions. However, engagement of the T cell inhibitory receptors CTLA-4 and PD-1 with their ligand can also induce intrinsic tolerance (108). The role of PD-1 and its contribution to T cell tolerance is reviewed in the next section.

Programmed death-1 (PD-1) and its role in T cell tolerance

Programmed death-1 (PD-1) was originally identified by a subtractive hybridization technique during programmed cell death of murine T cell hybridoma 2B4.11 cells and a murine hematopoietic progenitor cell line LyD9 (113). It is a member of the B7/CD28 family of costimulatory molecules and functions to negatively regulate T cell activation. It has two ligands, programmed death 1 ligand-1 (PD-L1), whose expression is on the epithelium and on all cells of hematopoietic origin, and programmed death 1 ligand-2 (PD-L2), whose expression is restricted to macrophages and dendritic cells (114, 115). Following ligand interaction, PD-1 signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) to down regulate T cell proliferation and limit cytokine production (114) (**Fig. 1-5 A**).

PD-1 is analogous to cytotoxic T-lymphocyte antigen-4 (CTLA-4) as a T cell inhibitory receptor yet their individual contributions to T cell regulation are drastically different. CTLA deficient mice develop a rapid lymphoproliferative disease and die prematurely at 3-4 weeks of age (116). In comparison, PD-1 deficient C57BL/6 (B6) mice develop a late-onset lupus-like autoimmunity with arthritis, glomerulonephritis and autoantibody production against cardiac troponin I resulting in cardiomyopathy (117, 118). Furthermore, $Rag^{-/-}$ recipients of fetal PD-$1^{-/-}$ HSCs developed lethal multi-organ autoimmunity and displayed lymphocytic infiltration in multiple organs including the liver, kidney, pancreas, eyes, heart, lung and esophagus (119).

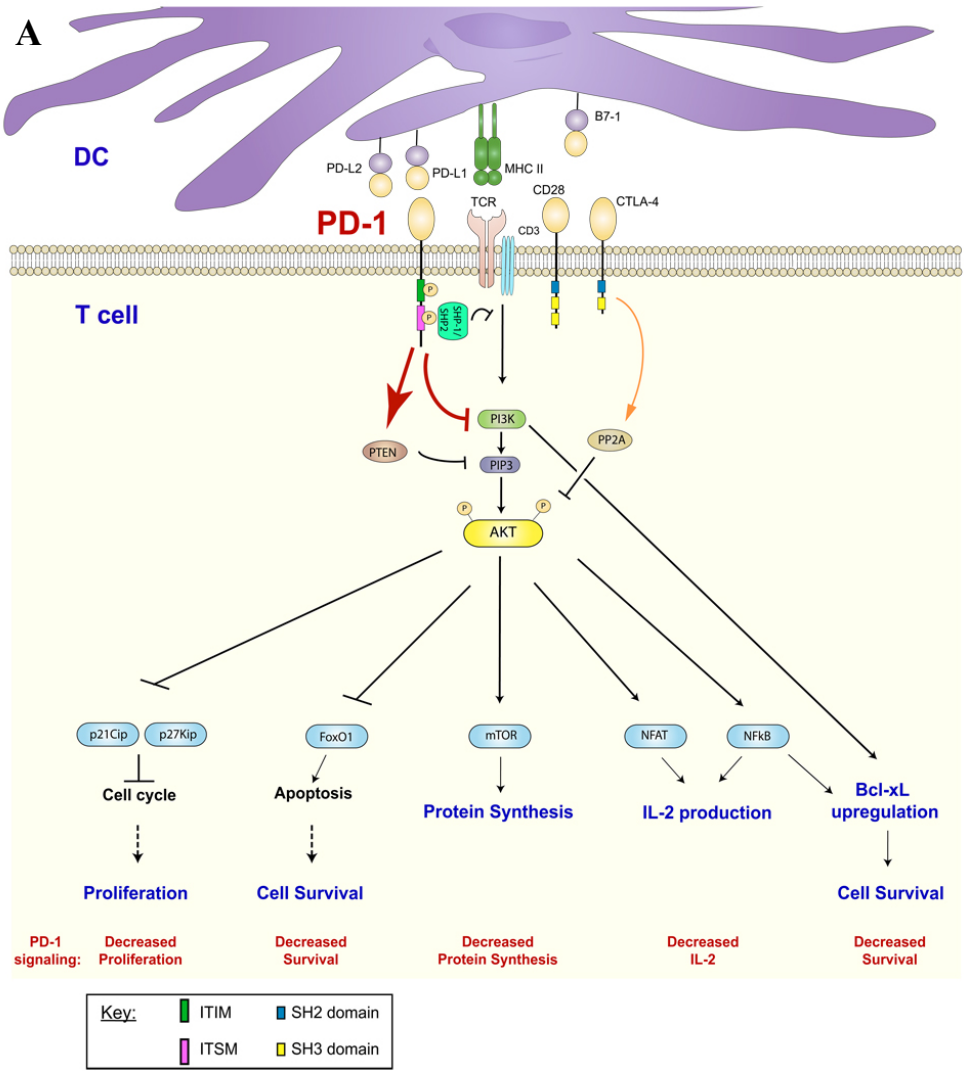


Fig. 1-5. Downstream signaling of the T cell inhibitory receptor PD-1 negatively regulates T cell receptor signaling. **A.** Signaling model following ligation of the inhibitory receptor PD-1 expressed on the surface of T cells. PD-1/PD-L1 or PD-L2 interaction and downstream signaling abrogates TCR signaling to cause decreased proliferation, survival, protein synthesis and IL-2 production. From Franciso *et. al.* (2010) The PD-1 pathway in tolerance and autoimmunity. Immunological Reviews **236**: 219-242, Figure 2.

The contribution of PD-1 to T cell development and central tolerance is not entirely understood. Within the thymus, PD-1 expression is induced during the DN thymocyte stage in adult mice. Experimental studies analyzing B6 thymic tissue directly *ex vivo* found slight induction of PD-1 at the DN1 thymocyte stage later increasing to a larger proportion of PD-1+ thymocytes during the DN4 stage (120). PD-1 expression is induced following TCR ligation within developing DP thymocytes (114). In the absence of PD-1, the increase in apoptotic Annexin V+ DP thymocytes of 2C TCR transgenic/Rag $2^{-/-}$/PD-$1^{-/-}$ mice suggests that PD-1 can regulate selection events (121). However, evidence from HY^{cd4} PD-1 knock-out male mice definitively shows that PD-1 is not required for negative selection (119).

The initial implication of the involvement of PD-1 in tolerance and autoimmunity was recognized with the PD-1 knock-out mouse studies (117). Studies examining tolerance induction of CD8+ T cells by resting dendritic cells showed that the tolerizing signal required PD-1, since PD-1 deficient CD8+ T cells were primed and activated (122). The transfer of OT-1 wildtype or OT-1 PD-1 knock-out T cells into RIP-mOVA recipients directly examined the role of PD-1 in T cell regulation. The OT-1 PD-1 knock-out T cell recipients developed diabetes, displaying T cell expansion within the pancreatic lymph nodes, IFN-gamma production and infiltration into the pancreas whereas OT-1 wildtype T cell recipients remained healthy (123). Therefore, PD-1 appears to be required for the intrinsic inhibition of antigen-specific T cells within these model systems.

With my thesis work, I wanted to determine the mechanisms utilized within the thymus that reduce the appearance of autoreactive T cells and how these autoreactive cells come to be present within the periphery. Furthermore, I wanted to examine the characteristics of the self-reactive T cells present within the periphery that have escaped clonal deletion. These questions were addressed with the use of the T cell receptor transgenic mouse model, HY^{cd4}.

T cell receptor (TCR) Transgenic Mouse Models of Negative Selection

Non-TCR transgenic mouse models exhibit endogenous rearrangement of TCRs, generating a polyclonal T cell repertoire. These models are effective for studying positive selection events but are not as useful for analyzing negative selection. Due to the low frequency of antigen-specific T cells undergoing negative selection within non-transgenic mice, it is difficult to determine the contribution of specific genes to T cell development (124). T cell receptor (TCR) transgenic mouse models were developed to generate a significant proportion of the T cell population with a defined specificity. Therefore, TCR transgenic model systems can be used to study negative selection and the contribution of individual genes to this process. TCR transgenic model systems were generated to also express cognate antigen transgenically while others, such as the HY TCR transgenic model, have an endogenously expressed cognate antigen. While the various TCR transgenic mouse models, including the HY model, have contributed to the better understanding of T cell development and clonal deletion, their use does have limitations. Most TCR transgenic models express the TCR too soon, resulting in premature clonal deletion during the DN thymocyte stage. In comparison, non-TCR transgenic mice undergo clonal deletion at the DP thymocyte stage since this is when the αβ TCR is first expressed. Due to these developmental differences, it has been difficult to definitively identify genes involved in clonal deletion (125). However, the use of the HY^{cd4} physiological mouse model (described below) to examine negative selection has allowed for clearer studies of the genes involved in negative selection (126).

The HY^{cd4} mouse model of negative selection

The HY^{cd4} mouse model was developed to examine thymocyte selection events and can be utilized to analyze the molecular events mediating clonal deletion within a physiological setting that most other TCR transgenic mouse models are incapable of providing. Based on a Cre/loxP conditional expression strategy, the

TCRα chain from the HY TCR (126) is expressed at the DP thymocyte stage of development under control of the CD4 promoter. Therefore, DP thymocytes undergo T cell development in a physiological manner (**Fig. 1-6 A**) (74). The HY male antigen specific TCR is restricted by the Class I major histocompatibility complex (MHC) H-2D^{b} and is recognized by the T3.70 monoclonal antibody specific for the Vα17 of the HY TCRα chain (127). Therefore, female mice that lack HY male antigen expression are models of positive selection and develop CD8SP thymocytes whereas male mice expressing HY male antigen are models of negative selection and do not have a CD8SP thymocyte population. This physiological model provides a simplified method of comparing female and male mice to critically define the molecular events that mediate negative selection within DP thymocytes (126).

In an effort to understand the molecular mechanism of negative selection, a microarray analysis of HY^{cd4} DP thymocytes was performed. The microarray analysis of the DP thymocytes from HY^{cd4} male mice revealed 388 genes uniquely regulated by high affinity antigen encounter (128). Comparison of the microarray results to two other studies previously performed in MHC Class I- and Class II- restricted model systems and qRT-PCR confirmed the list of genes induced following high affinity antigen encounter. Genes of interest that were commonly up regulated during clonal deletion were PD-1, Gadd45β, Nur77 and Bim (128). The functional roles of PD-1 and Gadd45β are examined within this thesis in C57BL/6 (B6) wildtype and the transgenic HY^{cd4} model system.

Presence of self-reactive T3.70+ CD8+ T cells in the periphery of HY^{cd4} male mice

Within HY^{cd4} male mice, the thymus contains DP thymocytes but lacks a CD8SP thymocyte population, suggesting negative selection is intact. However, central tolerance is not perfect and the peripheral compartment of HY^{cd4} male mice

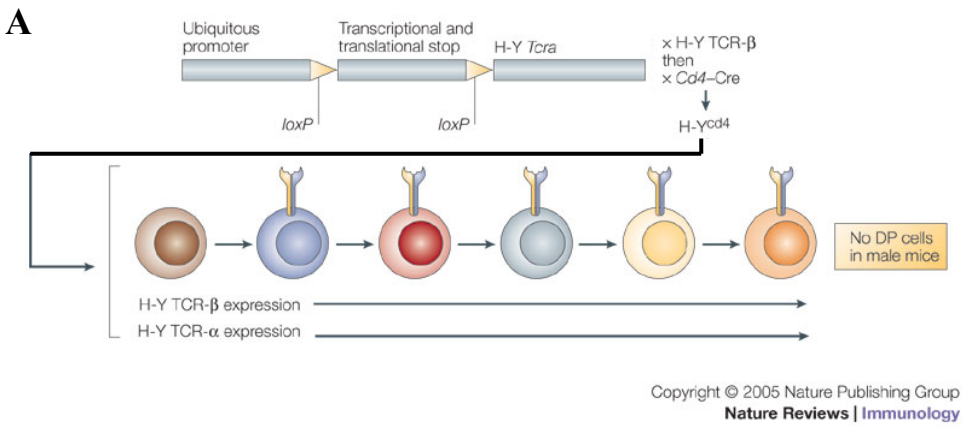


Fig. 1-6. Model of transcriptional regulation of HY TCRα chain by a CD4-Cre construct in the physiological transgenic HY^{cd4} mouse model. **A.** Detailed figure outlining the Cre/lox conditional expression strategy that regulations the TCRα chain expression from the HY TCR in HY^{cd4} mice. Physiological expression of the HY TCRα chain at the DP thymocyte stage allows for study of T cell selection events within HY^{cd4} mice. Figure has been modified from Hogquist, K.A. *et. al.* (2005) Central tolerance: learning self control in the thymus. Nature Reviews Immunology **5**: 772-782, Box 1.

contains a high frequency of T3.70+ CD8+ T cells that do not cause overt autoimmunity. Within the classical HY model system, it was also shown that a large proportion of self-reactive cells undergo negative selection within the thymus. Despite the removal of these cells from the T cell repertoire, the spleen contained T cells that had a high density of TCR and low density of CD8 coreceptor expression and were presumably spared from negative selection (127). Based on this evidence, we began to consider that the appearance of these self-reactive T cells within the periphery was due to something else other than the timing of TCR expression.

We began to consider alternative reasons for the presence of the peripheral T3.70+ CD8+ T cells within HY^{cd4} male mice and thought it could be a feature of the HY TCR. It was considered that the presence of T3.70+ CD8+ T cells within HY^{cd4} male mice was due to inefficient negative selection in the neonatal thymus. In classical HY mice, both HY female and male neonatal mice had T3.70+ T cells within the lymph nodes, but not the spleen, at 4 days post-birth (129). Therefore, it is possible that the antigen-specific T3.70+ T cells are escaping negative selection and entering and persisting within the periphery.

Rationale for the HY^{cd4} neonatal mice studies

Given the evidence of antigen-specific T3.70+ T cells within the periphery of HY neonatal mice, we analyzed T cell development within HY^{cd4} neonatal mice. We wanted to understand the ontogeny of the antigen-specific T3.70+ CD8+ T cell population within HY^{cd4} male mice and wondered if PD-1 contributed to their regulation during neonatal development. To address this question, thymic and splenic tissue of HY^{cd4} neonatal mice of 3, 5 and 11 days post-birth were analyzed for the phenotype of T3.70+ antigen-specific cells in the presence or absence of PD-1. It was hypothesized that neonatal T cell numbers and phenotype would not differ in the presence or absence of PD-1 given that PD-1 is not required for negative selection in HY^{cd4} adult mice (119). The experimental studies of HY^{cd4}

wildtype and PD-1 deficient neonatal mice, as well as B6 neonatal mouse controls, are presented in Chapter 3.

In addition to the neonatal mouse studies, it was of interest to determine the molecular events that mediate negative selection and the proteins involved in this process. Microarray studies of DP thymocytes from HY^{cd4} adult male mice showed the induction of Gadd45β expression following high affinity antigen encounter. Therefore, it appeared that Gadd45β might have a role in negative selection within the HY^{cd4} male mouse model.

Growth arrest and DNA-damage-inducible beta (Gadd45β) and its pro- and anti-apoptotic roles in different cell types

Microarray analysis of DP thymocytes from HY^{cd4} male mice identified Gadd45β as one gene up regulated during negative selection (128). Other microarray analyses also found Gadd45β to be highly expressed in other models of negative selection (130, 131). Therefore we became interested in understanding the role of Gadd45β in thymocyte negative selection within the HY^{cd4} model system.
The Gadd45 family of proteins is known for their role in the genotoxic stress response. It is composed of three family members: Gadd45α, Gadd45β and Gadd45γ. All isoforms are highly acidic, relatively small at 18 kDa in size, highly conserved evolutionarily with sequence homology of 55-57% and localize primarily to the nucleus (132). The structural similarity suggests these proteins have similar function, however, various experimental studies have shown that is not the case. It has been shown that Gadd45α has a role in cell cycle regulation; it associates with p53 and cdc2 complex to inhibit the kinase activity and downstream signaling of the cdc2/Cyclin B1 complex. In comparison, Gadd45β and Gadd45γ have been shown to have differential roles in cellular survival and apoptosis (133) (**Fig. 1-7 A**).

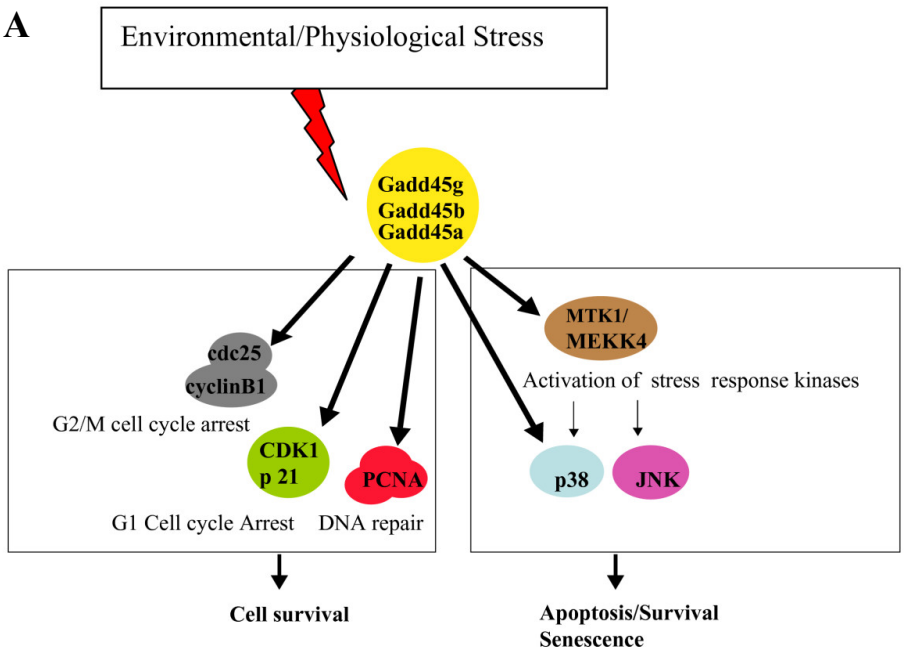


Fig. 1-7. The Gadd45 family of proteins are involved in cell survival and apoptosis responses. **A.** A detailed model showing the response of the Gadd45 protein family, Gadd45α, Gadd45β and Gadd45γ following environmental or physiological stress. The Gadd45 proteins can be involved in cellular survival or apoptosis/senescence depending upon the cell type. Evidence has been presented for Gadd45β to be involved in both roles within varying cell types. From Liebermann, D.A. and B. Hoffman. (2008) Gadd45 in stress signaling. Journal of Molecular Signaling **3**: 1-8 (doi: 10.1186/1750-2187-3-15), Figure 2.

The pro-apoptotic role of Gadd45β

Gadd45β was first discovered and shown to have a pro-apoptotic role in M1D+ myeloid leukemia cells treated with IL-1 and IL-6 and initially called myeloid differentiation immediate early gene MyD118 (134). Experimental studies within hepatocytes and hepatocellular carcinomas have shown that Gadd45β is involved in the downstream activation of the MAPKs p38 and JNK and apoptosis induction. Within the murine hepatocyte AML12 cell line, Gadd45β potentiates Fas-induced apoptosis by promoting the interaction between the MAPK p38 and retinoblastoma tumor suppressor (Rb). This activity of Gadd45β enhances Rb phosphorylation and activation by increasing p38 and Rb association (135). Using the same AML12 cell line system, Gadd45β is upregulated during transforming growth factor beta (TGF-β) induced apoptosis in a Smad-dependent manner. Ectopic expression of Gadd45β was sufficient to induce p38 activation and promote apoptosis, while anti-sense Gadd45β blocked apoptosis induction (136). Furthermore, Gadd45β was induced following treatment of hepatocellular carcinoma cell lines with the anti-cancer drug sorafenib, resulting in downstream activation of JNK. Therefore, Gadd45β had an apoptosis-inducing role within this carcinoma following sorafenib treatment (137).

The anti-apoptotic role of Gadd45β

An abundance of other experimental evidence has shown Gadd45β has an anti-apoptotic role within various tissues and cell lines. Gadd45β functions in an anti-apoptotic manner by promoting cell survival during stress and regulating the cell cycle. During the first 12 hours of hyperosmotic stress of a murine renal inner medullary-derived cell line, Gadd45α and Gadd45β mediated activation of caspase 3 and 7. Following 12 hours, any further pro-apoptotic activity was prevented by Gadd45α/β overexpression, which the authors attributed to the role of Gadd45α/β in modulating the stress response to promote cellular survival (138). Furthermore, the role of Gadd45α and Gadd45β in cell cycle control was

demonstrated when HSCs from the bone marrow of Gadd45α and Gadd45β -/- mice failed cellular arrest at the G2/M checkpoint following treatment with the anti-cancer drugs ultra violet radiation chemotherapy (UVC) and etoposide (VP-16) treatment but not to daunorubicin. Cell cycle arrest of the Gadd45α and Gadd45β deficient HSCs post-daunorubicin treatment suggests that specific cellular checkpoints may be dependent or independent of Gadd45α and Gadd45β (139).

The pro-survival activity of Gadd45β appears to be mediated through the MAPK pathway. Its expression is regulated by the NF-κB subunit RelA in HeLa cells (140) and its induction by NF-κB is required to prevent apoptosis of 3DO T cell hybridoma cells by JNK signaling (141). Within an *in vivo* model following hepatectomy, hepatocytes from Gadd45β $^{-/-}$ mice had decreased survival and increased programmed cell death during liver regeneration. Apoptosis of Gadd45β knock-out hepatocytes was due to increased JNK activation, observed by western blot. Gadd45β may prevent apoptosis through JNK activity, because a JNK-2 null mutation restored the regeneration of the liver tissue (142). Gadd45β may regulate JNK by directly binding to the upstream regulator of JNK, MAPK kinase 7 (MKK7). Gadd45β-MKK7 binding was shown to prevent MKK7 catalytic activity in activating JNK in an *in vitro* pull down assay with 3DO T cells (143). Furthermore, within hepatocytes, Gadd45β inhibits JNK by associating with constitutive active/androstane receptor (CAR) (144).

The function of Gadd45β in T cells

Gadd45β has been shown to contribute to the effector response and apoptotic pathway in T cells. It appears to alter downstream signaling of the MAPK pathway by directly associating with the upstream activator of JNK, MKK7 and may bind in a yet unidentified way with the upstream activator of p38, MEKK4 (MAP kinase kinase kinase 4) (145). MEKK4 $^{-/-}$ CD4+ T cells displayed reduced p38 activity and IFN-γ production, which was restored following retroviral

transduction of Gadd45β expression (146). Analysis of Gadd45β $^{-/-}$ mice has provided further evidence of its role in regulating T cell activity. Gadd45β deficient effector T_H1 cells had reduced p38 activation following short and long-term *in vitro* anti-CD3 stimulation, suggesting Gadd45β is required for sustained p38 activation following TCR stimulation. Gadd45β was also shown to be required for the effector inflammatory response of peripheral CD4+ T cells. Following *in vitro* stimulation with anti-CD3 for 24 hours, Gadd45β deficient T cells had impaired downstream activation of the MAPKs ERK, p38 and JNK. ELISA assays of the T cell supernatants revealed reduced production of IL-2 and IFN-γ in T_H0, T_H1 and T_H2 cells. Furthermore, Gadd45β is also required for the inflammatory response of T cells *in vivo*. Gadd45β deficient animals had a smaller proportion of IFN-γ producing T cells directly *ex vivo* 7-days post intravenous injection with 5 x 10^3 live *Listeria monocytogenes*. Therefore, it appears that Gadd45β is required for the downstream TCR signaling and inflammatory responses of T cells to both *in vitro* and *in vivo* stimulation (147). Finally, Gadd45β and Gadd45γ double knock-out mice rapidly succumbed to a lymphoproliferative disorder and systemic lupus erythematosus, demonstrating the importance of these proteins to T cell tolerance (148).

Rationale for examining the role of Gadd45β in clonal deletion

Microarray analysis has identified Gadd45β as one gene up regulated during negative selection of DP thymocytes within the HY^{cd4} male mouse model (128). Given the evidence demonstrating the contribution of Gadd45β to the apoptotic pathway through the MAPKs p38 and JNK and the autoimmune disorders in Gadd45β deficient mice (147, 148), we became interested in examining the contribution of Gadd45β to T cell development within the non-transgenic B6 background and negative selection within the HY^{cd4} mouse model. B6 wildtype and Gadd45β knock-out mice and HY^{cd4} Gadd45β wildtype and knock-out mice were generated to assess any differences within the thymic and peripheral T cell subsets. It was hypothesized that Gadd45β knock-out mice would exhibit reduced

clonal deletion and impaired T cell activity given the regulatory role of Gadd45β on p38 and JNK activation and the contribution of those MAPKs to clonal deletion. The experimental studies of B6 Gadd45β wildtype and knock-out mice and HY^{cd4} Gadd45β wildtype and knock-out mice are analyzed in Chapter 4.

Scope of the experimental analysis presented in this thesis

Within the HY^{cd4} mouse model, despite robust negative selection within the thymus, there is an antigen-specific CD8+ T cell population within the periphery (89). This observation is similar to what has been observed within the classical HY mouse model (127). It was considered that the neonatal stage of development was the origin of this self-reactive T cell population. Therefore, the ontogeny of the T3.70+ T cells within the thymic and splenic compartments of HY^{cd4} neonatal mice were analyzed. Additionally, PD-1 deficient neonates were compared to wildtype to determine if the inhibitory receptor contributed to T cell development during the neonatal stages. These findings are presented in Chapter 3.

Previous studies identified multiple genes potentially responsible for regulating negative selection of T cells, one of which was Gadd45β. In order to understand the contribution of Gadd45β to T cell development, B6 Gadd45β wildtype and knock-out mice were compared and HY^{cd4} Gadd45β wildtype and knock-out mice were compared. These findings are presented in Chapter 4.

Finally, one notable hallmark of the HY^{cd4} male mouse is down regulation of CD8 coreceptor. It has been thought that this process is due to TCR/CD8 internalization following peptide-MHC Class I encounter within the periphery (149). However, it was also considered that CD8 coreceptor levels might be actively modulated to prevent reactivity of this antigen-specific CD8+ T cell population to the ubiquitously expressed HY male antigen. Therefore, it was hypothesized that antigen-specific HY^{cd4} CD8+ T cells are regulated within the periphery by the IL-7 dependent mechanism, coreceptor tuning. *In vitro*

experiments analyzing coreceptor tuning and its possible contribution to peripheral tolerance of HY^{cd4} male CD8+ T cells is outlined in the Chapter 7 - Appendix.

Chapter 2: Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from Jackson Laboratories or NCI Frederick or from transgene negative littermates from RIP-mOVA mice. Gadd45β $^{-/-}$ mice were obtained from Frank Gonzalez at the National Institutes of Health (NIH). HY^{cd4} mice were generated as previously described (126). Breeding HY^{cd4} mice with Gadd45β $^{-/-}$ mice generated HY^{cd4} Gadd45β $^{-/-}$ mice. PD-1 $^{-/-}$ mice were obtained from Dr. Tasuku Honjo at Kyoto University and bred with HY^{cd4} mice to generate HY^{cd4} PD-1 $^{-/-}$ mice. For neonatal mice, the date of a litter's birth was defined as Day 0. All mice were bred and maintained within our colony at the University of Alberta. They were treated in accordance with protocols approved by the University of Alberta Animal Care and Use Committee and adult mice were used between 4 and 12 weeks of age for experiments.

Bone Marrow Chimeras

Bone marrow from congenic HY^{cd4} wildtype (CD45.1/2) and PD-1 knock-out (CD45.2) male mice was harvested and mixed with B6 (CD45.2) wildtype bone marrow in a 1:1:3 ratio. Bone marrow was then injected into lethally irradiated B6 wildtype male recipients and reconstituted for 10 weeks. Mice were then euthanized to analyze the thymus, spleen and lymph node tissue.

Experimental Protocols

Tissue Collection

Thymus, spleen and lymph nodes were harvested from euthanized adult mice and neonatal mice, and gently ground into a wire mesh screen to create a single cell suspension. Cells were suspended in Hank's Balanced Salt Solution (HBSS)

unless the experimental procedure required sterile culture in which case cells were suspended in sterile RP10 (RPMI-1640 media with 10% fetal calf serum [FCS] and 5% supplementary completum [SC]; SC contains 5% RPMI-1640 media, 5 mM HEPES, 50 mg/mL penicillin/streptomycin, 2 mM L-glutamine, 50 mM 2-mercaptoethanol and 50 mg/mL gentamicin sulfate. Cellular concentration of adult mouse tissue was determined by diluting the cell suspension 1:1 in 10% Trypan Blue and live cell counting on a haemocytometer (FisherScientific). Neonatal mouse tissue cellular concentrations were determined with the use of CountBright counting beads – the detailed protocol is provided within the Flow Cytometry section. Cells were maintained in sterile RP10 in preparation prior to flow cytometric staining or *in vitro* culture.

Coating of well plates with functional grade anti-CD3 and anti-CD28 antibodies

Functional grade anti-CD3 was diluted to 10 μM and anti-CD28 to 5 μM in phosphate buffered saline (PBS) and added to the desired well plates. The plate(s) were incubated at 4°C for 16 hours or 37°C for 2 hours. Following incubation, the antibody solution was aspirated away from the wells of the plate, the wells were washed twice with PBS and the responder cells were added at 2 million cells/mL. Cells were incubated at 37°C and 5% CO_2 for the desired amount of time and assayed according to the experimental protocol. Functional grade anti-CD3 and anti-CD28 antibodies were purchased from eBioscience.

Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Labeling of Cells

Cells were resuspended at 10 million cells/mL in sterile PBS. Cells were then stained with carboxyfluorescein diacetate succinimidyl ester (CFSE), Molecular Probes, at a concentration of 1.25μM. The CFSE-cell suspension was immediately mixed by inverting the tube multiple times and incubated in a 37°C water bath for 10 minutes. The CFSE-cell suspension was mixed twice during the

10-minute incubation to ensure uniform labeling of the cells. Topping off the tube with sterile RP10 quenched the mixture. After centrifugation, the cells were then resuspended in 5mL of sterile RP10 and counted with a hemocytometer. Cells are then resuspended at 10 million cells/mL.

In vitro Culture for Activation Induced Cell Death of Gadd45β -/- Thymocytes

Congenic B6 Ly5.2 splenocytes were loaded with the 0 μg, 0.01 μg, 0.1 μg and 1.0 μg of anti-CD3 antibody and incubated for 30 minutes in a 37°C and 5% CO_2 incubator. B6 and Gadd45β $^{-/-}$ thymocytes were then cultured with the B6 Ly5.2 anti-CD3 loaded splenocytes in a 4:1 ratio. Cultures were placed in a 37°C 5% CO_2 incubator for 16 hours. Cells were then harvested and assessed by flow cytometry.

In vitro Culture for Proliferation of Gadd45β $^{-/-}$ Splenocytes

B6 splenocytes were loaded with 0 μg, 0.1 μg and 2.0 μg of anti-CD3 antibody and incubate for 30 minutes in a 37°C and 5% CO_2 incubator. Congenic B6 and Gadd45β $^{-/-}$ splenocytes were CFSE labeled. CFSE labeled splenocytes were then co-cultured *in vitro* with anti-CD3 loaded splenocytes for 48 and 72 hours in a 37°C 5% CO_2 incubator. Following *in vitro* culture, cells were then harvested and examined by flow cytometry for apoptotic marker expression and for CFSE dilution to assess cellular proliferation.

Loading of B6 splenocytes with smcy peptide (HY male antigen)

The stock concentration of *smcy* peptide (peptide sequence: KCSRNRQYL, ProImmune) is 10 mM and was diluted to the desired concentrations (10 nM, 25 nM, 50 nM, 100 nM and 1 μM) in sterile RP10. The required volume of the *smcy* peptide was added to B6 splenocytes at a concentration of 2 million cells/mL. This co-culture was then incubated at 37°C and 5% CO_2 for a minimum of 30

minutes. During incubation, the responder cells were aliquoted into a well plate at a concentration of 2 million cells/mL. Following incubation, the *smcy*-peptide loaded B6 splenocytes was then aliquoted into the wells containing the responder cells at a ratio of 1:4. After the desired time of cell stimulation, the cells were removed from the incubator and assayed according to the desired experimental protocol.

Flow Cytometry

Cell surface staining

A volume of 200 μL of thymic, splenic or lymph node cells was aliquoted for each experimental sample in a 96-well plate. For the neonate experiments, a 100 μL volume was used for each thymic sample and 50 μL was used for each splenic sample. Prior to antibody staining, thymic and peripheral cells were treated with anti-Fc receptor antibody (supernatant from 24G.2 hybridoma cells) at a 1:200 ratio in FACS buffer (phosphate buffered saline (PBS), 1% fetal bovine serum and 0.02% sodium azide) for 15 minutes at 4°C. Cells were then washed once with FACS buffer. All antibody staining was done in a 1:200 concentration in FACS buffer except for anti-Bcl-2 staining which was done in a 1:10 concentrations as recommended by the manufacturer. Once surface antibodies were added, cells were incubated for 30 minutes at 4°C and washed two times with FACS buffer. After the last wash, cells were resuspended in 200 μL of FACS buffer and transferred to FACS tubes. The following antibodies were purchased from eBioscience: CD4, CD8α, CD8β, TCRβ, CD3ε, γδ TCR, HY TCR (T3.70), Vβ8 TCR, CD19, NK1.1, CD69, PD-1, CD24, CD25, CD44, CD45.2, CD62L, CD127, CD122, CD5, CD103 and Annexin V. Fluorochome conjugated streptavidin molecules were also purchased from eBioscience.

Intracellular staining

Surface antibody staining was done prior to intracellular staining for proteins of interest except Bim. Cells were fixed and permeabilized with the BD Cytofix/Cytoperm^{TM} Fixation/Permeabilization Kit as per the manufacturer's instructions prior to staining for cleaved caspase 3, Bcl-2 and Bim. The cleaved caspase 3 antibody (D175) was purchased from Cell Signaling Technology. Intracellular Bcl-2 and Bcl-2 isotype staining was done with the PE Hamster anti-mouse Bcl-2 set from BD Pharmingen. Anti-Bim$_{S/EL/L}$ (Clone 10B12) monoclonal antibody was purchased from Alexis Biochemicals. The anti-rabbit secondary antibodies were purchased from Invitrogen. Cells that were assayed for Nur77 expression were fixed and permeabilized with the Foxp3 Staining Buffer Set according to the manufacturer's instructions. The Nur77 antibody was purchased from eBioscience.

CountBright Absolute Counting Beads

The thymus and spleen from B6 and HY^{cd4} neonatal mice were small to count with the hemocytometer, therefore CountBright Absolute Counting beads from Invitrogen were used. The beads were used as an indirect method of determining the cellularity of the thymic and spleen tissue from the neonatal mice. Comparison of the number of beads acquired to the number of beads originally added provided a ratio that could be applied to determine the original cellularity of each tissue.

Prior to acquisition of the samples on the flow cytometer, 20 µL of CountBright Absolute Counting Beads was added to each sample which equates to 20 000 beads. In order to observe the beads on the flow cytometer, the side scatter voltage was reduced and the beads were visible when examining the forward and side scatter of the cells. During acquisition of the samples, the beads were also collected. The number of beads acquired was then determined based on the bead

gating during flow cytometry analysis of each neonatal thymus and spleen sample. Based on the number of beads acquired and the number of beads added, a ratio can be determined: # beads acquired/20 000. Once the number of live cells is determined within a thymus or spleen sample, and the number of times the sample was divided for staining, the cellularity of the tissue can be determined based on the following formula:

$$\frac{\text{Number of Live Cells}}{\text{Ratio of Beads Acquired vs. Added}} \times \text{Number of Times the Sample was Divided}$$

Based on the calculated cellularity for the thymus and spleen samples, the absolute number of various T cell populations could be determined from B6 and HY^{cd4} neonatal mice.

Data Collection and Analysis

The thymus, spleen and lymph node samples from various mouse models were examined on a FACS Canto II (BD Biosciences) or a LSR II (BD Biosciences) flow cytometer. FCS 2.0/3.0 files were exported and analyzed using FlowJo software (Tree Star Inc.) for data analysis.

Statistical Analysis

Prism (GraphPad Software) was used to generate graphs and perform statistical analysis. A two-tailed unpaired student's *t* test with a 95% confidence interval was used to determine the *p* values.

Chapter 3: Results

Examining the Ontogeny of Antigen-Specific T3.70+ T cells in HY^{cd4} Neonatal Mice

The thymus of the HY^{cd4} male mouse model contains a substantial proportion of self-reactive DP thymocytes dulling the surface expression of CD4 and CD8 coreceptors. The absence of a CD8SP thymocyte population suggests that these T cells are removed by negative selection mechanisms. However, despite robust selection events within the thymus, there is a large proportion of self-reactive T cells within the periphery of HY^{cd4} male mice. In order to understand the ontogeny of this self-reactive T cell population, the T cell compartments of HY^{cd4} neonatal female and male mice were examined at Days 3, 5 and 11 post-birth.

Characterization of the B6 and HY^{cd4} Adult Mouse Controls

HY^{cd4} mice contain a substantial proportion of antigen-specific T3.70+ T cells

The HY^{cd4} mouse model is a physiological T cell receptor (TCR) transgenic mouse model of T cell selection and can be utilized to study the molecular mechanisms responsible for mediating selection events. A substantial proportion of T cells within HY^{cd4} mice express the transgenic TCR and the remaining T cells have endogenously rearranged TCR chains. The self-reactive TCR, HY TCR, is restricted by the MHC Class I molecule H-2D^{b}, therefore all HY TCR expressing T cells develop into the CD8+ T cell lineage. The cognate antigen for the HY TCR is the HY male antigen and it is not expressed within HY^{cd4} female mice; therefore, HY^{cd4} female mice are a model of T cell positive selection. In contrast, HY male antigen is expressed within HY^{cd4} male mice, therefore HY TCR expressing thymocytes encounter their high affinity antigen and are removed from the repertoire. Due to this activity, HY^{cd4} male mice are a model of negative selection. Despite the enforcement of central tolerance, HY^{cd4} male mice have a

self-reactive T3.70+ T cell population within the periphery. It is currently unclear what the origin of the T3.70+ T cell population is and we considered that these cells were escaping negative selection.

Directly *ex vivo* DP thymocytes from HY^{cd4} male mice undergoing negative selection express high levels of the activation marker CD69, and many thymocytes express the inhibitory molecule PD-1. However within the periphery, analysis shows that the majority of CD8+ T cells are CD69- PD-1-, suggesting that these cells are not overtly activated within the periphery. It is currently unclear if CD69 and PD-1 expression are turned off prior to export from the thymus or within the periphery.

We wanted to understand the ontogeny of these peripheral antigen-specific T cells within HY^{cd4} male mice and determine whether or not they expressed or could be regulated by PD-1. In order to address these questions, I assayed HY^{cd4} wildtype and PD-1 knock-out neonatal mice, at Day 3, 5 and 11 post-birth, to characterize the antigen-specific T3.70+ T cell populations of the thymus and spleen. I also wanted to determine the impact of PD-1 deficiency on the neonatal T cell repertoire in comparison to B6 adult female and HY^{cd4} adult male controls. B6 neonates were also analyzed at Day 3, 5 and 11 post-birth to get a baseline of polyclonal T cell development.

HY^{cd4} male mice lack a CD8SP thymocyte population due to negative selection

In order to analyze the monoclonal T cell population of HY^{cd4} mice, the antigen-specific thymocytes were first segregated from other cell types based on HY TCR surface expression. HY TCR expressing thymocytes were separated by T3.70 antibody binding and marked as T3.70- and T3.70+. Therefore, within the B6 adult female mouse, nearly all of the cells in the thymus were T3.70- (**Fig. 3-1 A**). In comparison, approximately 30-50% of thymocytes were T3.70+ within the thymus of the HY^{cd4} female mouse, whereas that proportion was generally higher

in a HY^{cd4} male mouse thymus (**Fig. 3-1 A**). Within both the HY^{cd4} female and male thymi, there was a proportion of T3.70- cells; this subset is composed of T cells expressing endogenously rearranged TCRα chains to form the polyclonal, non-transgenic T cell population of the HY^{cd4} mice.

Total wildtype thymocytes and antigen-specific T3.70+ thymocytes were further analyzed for CD4 and CD8 coreceptor expression. Based on CD4 and CD8 coreceptor expression, the thymocyte subsets are identified as follows: CD4- CD8-, double negative thymocytes (DNs); CD4+ CD8+, double positive thymocytes (DPs); CD4+ CD8-, CD4 single positive thymocytes (CD4SPs); and CD4- CD8+, CD8 single positive thymocytes (CD8SPs). Within the thymus of the B6 adult female mouse, DN thymocytes accounted for the smallest proportion of thymocytes whereas the DP thymocyte compartment was the largest in proportion (**Fig. 3-1 B**). The individual single positive thymocyte subsets, CD4SP and CD8SP accounted for about 20% of total thymocytes.

When the B6 adult female control was compared to the HY^{cd4} mice, the stark contrast of the T3.70+ thymocyte populations was evident. The thymus of the HY^{cd4} female mouse had very few DN thymocytes but had a similar proportion of DP thymocytes compared to the B6 female control. A few cells were present in the CD4SP thymocyte compartment within the HY^{cd4} female mouse. However, a sizeable population of antigen-specific CD8SP thymocytes were present, consistent with positive selection (**Fig. 3-1 B**). When the thymus of the HY^{cd4} male mouse was analyzed, the thymocyte subsets looked different than the HY^{cd4} female. The HY^{cd4} male mouse had a large, broadening DP thymocyte population that spilled over into the DN thymocyte gate (**Fig. 3-1 B**). The CD8SP thymocyte population was absent in HY^{cd4} male mice, suggesting these cells were removed by negative selection.

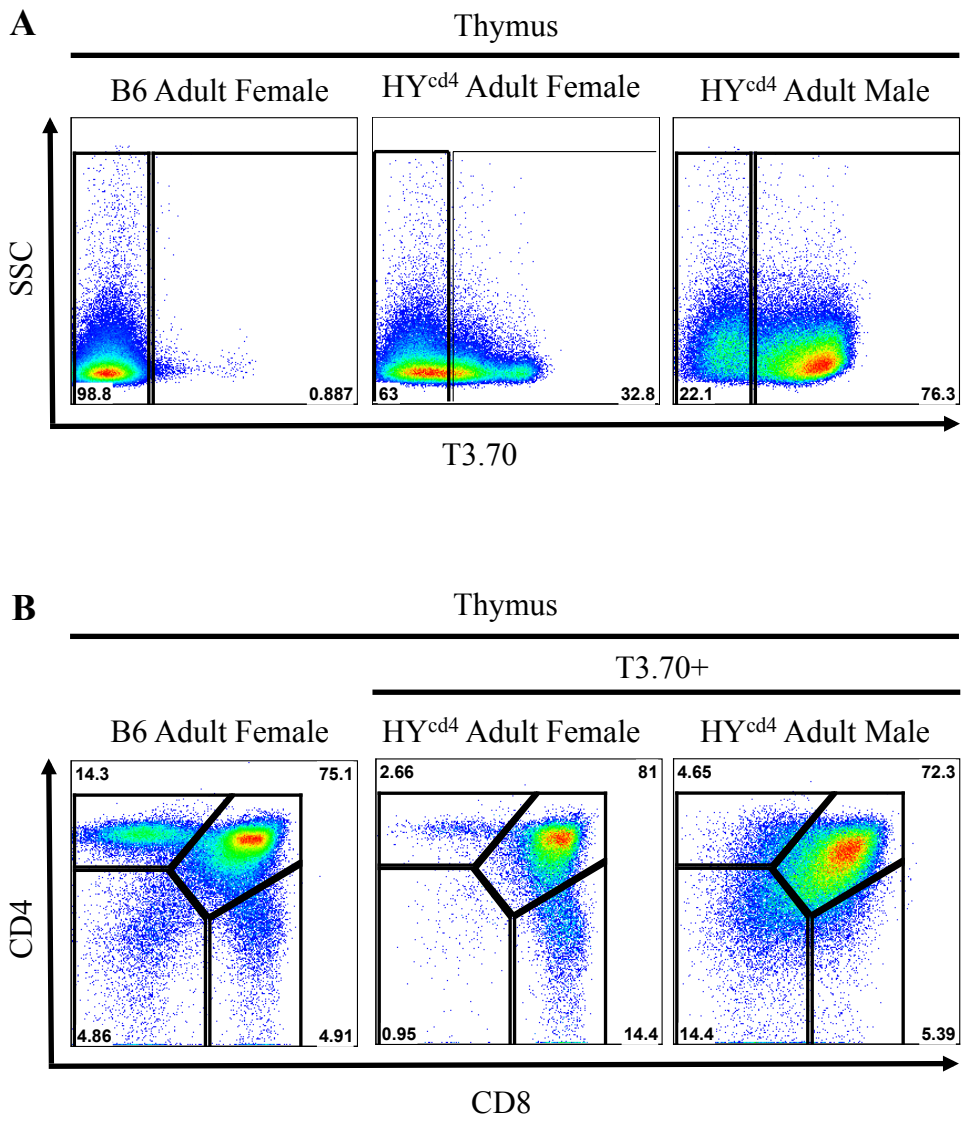


Fig. 3-1. The thymic population of HY^{cd4} adult male mice contained a broadening T3.70+ DP thymocyte population that differed in phenotype to HY^{cd4} and B6 adult female mice. **A.** Representative flow cytometric plots of T3.70 TCR expression on total thymocytes from B6 adult female, HY^{cd4} adult female and male mice. **B.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression on total thymocytes of the B6 adult female and T3.70+ thymocytes of the HY^{cd4} adult female and male mice. Representative of B6 n = 10; HY^{cd4} F n = 9; HY^{cd4} M n = 9.

Negative selection is leaky and not intact within HY^{cd4} adult male mice

The spleen was analyzed to assess the polyclonal T cell populations of the B6 adult female mouse compared to the antigen-specific T3.70+ T cell populations of the HY^{cd4} female and male mice. Similar to the thymus, the spleen T cell populations were initially gated by HY TCR expression with the T3.70 antibody. Within the B6 adult female spleen, approximately 98% of cells were T3.70- and the remainder was T3.70+ (**Fig. 3-2 A**). This small proportion of T3.70+ cells was due to endogenous TCRα chain rearrangement and was quantifiable since the monoclonal T3.70 antibody recognizes the TCRα chain. Furthermore, the HY^{cd4} adult female spleen had a similar proportion of T3.70- cells to the B6 adult female, at approximately 95% of splenocytes. However, there was a distinct population that was T3.70+ accounting for 3-5% of splenocytes (**Fig. 3-2 A**). As HY^{cd4} female mice are a model of positive selection, it was expected to see this population of T3.70+ T cells. As previously published, contrary to expectations, there was a T3.70+ T cell population within the HY^{cd4} male mouse spleen, accounting for 10-15% of splenocytes (**Fig. 3-2 A**) (89). Although negative selection was intact, based on the absence of a thymic T3.70+ CD8SP population, the presence of T3.70+ T cells within the spleen suggested that negative selection is imperfect and some T cells may have escaped clonal deletion.

The T3.70- population of the B6 adult female and the T3.70+ populations of the HY^{cd4} female and male mice were then assessed for CD4 and CD8 coreceptor expression to distinguish individual T cell subsets. Within the B6 adult female control, the spleen was not gated with other T cell markers; therefore the majority of the cells within the DN gate are non-T cells. As the B6 female has a polyclonal T cell repertoire, it was expected to see both CD4+ and CD8+ T cell populations within the spleen. The CD4+ T cell compartment accounted for about 15-20% of T3.70- splenocytes whereas the CD8+ T cell compartment accounted for 8-12% of T3.70- splenocytes (**Fig. 3-2 B**).

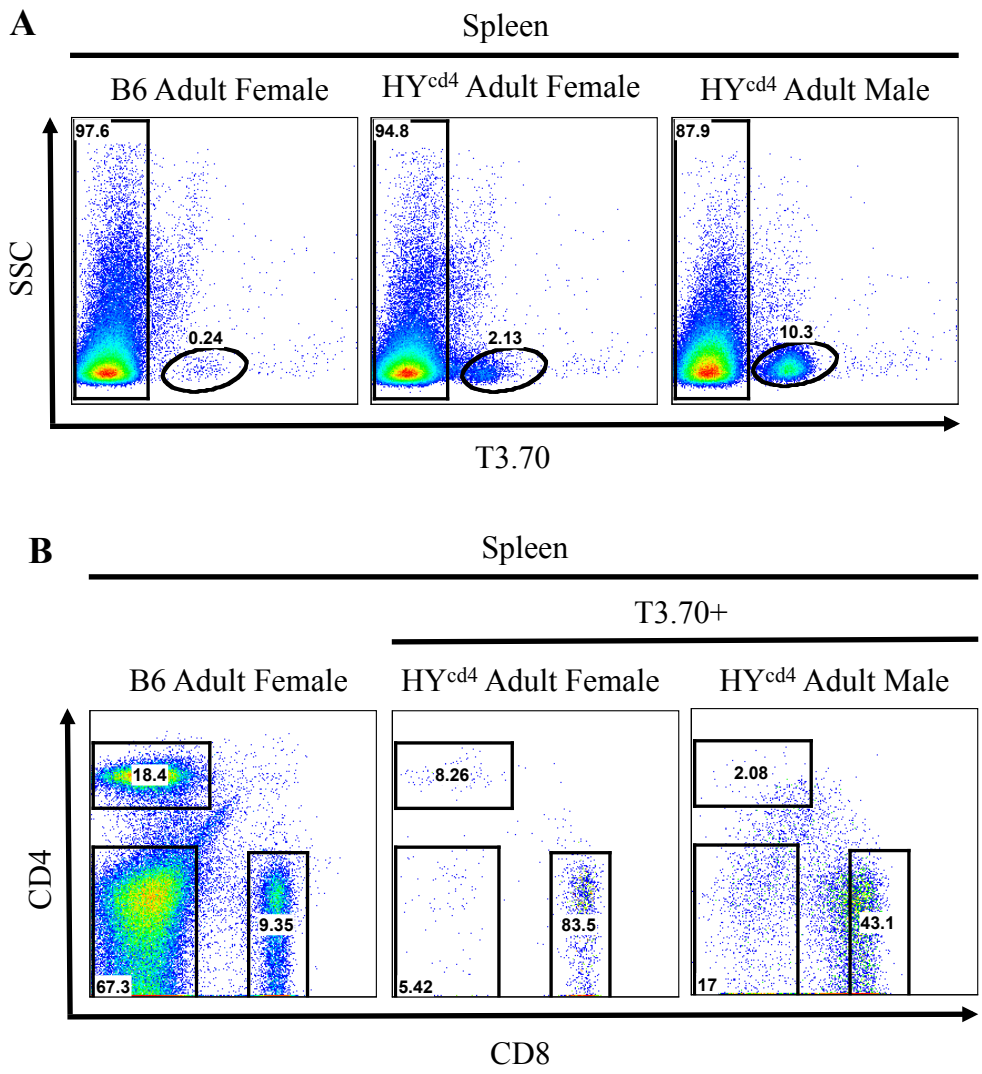


Fig. 3-2. The spleen of HY^{cd4} adult male mice contained a substantial proportion of T3.70+ CD8+ T cells compared to HY^{cd4} adult female mice despite negative selection in the thymus. **A.** Representative flow cytometric plots of T3.70 TCR expression on total splenocytes from B6 adult female, HY^{cd4} adult female and male mice. **B.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression on total splenocytes of the B6 adult female and T3.70+ splenocytes of the HY^{cd4} adult female and male mice. Representative of B6 n = 7; HY^{cd4} F n = 7; HY^{cd4} M n = 5.

Within the monoclonal T cell repertoire of HY^{cd4} mice, the T cell subsets differed from the B6 adult female mouse. The T3.70 TCR is MHC Class I restricted, therefore I expected to see only the presence of a CD8+ T cell population within HY^{cd4} female mice. As expected, the majority of the peripheral T3.70+ T cells were CD8+ T cells and a small proportion of cells were also found within the DN and CD4+ T cell gates (**Fig. 3-2 B**). The small percentage of CD4+ T cells within the T3.70+ splenocyte compartment could be due to cells expressing endogenous TCRα chains within HY^{cd4} female mice.

When the T3.70+ population of cells from the spleen of HY^{cd4} male mice was analyzed there was a substantial proportion of CD8+ T cells. Furthermore, a large population expressed lower levels of the CD8 coreceptor when compared to the HY^{cd4} female spleen, which caused about half of the population to fall out of the CD8+ T cell gate. Therefore, the proportion of CD8+ T cells was likely higher than 40-45%, but the gating was maintained for relative comparison to the B6 adult female control. Furthermore, there were small frequencies of T3.70+ T cells that fell within the CD4+ and DN T cell gates (**Fig. 3-2 B**). Therefore, it appeared that the antigen-specific T3.70+ T cell population that escaped negative selection within the thymus was largely composed of CD8+ T cells that had down regulated CD8 coreceptor.

B6 and HY^{cd4} wildtype and PD-1knock-out female and male neonatal thymi have altered thymocyte subsets compared to B6 and HY^{cd4} adult male controls

Despite the presence of the antigen-specific T cell population within the periphery of HY^{cd4} male mice, these cells do not cause overt autoimmunity (*unpublished data*). Therefore, we began to consider the ontogeny of the T3.70+ antigen-specific population and hoped that if we understood where these cells originated from that might provide insight into their tolerance regulation that is preventing autoimmunity. Therefore neonatal HY^{cd4} female and male mice were analyzed at Day 3, 5 and 11 post-birth, and compared to age-matched B6 neonates, to

determine when these T cells first appeared in the periphery and their surface expression of various T cell markers.

Day 11 B6 neonatal mice have a strange CD8SP intermediate T cell population

The thymic analysis of the T cell subsets within neonatal mice began by examining CD4 and CD8 coreceptor expression of the B6 neonatal mice. B6 neonates were examined first to determine a baseline of thymic T cell subsets within a polyclonal repertoire of a neonate and how they differed from a B6 adult mouse. They also served as a comparison to the transgenic, T3.70+ populations from HY^{cd4} neonates. Within the B6 neonates, the thymocyte populations appeared to be aggregated together more so than in the B6 adult female. A small proportion of cells were DN thymocytes whereas the majority of cells were DP thymocytes between Day 3 and Day 11 post-birth, similar to what was observed in the B6 adult female (**Fig. 3-3 A**). There were CD4SP and CD8SP subsets also present within the thymus of B6 neonates as early as Day 3 post-birth.

The thymocyte subsets of the Day 3 and Day 5 B6 neonates appeared similar but at Day 11 post-birth the subsets looked different. The CD8SP thymocyte population appeared to have shifted against the DN and DP thymocyte gates and had reduced CD8α coreceptor expression (**Fig. 3-3 B**). This observation was in stark contrast to the CD4 by CD8α coreceptor profiles observed within the B6 adult female (**Fig. 3-1 B**) and the population was only present in 3 out of 5 neonates (**Fig. 3-3 A**, **B**). When analyzed by CD8 coreceptor and TCRβ expression, the majority of cells were CD8+ TCRβ- and likely the immature single positive thymocyte population. A small proportion of cells were CD8+ TCRβ+ and therefore mature CD8SP thymocytes (**Fig. 3-3 B**).

The absolute numbers of the thymocyte subsets within B6 neonates were calculated to ensure there were no drastic changes in cell number during neonatal development that were not reflected by cell frequency. The absolute number of

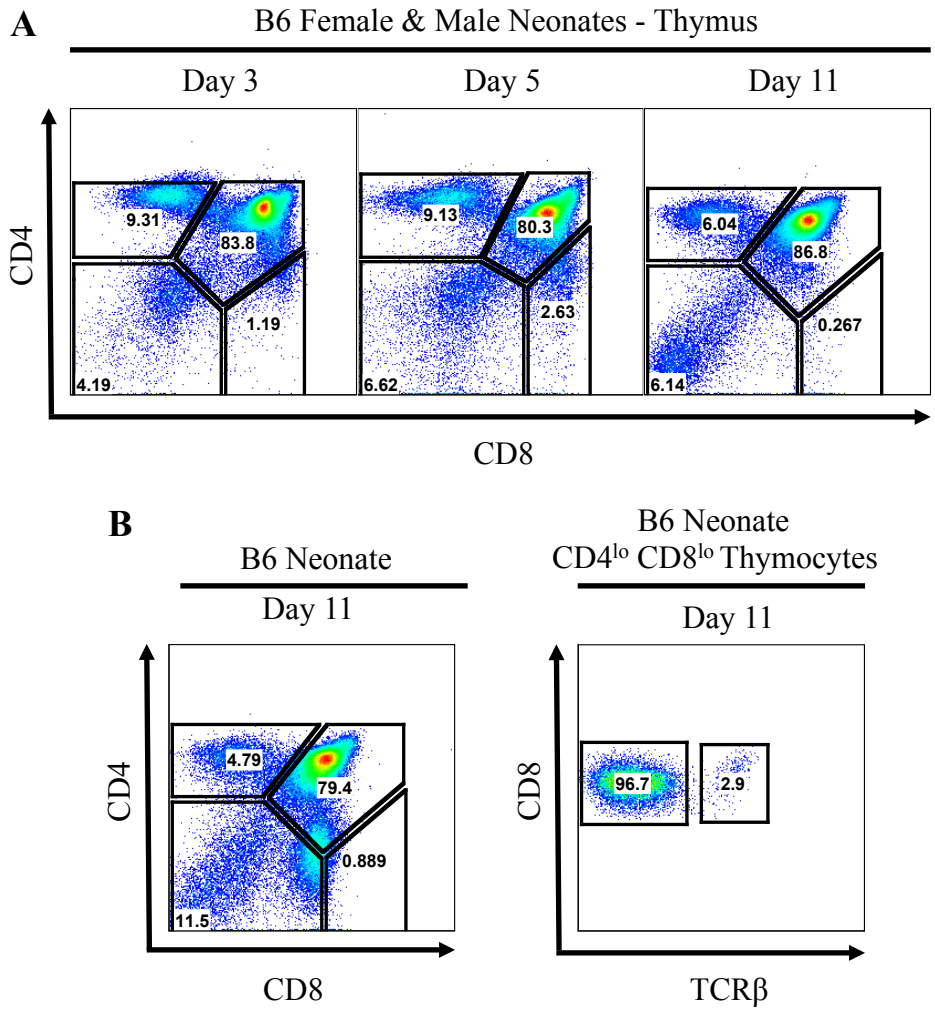


Fig. 3-3. B6 wildtype neonates contained substantial thymocyte populations at Day 3, 5 and 11 post-birth. **A.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression on total thymocytes of B6 neonates. Representative of B6 neonates Day 3 n = 5; Day 5 n = 4; Day 11 n = 5, 2 without interesting cell population. **B.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression on total thymocytes of Day 11 B6 neonates with CD4lo CD8lo population and of CD8 and TCRβ surface expression on the interesting CD4lo CD8lo thymocyte population. The CD8hi TCRβlo cells are immature single positive thymocytes and the CD8hi TCRβhi cells are mature CD8SP thymocytes. Representative of Day 11 B6 neonates n = 3.

DP thymocytes in Day 3 B6 neonates was approximately 2-3 x 10^6 cells and there was no statistically significant difference in number at Day 5 (**Fig. 3-4 A**). However, there was a statistically significant increase in the absolute number of DP thymocytes between Days 3 and 5 to Day 11 to approximately 6 x 10^7 cells at Day 11 post-birth. There was a similar trend of increasing thymocyte numbers within the CD4SP thymocyte compartment of B6 neonates. Again, there was not a statistically significant increase in the number of CD4SP thymocytes between Day 3 and Day 5 post-birth but there was a significant increase at Day 11 post-birth to approximately 3.5 x 10^6 cells, when compared to both Day 3 and Day 5 (**Fig. 3-4 B**). Finally, the absolute number of CD8SP thymocytes was smaller compared to the CD4SP thymocyte compartment within the B6 neonates. At Day 3, B6 neonates had approximately 50 000 CD8SP thymocytes, which significantly increased two-fold at Day 5. There was a statistically significant 4-5 fold increase in TCRβ+ CD8SP+ thymocytes from Day 3 and Day 11 B6 neonates. While it appears that the number of TCRβ+ CD8SP thymocytes increased between Day 5 and 11, this increase was not statistically significant (**Fig. 3-4 C**). This graphical data suggested that there were mature thymocytes present within the thymus in the early days post-birth. Interestingly, there are an abundance of CD4SP thymocytes compared to CD8SP thymocytes between Day 3 and Day 11 post-birth. Although the evidence suggest that positive selection of the polyclonal repertoire was intact, it appears that CD4SP thymocytes develop much earlier compared to CD8SP thymocytes in the few days post-birth.

HY^{cd4} female neonatal mice have delayed positive selection of T3.70+ thymocytes

The thymic analysis of the HY^{cd4} neonatal mice began with segregation of the T3.70+ and T3.70- thymocytes. Within HY^{cd4} wildtype female neonates, there was approximately 30-50% of T3.70+ thymocytes between Days 3 and 11 post-birth (**Fig. 3-5 A**). The HY^{cd4} PD-1 knock-out female neonates had a similar range in frequency of T3.70+ thymocytes between Days 3 and 11 (**Fig. 3-5 A**), suggesting that PD-1 deficiency did not impact antigen-specific thymocyte

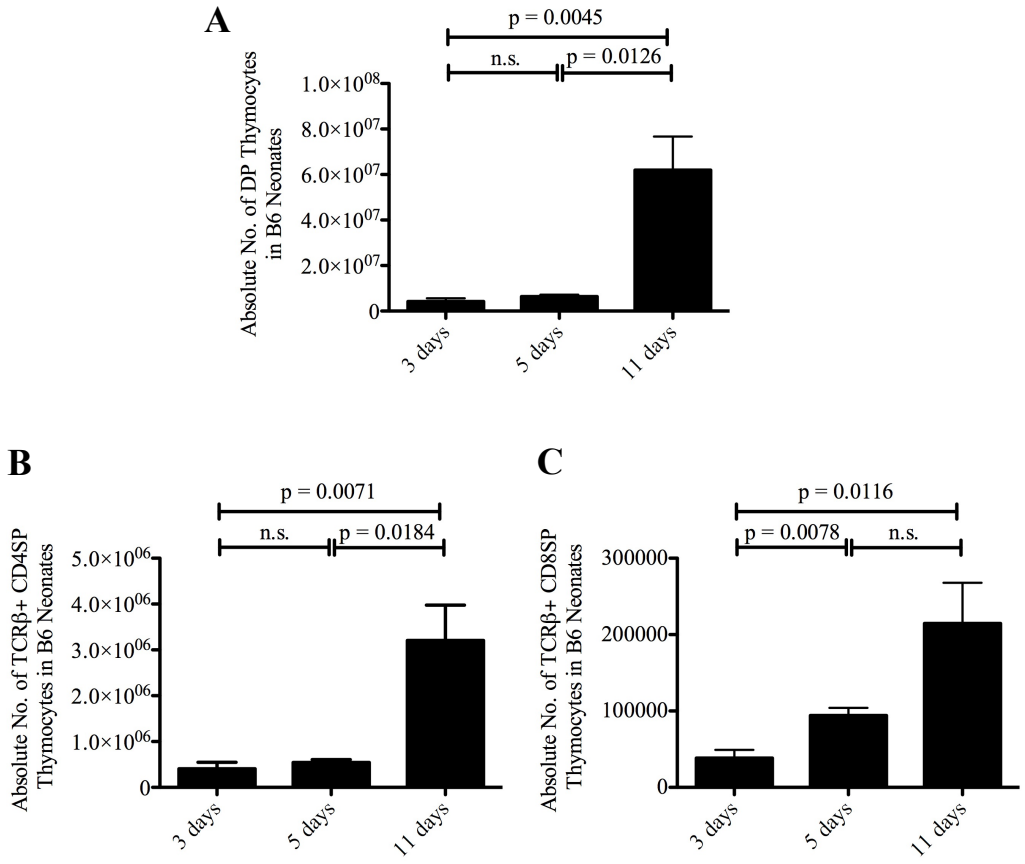


Fig. 3-4. The absolute number of DP, TCRβ+ CD4SP and TCRβ+ CD8SP thymocytes in B6 neonates increased most drastically at Day 11 post-birth compared to Day 3. **A.** Graphical analysis of the absolute number of DP thymocytes in B6 neonates. **B.** Graphical analysis of the absolute number of TCRβ+ CD4SP thymocytes in B6 neonates. **C.** Graphical analysis of the absolute number of TCRβ+ CD8SP thymocytes in B6 neonates. n.s.: not significant. Representative of B6 neonates Day 3 n = 5; Day 5 n = 4; Day 11 n = 5.

frequencies within the HY^{cd4} female neonates. The number of T3.70+ T cells increased drastically from Day 3 to Day 11 post-birth in both HY^{cd4} wildtype and PD-1 knock-out female neonates (**Fig. 3-5 B**). Within the HY^{cd4} wildtype Day 5 and Day 11 female neonates, there was a statistically significant increase in T3.70+ thymocyte number. However, the absence of PD-1 did not result in a statistically significant change in the cell numbers of any subset.

Next we assessed the CD4 and CD8 coreceptor profiles of T3.70+ thymocytes from wildtype or PD-1 knock-out HY^{cd4} female neonates. At Day 3 post-birth, the DP thymocyte subset accounted for the largest proportion of cells within the T3.70+ thymocyte subset in both HY^{cd4} wildtype and PD-1 deficient female neonates (**Fig. 3-6 A**). Interestingly, the CD8SP thymocyte population was incredibly small in both HY^{cd4} wildtype and PD-1 knock-out female neonates at Days 3 and 5 post-birth, suggesting positive selection of T3.70+ thymocytes was delayed within HY^{cd4} female neonates. However, at Day 11 post-birth, the HY^{cd4} wildtype female neonates began to show an interesting group of cells that had down regulated CD8 coreceptor and slightly reduced levels of CD4 coreceptor (**Fig. 3-6 A**). HY^{cd4} PD-1 knockout female neonates did not have this cell population within the DP thymocyte gate. One possibility is that these cells might be representative of positively selected DP thymocytes transitioning to the CD8SP thymocyte compartment. The majority of the flow cytometry data suggested that PD-1 deficiency with HY^{cd4} female neonates did not alter the frequency of T3.70+ DP and CD8SP thymocytes. The contribution of PD-1 to the interesting thymocyte population observed in Day 11 HY^{cd4} wildtype female neonates is currently unclear.

The absolute numbers of the DP and CD8SP thymocytes were calculated to determine if there were any differences in the absence of PD-1 that may not have been shown with cellular frequencies. The graphical analysis of the number of DP thymocytes within both HY^{cd4} wildtype and PD-1 knock-out female neonates showed that there were very few cells at Day 3 and 5 post-birth. However, there

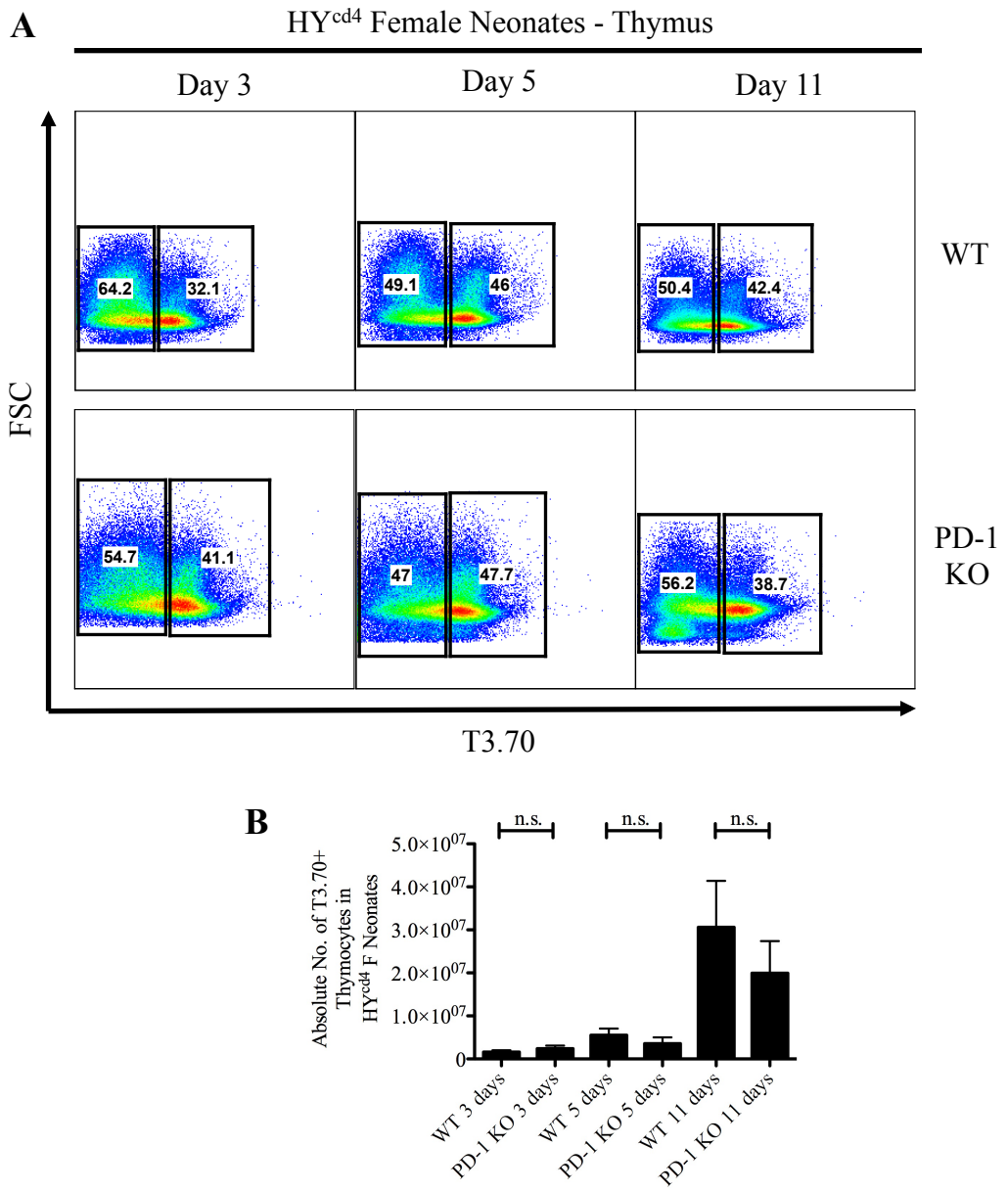


Fig. 3-5. HY^{cd4} female neonates have a sizeable proportion of T3.70+ thymocytes as early as Day 3 post-birth. **A.** Representative flow cytometric plots of T3.70 TCR expression on total thymocytes from HY^{cd4} wildtype and PD-1 knock-out female neonates. **B.** Graphical analysis of the absolute number of T3.70+ thymocytes from HY^{cd4} wildtype and PD-1 knock-out female neonates. n.s.: not significant. Representative HY^{cd4} wildtype female neonates: Day 3 n = 7; Day 5 n = 9; Day 11 n = 3. Representative of HY^{cd4} PD-1 knock-out female neonates: Day 3 n = 4; Day 5 n = 4; Day 11 n = 5.

was a drastic increase in DP thymocyte numbers at Day 11 post-birth when compared to Day 3 and 5. There was no statistically significant difference between the age-matched HY^{cd4} female neonates in the presence or absence of PD-1 (**Fig. 3-6 B**). A similar observation was made when the absolute number of CD8SP thymocytes was calculated. The number of CD8SP thymocytes was approximately 20 000 cells at Day 3 post-birth, in both HY^{cd4} wildtype and PD-1 knock-out female neonates. This number remained fairly consistent at Days 5 and 11 post-birth; and again, there was no statistically significant difference between age matched HY^{cd4} female neonates in the presence or absence of PD-1 (**Fig. 3-6 C**). In consideration of both the flow cytometric data and the graphical analysis together, the evidence suggested that positive selection of T3.70+ thymocytes was delayed within HY^{cd4} female neonates. At Day 11, there might have been the emergence of a positively selected T3.70+ thymocyte population within the HY^{cd4} wildtype female neonates that was absent in the HY^{cd4} PD-1 knock-outs. The significance of PD-1 deficiency on this interesting population being positively selected is currently unclear. Further analysis of HY^{cd4} female neonates at Day 15 and at the weaning age Day 21 is required to show that this population was destined to the CD8SP thymocyte subset and to determine whether or not this process is PD-1 dependent.

T3.70+ thymocytes of HY^{cd4} male neonatal mice undergo negative selection

Analysis of the thymocyte population within HY^{cd4} male neonates showed a similar, distinct T3.70+ population as was seen in the HY^{cd4} female neonates. HY^{cd4} wildtype male neonates had similar proportions of T3.70+ thymocytes between Day 3 and 11 post-birth ranging from 45-60% (**Fig. 3-7 A**). This range did not change in HY^{cd4} PD-1 knock-out male neonates, suggesting that PD-1 deficiency had no impact on the frequency of T3.70+ thymocytes. At Day 11, the frequency of T3.70+ thymocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates did not amount to the frequency of T3.70+ thymocytes seen in the HY^{cd4} adult male thymus, ranging between 70-80% (**Fig. 3-1 A**). Calculation of the

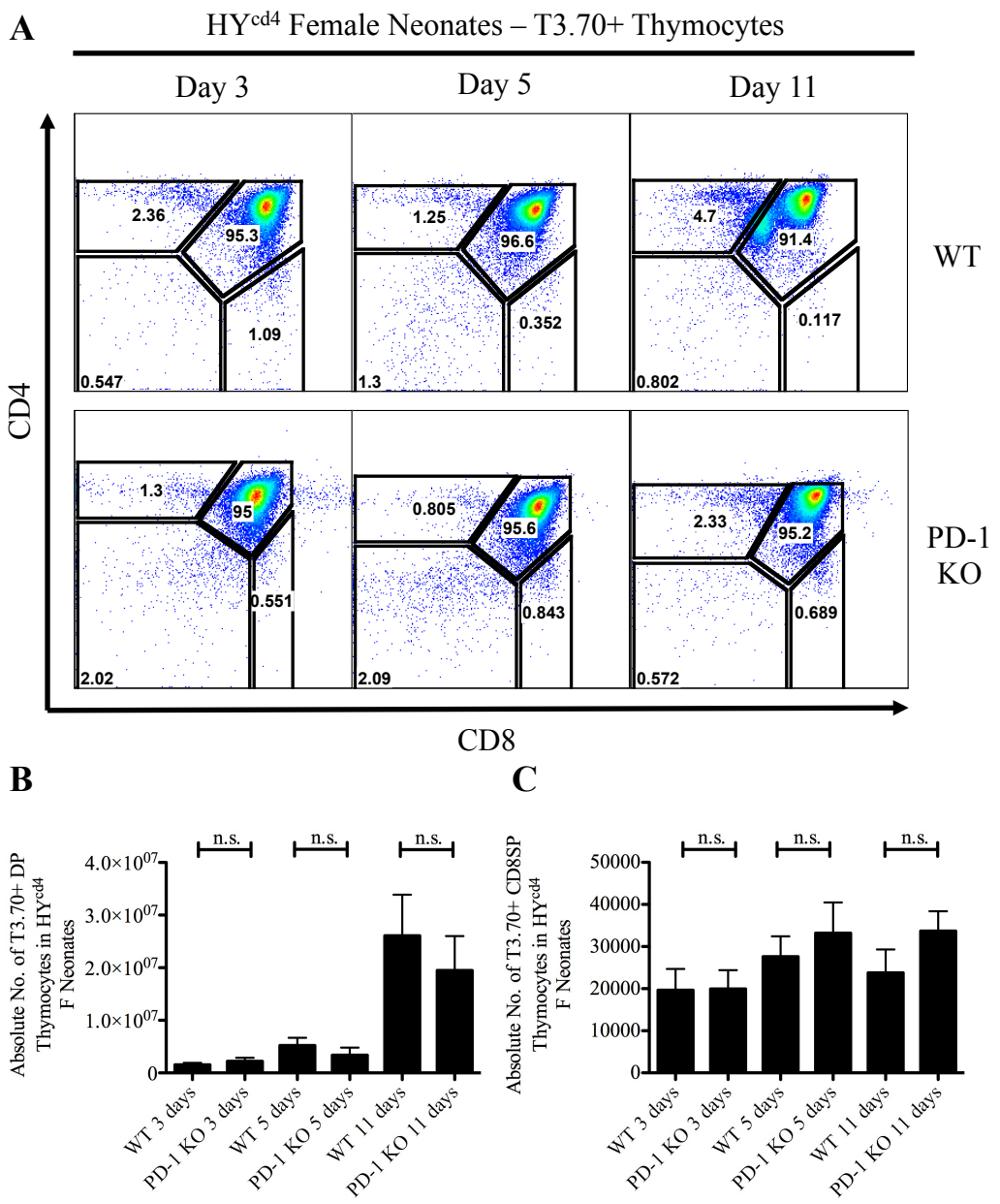


Fig. 3-6. HY^{cd4} female neonates lacked a substantial T3.70+ CD8SP thymocyte population between Days 3 and 11 post-birth. **A.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression on T3.70+ thymocytes from HY^{cd4} female neonates at Day 3, 5 and 11 post-birth. **B.** Graphical analysis of the absolute number of T3.70+ DP thymocytes in HY^{cd4} wildtype and PD-1 knock-out female neonates. **C.** Graphical analysis of the absolute number of T3.70+ CD8SP thymocytes in HY^{cd4} wildtype and PD-1 knock-out female neonates. n.s.: not significant. Representative HY^{cd4} wildtype female neonates: Day 3 n = 7; Day 5 n = 9; Day 11 n = 3. Representative of HY^{cd4} PD-1 knock-out female neonates: Day 3 n = 4; Day 5 n = 4; Day 11 n = 5.

absolute number of T3.70+ thymocytes from multiple replicates of HY^{cd4} wildtype and PD-1 knock-out male neonates showed a gradual increase in number from approximately 2 x 10^6 cells at Day 3 to approximately 1.5-1.7 x 10^7 cells at Day 11 post-birth. However, there was no statistically significant difference in the absence of PD-1 (**Fig. 3-7 B**). Therefore, the evidence suggested that PD-1 deficiency did not alter the proportion and absolute number of T3.70+ thymocytes in HY^{cd4} male neonates.

The T3.70+ thymocyte population from HY^{cd4} male neonates was further examined for the presence of a DP thymocyte population undergoing negative selection. HY^{cd4} wildtype and PD-1 knock-out male neonates were compared to determine if PD-1 deficiency had any effect on the generation of the thymocyte subsets within male neonates. At Day 3 post-birth, the DP thymocytes population was the largest in proportion of all the T3.70+ thymocyte subsets (**Fig. 3-8 A**). When HY^{cd4} wildtype male neonates were compared to HY^{cd4} PD-1 knock-out male neonates, there was no dramatic difference in proportion of DP thymocytes. Furthermore, the DP thymocyte population was broad and had reduced expression of both CD4 and CD8α coreceptors; this observation was also seen within HY^{cd4} adult males (**Fig. 3-1 B**). The broad DP thymocyte population was also seen in HY^{cd4} male neonates at Days 5 and 11 post-birth.

Graphical analysis of the absolute number of T3.70+ DP thymocytes showed a gradual increase between Days 3 and 11 post-birth whereas the CD8SP thymocyte number did not change dramatically post-birth. At Day 3 post-birth, there were approximately 1-2 x 10^6 DP thymocytes within the thymi of HY^{cd4} wildtype and PD-1 knock-out male neonates (**Fig. 3-8 B**). The DP thymocyte number increased approximately two-fold at Day 5 post-birth, and increased three-fold further at Day 11 post-birth. However, when the number of DP thymocytes was compared between age-matched HY^{cd4} wildtype and PD-1 knock-out male neonates, there was no statistically significant difference found in the absence of PD-1. Within the CD8SP compartment, there were few CD8SP thymocytes in

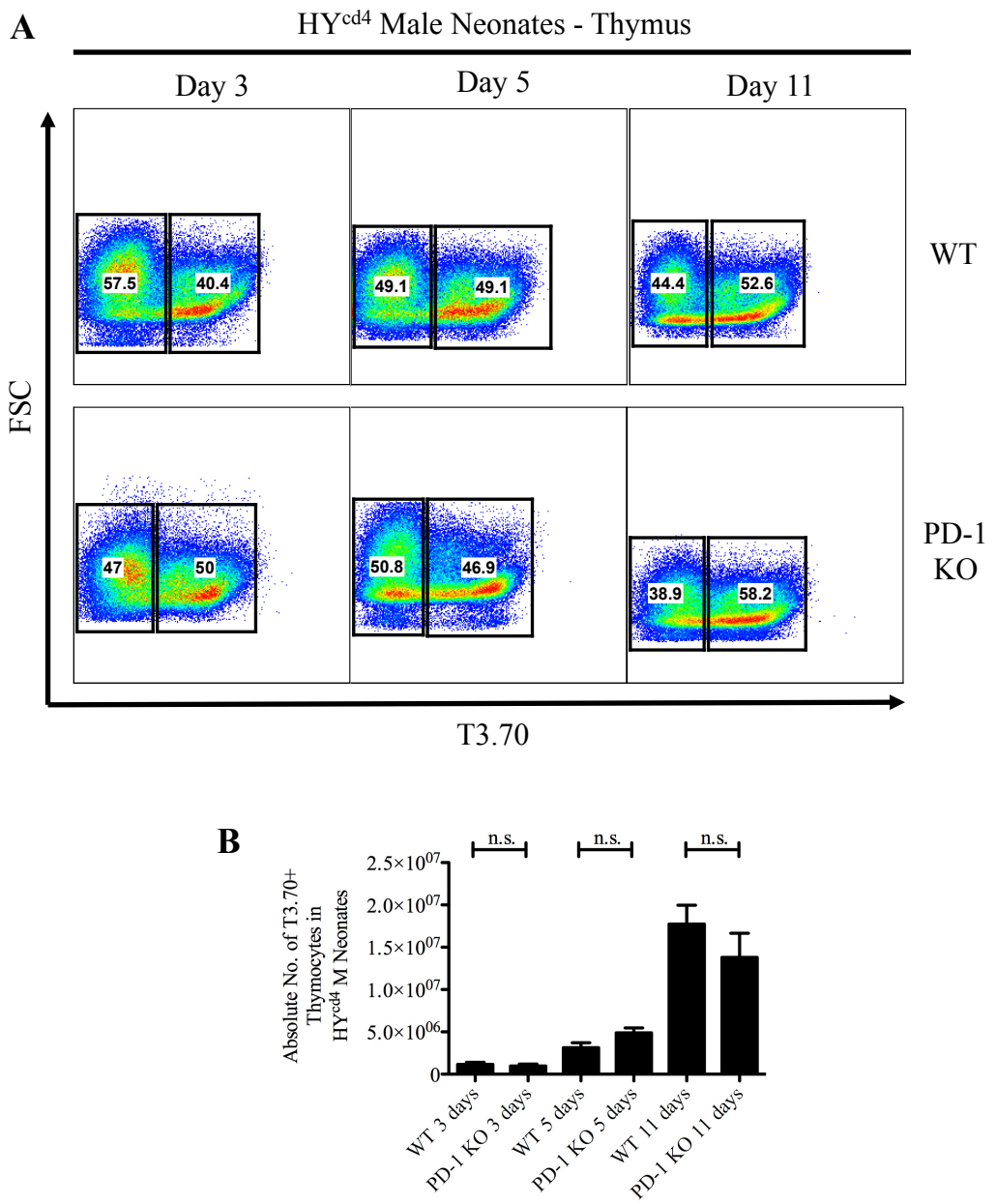


Fig. 3-7. Antigen-specific T3.70+ thymocytes accounted for approximately half of total thymocytes within HY^{cd4} wildtype and PD-1 knock-out male neonates. **A.** Representative flow cytometric plots of T3.70 TCR expression on total thymocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates. **B.** Graphical analysis of the absolute number of T3.70+ thymocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates. n.s.: not significant. Representative HY^{cd4} wildtype male neonates: Day 3 n = 10; Day 5 n = 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 knock-out male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

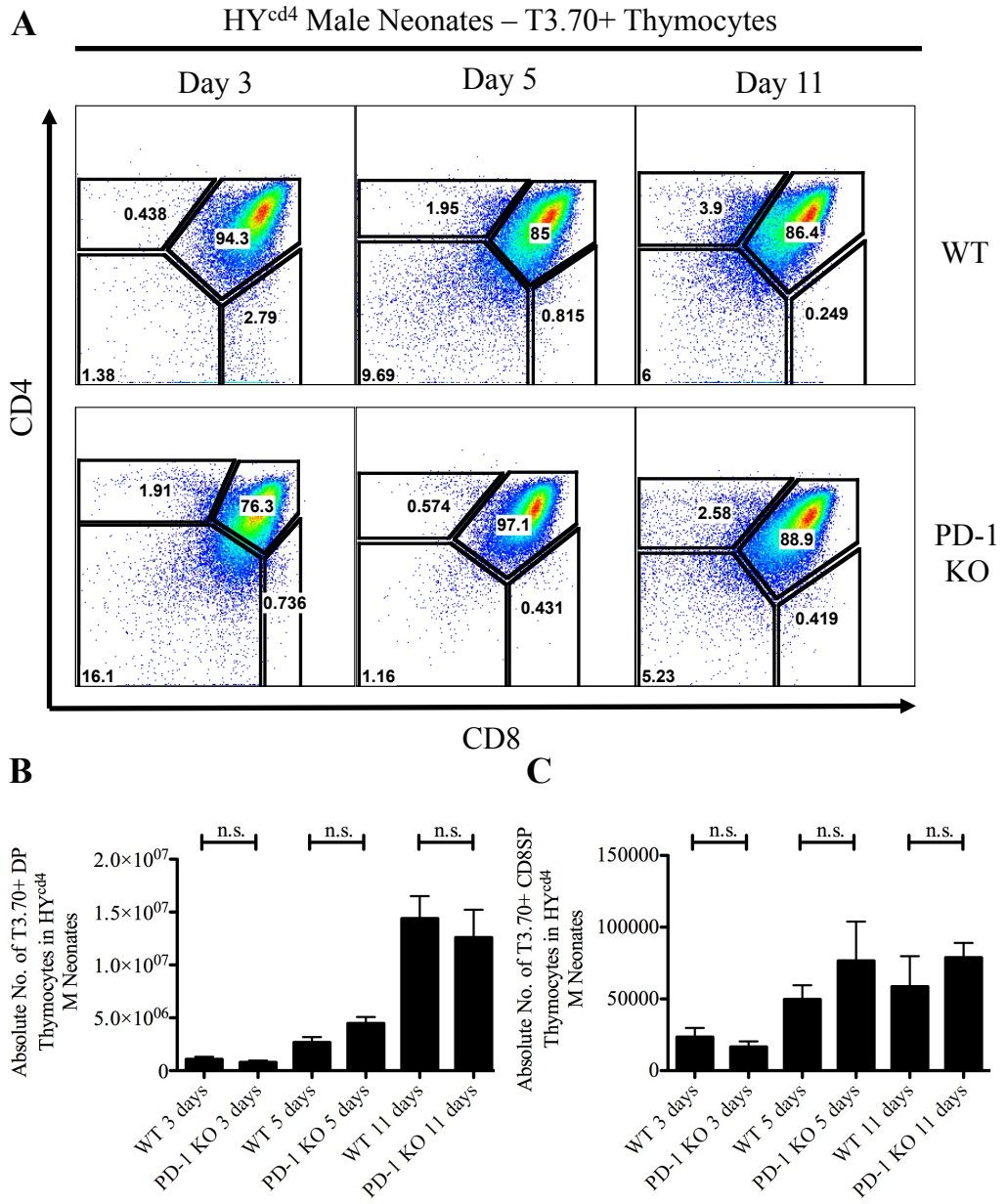


Fig. 3-8. HY^{cd4} PD-1 knock-out male neonates had similar frequencies and absolute numbers of T3.70+ DP and CD8SP thymocytes compared to HY^{cd4} wildtype male neonates. **A.** Representative flow cytometric plots of CD4 by CD8 coreeptor expression on T3.70+ thymocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates at Day 3, 5 and 11 post-birth. **B.** Graphical analysis of the absolute number of T3.70+ DP thymocytes in HY^{cd4} wildtype and PD-1 knock-out (KO) male neonates. n.s.: not significant. **C.** Graphical analysis of the absolute number of T3.70+ CD8SP thymocytes in HY^{cd4} wildtype and PD-1 knock-out (KO) male neonates. n.s.: not significant. Representative HY^{cd4} wildtype male neonates: Day 3 n = 10; Day 5 n = 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 KO male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

Day 3 HY^{cd4} male neonates and the numbers increased marginally until Day 11 (**Fig. 3-8 C**). PD-1 deficiency did not influence the number of CD8SP thymocytes. Based on the flow cytometric data and the graphical analysis, HY^{cd4} male neonates had a DP thymocyte population as early as Day 3 post-birth and these cells down regulated both CD4 and CD8 coreceptors as previously seen in HY^{cd4} male adults. Since CD4 and CD8α coreceptor down regulation is characteristic of T3.70+ DP thymocytes undergoing negative selection, it appeared that negative selection of T3.70+ thymocytes was intact as early as Day 3 within HY^{cd4} male neonates.

HY^{cd4} wildtype and PD-1 knock-out female and male neonatal T3.70+ DP thymocytes appear to undergo selection similarly to the respective controls

HY^{cd4} female and male neonatal T3.70+ DP thymocytes showed no differences in expression of molecular markers associated with selection compared to controls

In order to more fully understand positive and negative selection in neonates, I examined the expression of various molecules previously correlated with positive and negative selection. Surface expression of the activation receptor CD69 increases following TCR stimulus and downstream signaling; therefore, its expression is indicative of TCR engagement. CD69 expression on the surface of DP thymocytes from B6 neonates was directly compared to DP thymocytes of B6 adult female mice. CD69 surface expression of the B6 adult female DP thymocytes was also compared to the T3.70+ DP thymocytes of HY^{cd4} wildtype and PD-1 knock-out female neonates to examine any differences within positive selection of a restricted T cell repertoire. Within the B6 neonates, the DP thymocytes expressed similar levels of CD69 when compared to the B6 adult female controls (**Fig. 3-9 A**). This suggested that the polyclonal T cells of both B6 neonates and adults were expressing CD69 to similar extents.
In comparison, within the HY^{cd4} female neonates, the DP thymocytes expressed similar to reduced levels of CD69 compared to the B6 adult female control.

There appeared to be no difference in CD69 surface expression on DP thymocytes from HY^{cd4} female neonates compared to the B6 adult female control (**Fig. 3-9 B**). Furthermore, PD-1 deficiency did not alter CD69 surface expression in HY^{cd4} female neonates. These data supported the finding of delayed positive selection and are consistent with that conclusion. In order to determine how long positive selection is delayed within HY^{cd4} female neonates, analysis of the T3.70+ thymocytes from neonates at Day 15 post-birth and older would be required.

CD69 surface expression was also assessed on DP thymocytes from HY^{cd4} male neonates in order to determine if differences in thymocyte activation existed between the neonate and adult stages, and to appreciate if PD-1 contributed to this process. HY^{cd4} adult male DP thymocytes expressed higher levels of CD69 compared to the B6 female control, suggesting that these cells had encountered high affinity antigen within the thymus (**Fig. 3-10 A**). The DP thymocytes from HY^{cd4} male neonates expressed similar to slightly reduced levels of CD69 compared to the HY^{cd4} adult control. This overall trend did not differ in PD-1 deficient HY^{cd4} male neonates (**Fig. 3-10 A**). Based on similar CD69 surface expression trends, the data suggested that the DP thymocytes from HY^{cd4} male neonates appear to have encountered antigen similarly to the HY^{cd4} adult male controls.

Following TCR stimulation and downstream signaling, the expression of the activation receptor CD69 is followed shortly thereafter with the surface expression of the inhibitory receptor PD-1 (150, 151). PD-1 is another molecule associated with positive and negative selection events, and was therefore assessed on DP thymocytes from B6 and HY^{cd4} female and male neonates. B6 neonatal DP thymocytes expressed similar levels of PD-1 compared to B6 adult female controls at Days 3 to 11 post-birth (**Fig. 3-11 A**). Similarly, DP thymocytes from HY^{cd4} female neonates at Days 3, 5 and 11 had nearly identical levels of PD-1 when compared to B6 adult female controls (**Fig. 3-11 B**), suggesting that PD-1 was not induced in HY^{cd4} female neonatal DP thymocytes.

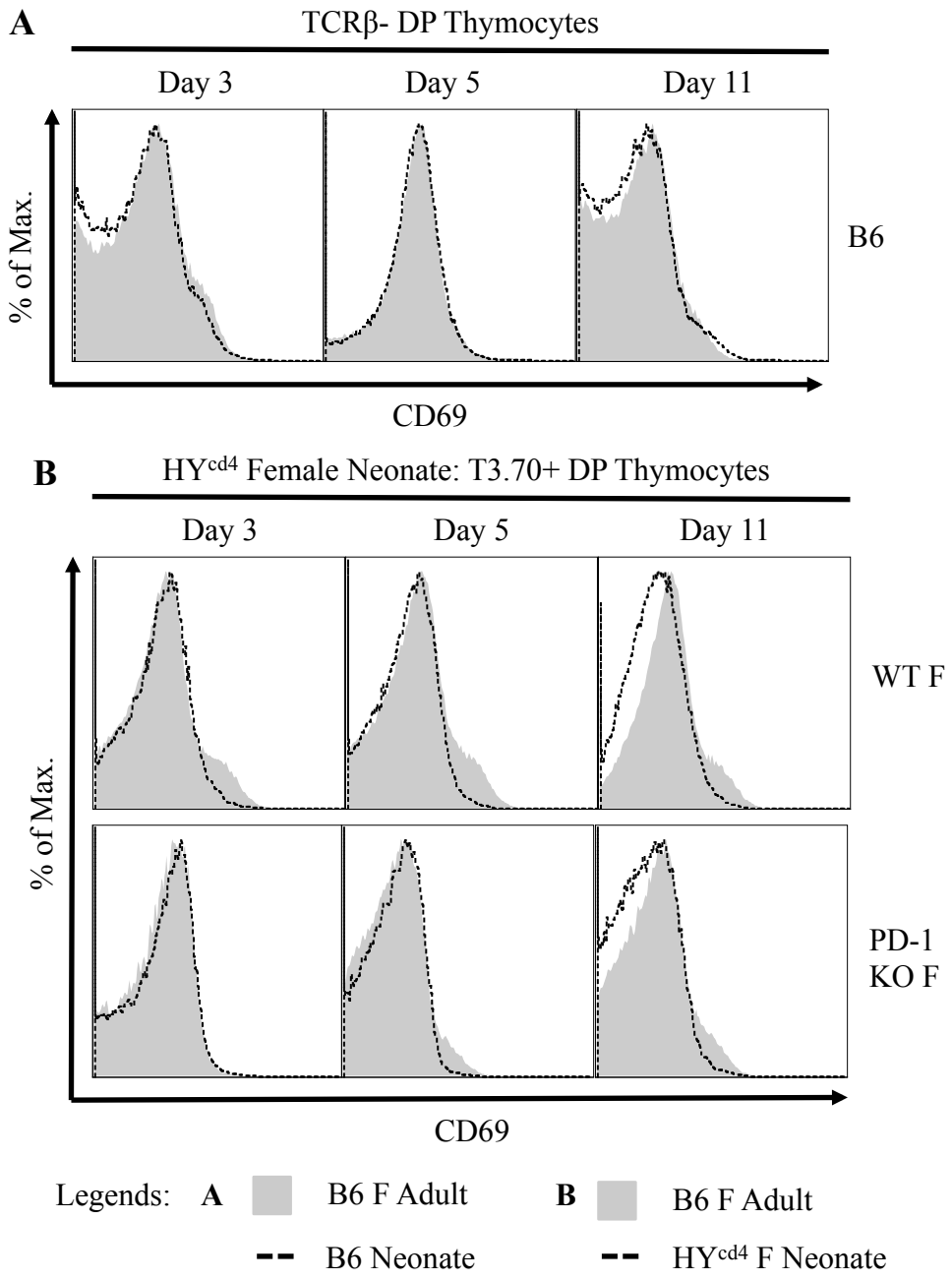


Fig. 3-9. B6 and HY^{cd4} female neonatal DP thymocytes expressed similar levels of the activation marker CD69 compared to B6 adult female control DP thymocytes. **A.** Histogram overlays of CD69 expression on TCRβ- DP thymocytes from B6 neonates compared to B6 adult females. n = 5; 4; 5. **B.** Histogram overlays of CD69 expression on T3.70+ DP thymocytes from HY^{cd4} female neonates compared to B6 adult female TCRβ- DP thymocytes. B6 female control (solid grey); HY^{cd4} female neonates (dashed). Representative of HY^{cd4} WT F n= 7; 5; 3. Representative of HY^{cd4} PD-1 KO F: n = 4; 4; 5.

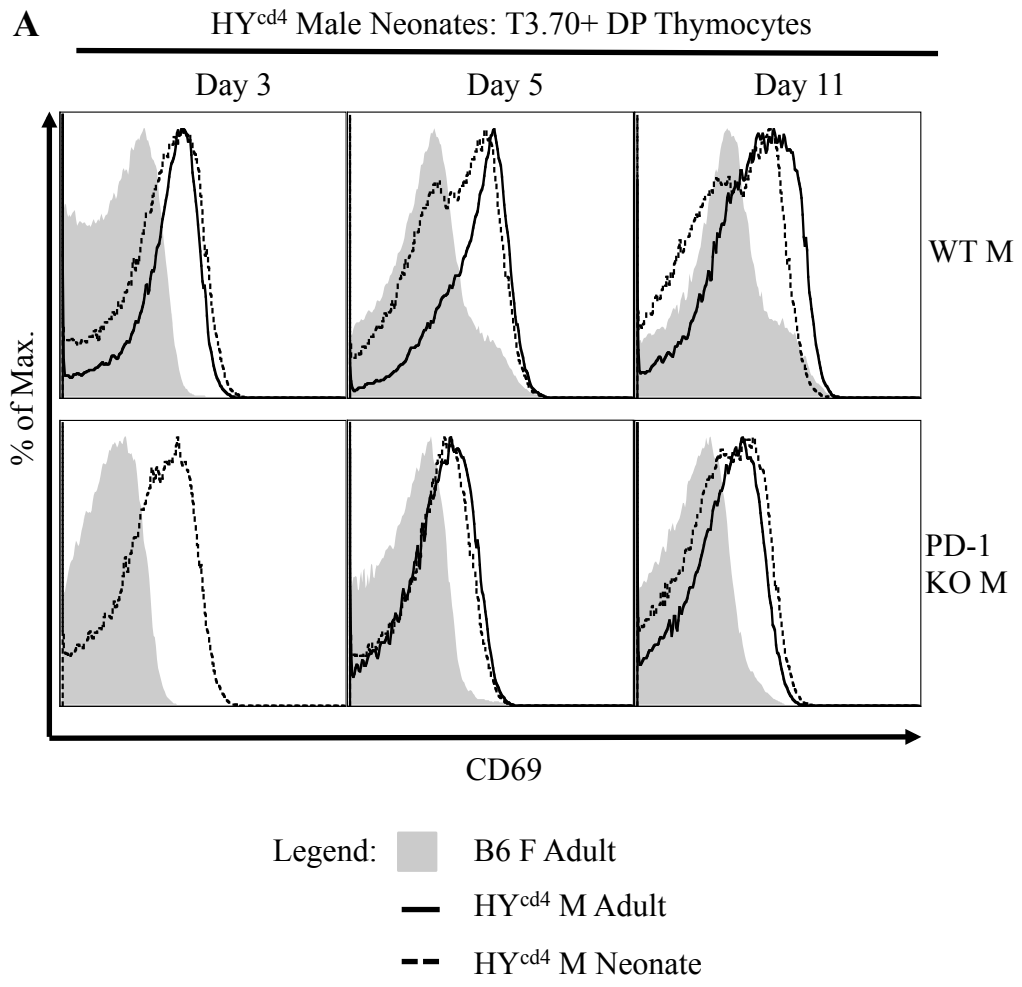


Fig. 3-10. HY^{cd4} male neonatal DP thymocytes expressed similar to reduced levels of the activation marker CD69 at Days 3 to 11 post-birth when compared to HY^{cd4} adult male control DP thymocytes. **A.** Histogram overlays of CD69 surface expression on T3.70+ DP thymocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates compared to T3.70+ DP thymocytes from HY^{cd4} adult male controls and TCRβ- DP thymocytes from B6 adult female controls. The solid grey represents the B6 adult female control, the solid line represents the HY^{cd4} adult male control and the dashed line represents the HY^{cd4} male neonate at the respective day post-birth. Representative of HY^{cd4} wildtype male neonates: Day 3 n = 10; Day 5 n = 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 knock-out male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

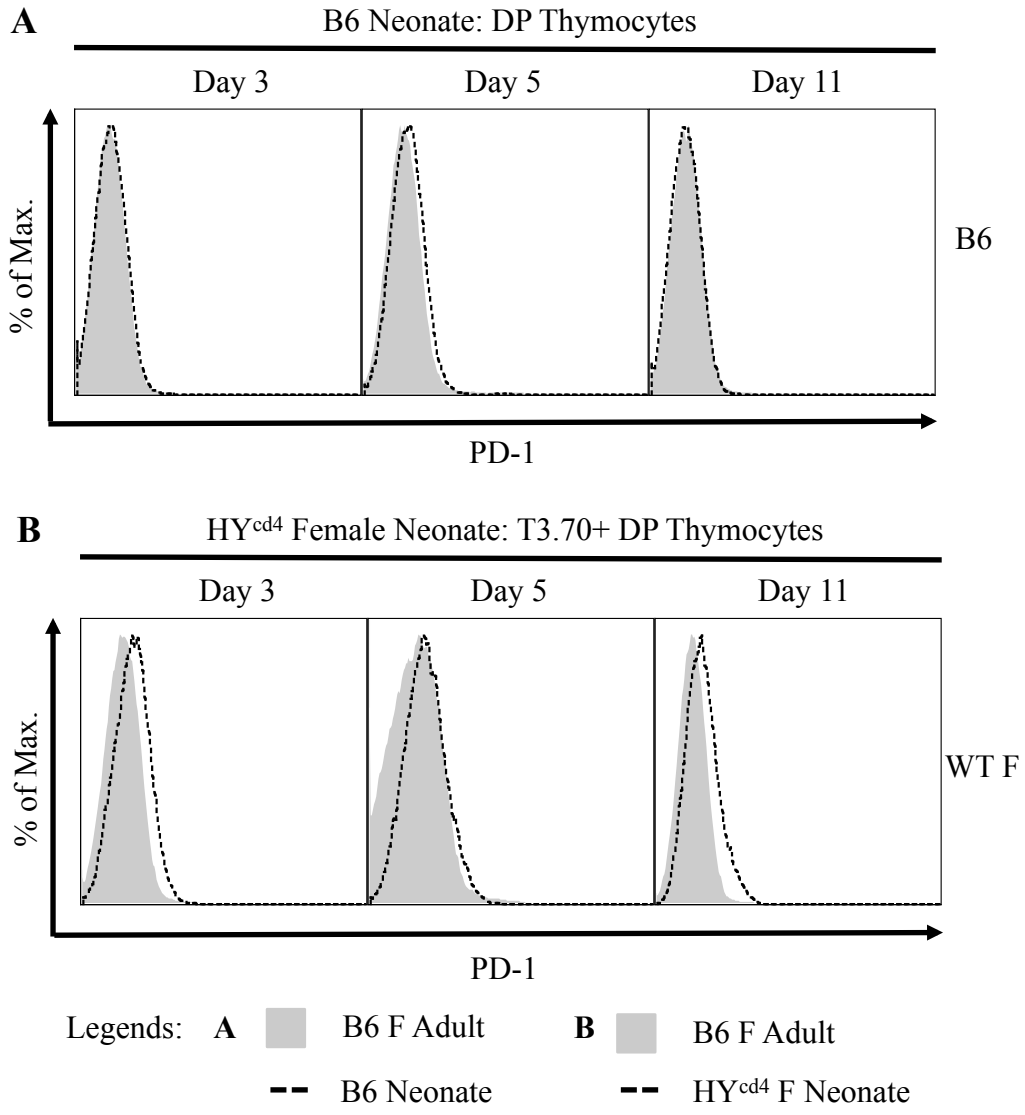


Fig. 3-11. Surface expression of the inhibitory receptor PD-1 was not induced on B6 or HY^{cd4} female neonatal DP thymocytes at Days 3 to 11 post-birth when compared to the B6 adult female control. **A.** Histogram overlays of PD-1 surface expression on DP thymocytes from B6 neonates compared to DP thymocytes from B6 adult female controls. Representative of B6 neonates Day 3 n = 5; Day 5 n = 4; Day 11 n = 5. **B.** Histogram overlays of PD-1 surface expression on T3.70+ DP thymocytes from HY^{cd4} wildtype female neonates compared to TCRβ- DP thymocytes from B6 adult female controls. The solid grey represents the B6 adult female control and the dashed line represents the B6 or HY^{cd4} female neonates at the specified day post-birth. Representative of HY^{cd4} WT female neonates Day 3 n= 7; Day 5 n = 5; Day 11 n = 3. Representative of HY^{cd4} PD-1 KO female neonates Day 3 n = 4; Day 5 n = 4; Day 11 n = 5.

In contrast to the B6 and HY^{cd4} female neonates, HY^{cd4} wildtype male neonates expressed similar to reduced levels of PD-1 on the cell surface compared to the HY^{cd4} adult male controls (**Fig. 3-12 A**). This expression level was higher than on the B6 adult female control DP thymocytes, which was expected, as these cells have encountered high affinity antigen within the thymus. Collectively the data suggests there were no drastic observable differences in PD-1 surface expression between B6 and HY^{cd4} wildtype neonatal DP thymocytes when compared to their respective adult controls. It appeared that DP thymocytes of HY^{cd4} male neonates were encountering high-affinity antigen within the thymus and becoming activated based on CD69 and PD-1 surface expression.

In order to determine whether the T3.70+ DP thymocytes from HY^{cd4} wildtype or PD-1 knock-out male neonates were undergoing negative selection, we examined the activation of caspase 3 in these cells. Caspase 3 cleavage is a hallmark of apoptosis and clonal deletion mediated by apoptosis is one form of negative selection (75). Therefore, intracellular caspase 3 cleavage can provide definitive evidence of negative selection by clonal deletion occurring within the DP thymocyte population of B6 and HY^{cd4} mice. In order to get a baseline level of intracellular active caspase 3, DP thymocytes from a B6 adult female and HY^{cd4} adult female and male mice were analyzed. The low frequency of active caspase 3 expressing DP thymocytes from the B6 adult female was similar to the HY^{cd4} adult female (**Fig. 3-13 A**). As we previously published, within HY^{cd4} adult male, approximately 1-2% of DP thymocytes expressed active caspase 3, which is drastically higher than the B6 adult female control (89).

The analysis of active caspase 3 expressing DP thymocytes continued within HY^{cd4} male neonates. The frequency of active caspase 3 expressing DP thymocytes in HY^{cd4} male neonates was between 1-2.5% and therefore similar to HY^{cd4} adult males (**Fig. 3-14 A**). HY^{cd4} PD-1 deficient DP thymocytes appeared to express higher levels of active caspase 3 compared to the HY^{cd4} wildtype neonates. However, graphical analysis of the frequency of active caspase 3

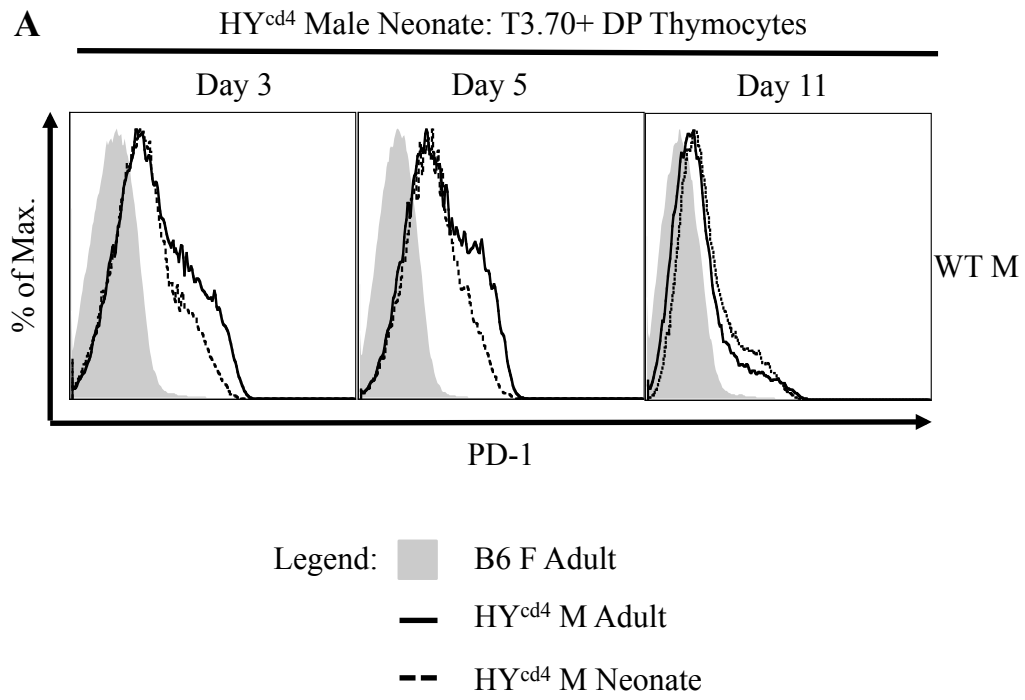


Fig. 3-12. HY^{cd4} male neonatal DP thymocytes expressed similar to slightly reduced levels of the inhibitory receptor PD-1 at Days 3 to 11 post-birth compared to DP thymocytes from the HY^{cd4} adult male controls. **A.** Histogram overlays of PD-1 surface expression on T3.70+ DP thymocytes from HY^{cd4} wildtype male neonates compared to T3.70+ DP thymocytes from HY^{cd4} adult male controls and TCRβ- DP thymocytes from B6 adult female controls. The solid grey represents the B6 adult female control, the solid line represents the HY^{cd4} adult male control and the dashed line represents the HY^{cd4} male neonate at the respective day post-birth. Representative of HY^{cd4} wildtype male neonates: Day 3 n = 10; Day 5 n = 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 knock-out male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

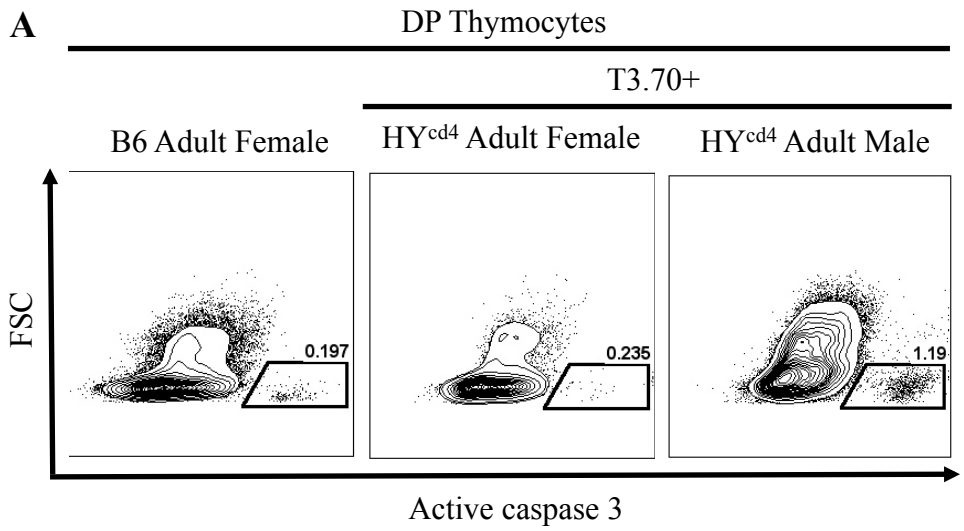


Fig. 3-13. HY^{cd4} adult male mice have higher frequencies of active caspase 3-expressing T3.70+ DP thymocytes, undergoing apoptosis, when compared to T3.70+ DP thymocytes from HY^{cd4} adult female mice and polyclonal DP thymocytes from B6 adult female mice. **A.** Representative flow cytometric plots of active caspase 3 expressing DP thymocytes from a B6 adult female, HY^{cd4} adult female and HY^{cd4} adult male. Representative of B6 n = 10; HY^{cd4} F n = 9; HY^{cd4} M n = 9.

expressing DP thymocytes only showed a significant difference between HY^{cd4} wildtype and PD-1 knock-out male neonates at Day 3 post-birth (**Fig. 3-14 B**). This data suggested that HY^{cd4} male neonate DP thymocytes capable of expressing PD-1 were protected from undergoing apoptosis at Day 3 post-birth. Furthermore, the proportion of active caspase 3 expressing DP thymocytes was significantly lower in Day 3 HY^{cd4} wildtype male neonates compared to the HY^{cd4} adult male, and significantly higher in Day 3 and Day 5 HY^{cd4} PD-1 knock-out male neonates compared to the control (**Fig. 3-14 A, B**). This may also suggest that in the early days post-birth, DP thymocytes from HY^{cd4} wildtype male neonates may undergo less apoptosis compared to HY^{cd4} male adults, whereas DP thymocytes lacking PD-1 undergo more apoptosis compared to HY^{cd4} male adults. Despite these differences in the HY^{cd4} male neonates, the surface expression of CD69 and PD-1 and intracellular active caspase 3 on DP thymocytes from HY^{cd4} male neonates suggested that the DP thymocytes were undergoing negative selection within the thymus.

B6 and HY^{cd4} male neonatal spleens did not differ from B6 adult female and HY^{cd4} adult male controls, whereas HY^{cd4} females lacked splenic T3.70+ T cells

B6 neonatal spleens contained growing populations of TCRβ+ splenocytes

The neonatal mouse studies continued with detailed analysis of the T cell subsets within the spleen of B6 and HY^{cd4} female and male neonates. Analysis of the splenic T cell subsets began with the B6 neonates. Expression of CD4 and CD8 coreceptor expression provided a baseline of the peripheral T cell populations in a non-transgenic background. Within B6 neonates, the vast majority of T cells expressed CD4 with some expressing CD8 coreceptor and few being DN T cells (**Fig. 3-15 A**). There was a small frequency of T cells within the DP T cell gate that reduced in frequency between Days 3, 5 and 11. On Days 5 and 11, there was an increase in the frequency of CD8+ T cells with a corresponding decrease in CD4+ T cells. The frequency of DN T cells also appeared to increase between

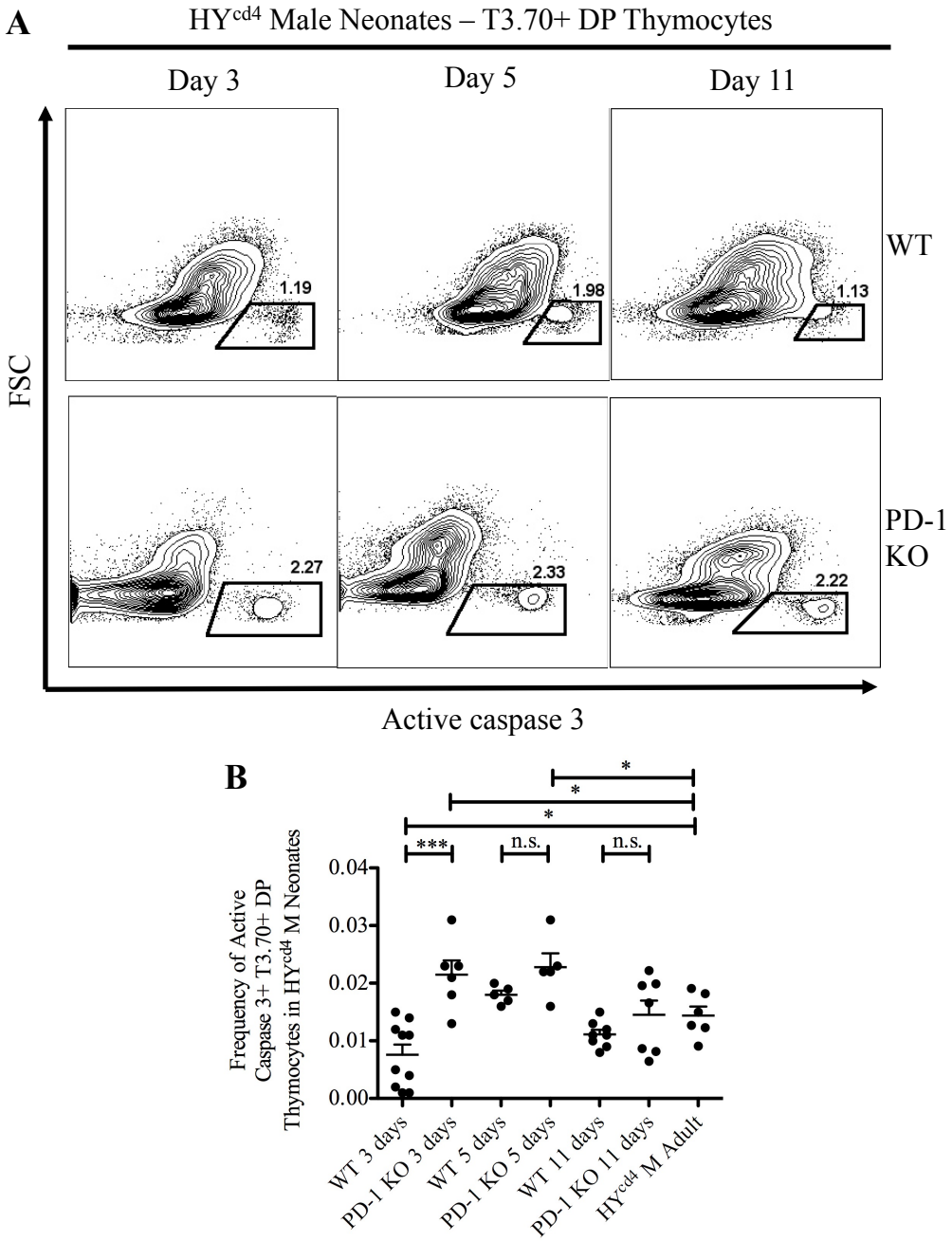


Fig. 3-14. Surface expression of PD-1 appeared to protect HY^{cd4} wildtype male DP thymocytes from undergoing apoptosis at Day 3 post-birth. **A.** Representative flow cytometric plots of active caspase 3 expressing T3.70+ DP thymocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates at Day 3, 5 and 11 post-birth. **B.** Graphical analysis of the frequency of active caspase 3 expressing DP thymocytes from HY^{cd4} wildtype and PD-1 knock-out neonates. * = 0.0195 (WT Day 3); 0.0342 (PD-1 KO Day 3) and 0.0140 (PD-1 KO Day 5), respectively. *** = 0.0003. n.s.: not significant. Representative of HY^{cd4} WT male neonates: Day 3 n = 10; Day 5 n= 5; Day 11 n = 8. Representative of HY^{cd4} PD-1 KO male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

Days 5 and 11 post-birth. The absolute number of TCRβ expressing DP, CD4+ and CD8+ T cells within the B6 neonates were quantified at Days 3, 5 and 11. This allowed for closer examination of the individual T cell subsets that may have increased in size between Days 5 and 11. The absolute number of DP T cells was approximately 1000 cells at Days 3 and 5 post-birth; however, there was a significant 5-6 fold increase in DP T cell number at Day 11 post-birth (**Fig. 3-15 B**). A similar trend was observed within both the CD4+ and CD8+ T cell compartments. The absolute number of CD4+ T cells was well below 50 000 cells at Days 3 and 5 post-birth within B6 neonates; that number increased significantly to approximately 350 000 cells at Day 11 post-birth (**Fig. 3-15 C**). Similarly, the number of CD8+ T cells was well below 4 000 cells at Day 3 and but significantly increased 1.5-fold to approximately 6 000 cells at Day 5. Furthermore, there was an even larger statistically significant 20-fold increase to approximately 125 000 CD8+ T cells at Day 11 post-birth (**Fig. 3-15 D**). Therefore, this data showed there were statistically significant increases in the absolute number of splenic CD4+ and CD8+ T cells between B6 neonates at Days 3, 5 and 11 post-birth, and is consistent with increased numbers of thymic CD4SP and CD8SP over that time frame.

HY^{cd4} female neonatal mice have delayed positive selection and thymic export

Prior to analyzing the peripheral T cell subsets from HY^{cd4} female and male neonates, the antigen-specific T cells were first gated by T3.70 expression in a similar manner to the peripheral HY^{cd4} adult splenocytes shown earlier in this thesis (**Fig. 3-2 A**). The thymic flow cytometry data of HY^{cd4} female neonates suggested that positive selection of T3.70+ T cells was delayed; therefore splenocytes were examined for T3.70 expression. If positive selection was delayed, we expected to see the absence of an antigen-specific T3.70+ peripheral T cell population within HY^{cd4} female neonates. As previously shown, HY^{cd4} adult female mice have an antigen-specific T3.70+ T cell population within the spleen, accounting for 2-3% of total splenocytes (**Fig. 3-2 A**). In contrast, the

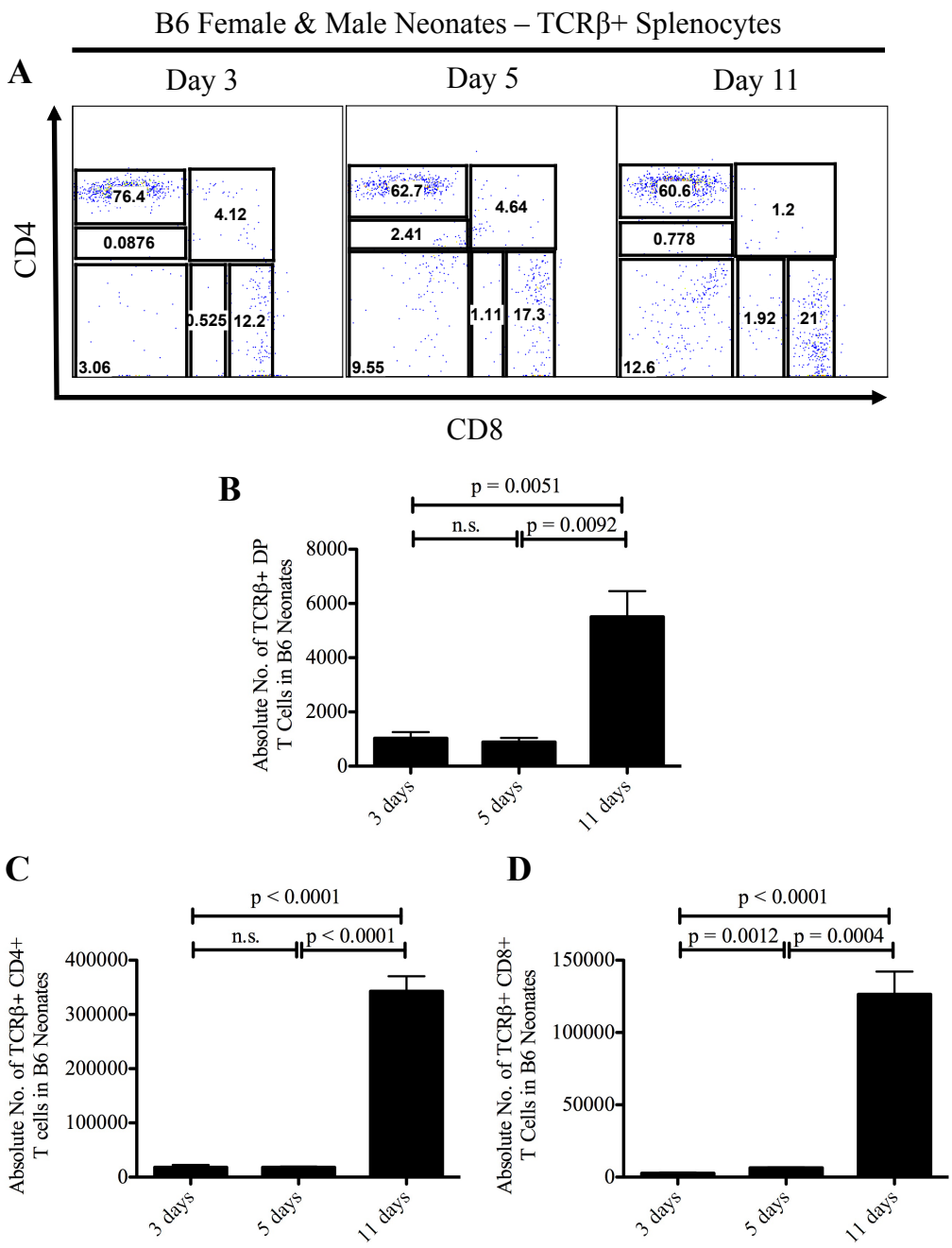


Fig. 3-15. The frequency and absolute number of TCRβ+ CD4+ and CD8+ T cells within the spleen of B6 neonates gradually and significantly increased between Days 3, 5 and 11 post-birth. **A.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression on total TCRβ+ splenocytes of B6 neonates. **B.** Graphical analysis of the absolute number of TCRβ+ DP T cells from the spleens of B6 neonates. **C.** Graphical analysis of the absolute number of TCRβ+ CD4+ T cells from the spleens of B6 neonates. **D.** Graphical analysis of the absolute number of TCRβ+ CD8+ T cells from the spleens of B6 neonates. n.s.: not significant. Representative of B6 neonates Day 3 n = 5; Day 5 n = 4; Day 11 n = 9.

proportion of T3.70+ splenocytes within HY^{cd4} female neonates at Day 3 was very low (**Fig. 3-16 A**). There was no increase in frequency of T3.70+ splenocytes between Days 5 and 11 post-birth, and PD-1 deficient neonates also lacked a T3.70+ T cell population. Graphical analysis of the absolute number of T3.70+ splenocytes showed very few T cells at Days 3 and 5 post-birth. At Day 11 post-birth there was a 2 to 4-fold increase in T3.70+ splenocyte number to approximately 20 000 cells (**Fig. 3-16 B**). PD-1 deficiency had no statistically significant difference on T3.70+ T cell number. Therefore, this data suggested that positive selection of T3.70+ T cells was not yet established at Day 3 to Day 11 post-birth and PD-1 deficiency had no impact on this population. Furthermore, due to the low frequency of T3.70+ T cells within the spleen of HY^{cd4} female neonates, it was difficult to make conclusions about the frequency and absolute number of CD4+ and CD8+ T cells. Therefore, the flow cytometry data of CD4 and CD8 coreceptor expression and absolute numbers from the spleens of HY^{cd4} female neonates is not shown in this thesis.

Negative selection is imperfect within HY^{cd4} male neonatal mice

While the HY^{cd4} female neonates appeared to have delayed positive selection of T3.70+ T cells, the DP thymocyte subset of HY^{cd4} male neonates had CD4 and CD8 coreceptor down regulation, characteristic of negative selection in the HY^{cd4} adult male mouse. However, as previously shown, there was the presence of an self-reactive, T3.70+ T cell population within the spleen of HY^{cd4} adult males, suggesting some thymocytes escaped clonal deletion. Therefore, we wanted to examine the spleens of HY^{cd4} male neonates to determine if an antigen-specific T3.70+ T cell population was present. Furthermore, HY^{cd4} wildtype and PD-1 knock-out male neonates were compared to examine if PD-1 deficiency had any impact on thymocyte escape from negative selection. A small proportion of antigen-specific T3.70+ T cells were present in HY^{cd4} male neonates as early as Day 3 post-birth and ranged from 1-2.5% (**Fig. 3-17 A**). PD-1 deficiency did not alter the frequency of T3.70+ thymocytes. Surface expression levels of T3.70

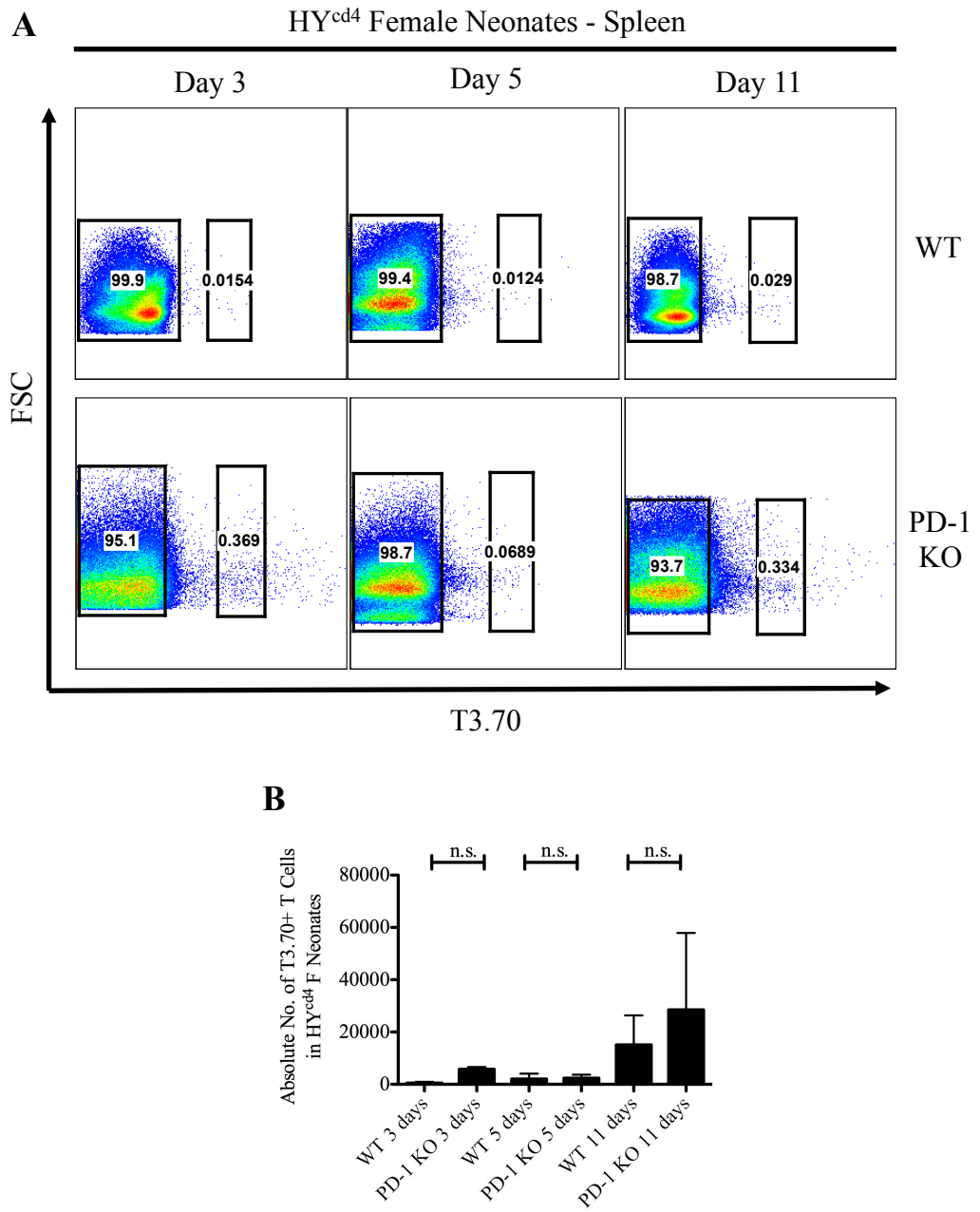


Fig. 3-16. The spleen of HY^{cd4} wildtype and PD-1 knock-out female neonates had very small frequencies and numbers of T3.70+ T cells at Days 3, 5 and 11 post-birth. **A.** Representative flow cytometric plots of T3.70 TCR expression on total splenocytes from HY^{cd4} wildtype and PD-1 knock-out female neonates. **B.** Graphical analysis of the absolute number of T3.70+ splenocytes from HY^{cd4} wildtype and PD-1 knock-out female neonates. n.s.: not significant. Representative of HY^{cd4} wildtype female neonates: Day 3 n = 7; Day 5 n = 9; Day 11 n = 3. Representative of HY^{cd4} PD-1 knock-out female neonates: Day 3 n = 4; Day 5 n = 4; Day 11 n = 5.

appeared to be higher in HY^{cd4} PD-1 knock-out male neonates compared to wildtype at Days 3 and 5 post-birth. However, there was no significant difference between HY^{cd4} wildtype and PD-1 knock-out male neonates on the respective days.

Graphical analysis of the absolute numbers of T3.70+ T cells in the spleen of HY^{cd4} male neonates showed an increase in T cell number between Days 3 and 11 post-birth. A marginal increase in T3.70+ T cell number was observed between Day 3 and Day 5 post-birth (**Fig. 3-17 B**). However, at Day 11, the HY^{cd4} male neonates had a 3-4 fold increase in the absolute number of T3.70+ T cells within the spleen in comparison to Day 3 and 5. PD-1 deficiency had no significant difference in T3.70+ T cell number (**Fig. 3-17 B**). Therefore, based on this evidence, we concluded that T3.70+ T cells were present within the spleen of both HY^{cd4} wildtype and PD-1 knock-out male neonates as early as Day 3 post-birth. The presence of T3.70+ T cells within the spleen suggested that at least some antigen-specific T3.70+ T cells escaped negative selection during the neonatal development stage. Finally, PD-1 deficiency had no impact on the frequency and absolute number of T3.70+ T cells within the spleen of HY^{cd4} male neonates.

In order to more fully understand the ontogeny of the antigen-specific T cells in the spleen, we examined the splenic T cell subsets of HY^{cd4} male neonates by CD4 and CD8 coreceptor expression. HY^{cd4} wildtype male neonates were compared to HY^{cd4} PD-1 knock-out male neonates to determine if PD-1 deficiency altered the frequency and absolute number of splenic T cell subsets. The flow cytometry data of the HY^{cd4} wildtype and PD-1 knock-out male neonates showed a very interesting and substantial DP T cell population within the spleen at Day 3 post-birth (**Fig. 3-18 A**). The frequency of this DP T cell population gradually decreased from Day 3 to Day 11 in HY^{cd4} male neonates. There was also a robust CD8+ T cell population that appeared to gradually down regulate CD8 coreceptor from Days 3 to 11 post-birth (**Fig. 3-18 A**). Therefore, the CD4 by CD8 plots suggested that DP and CD8+ T cells were present within

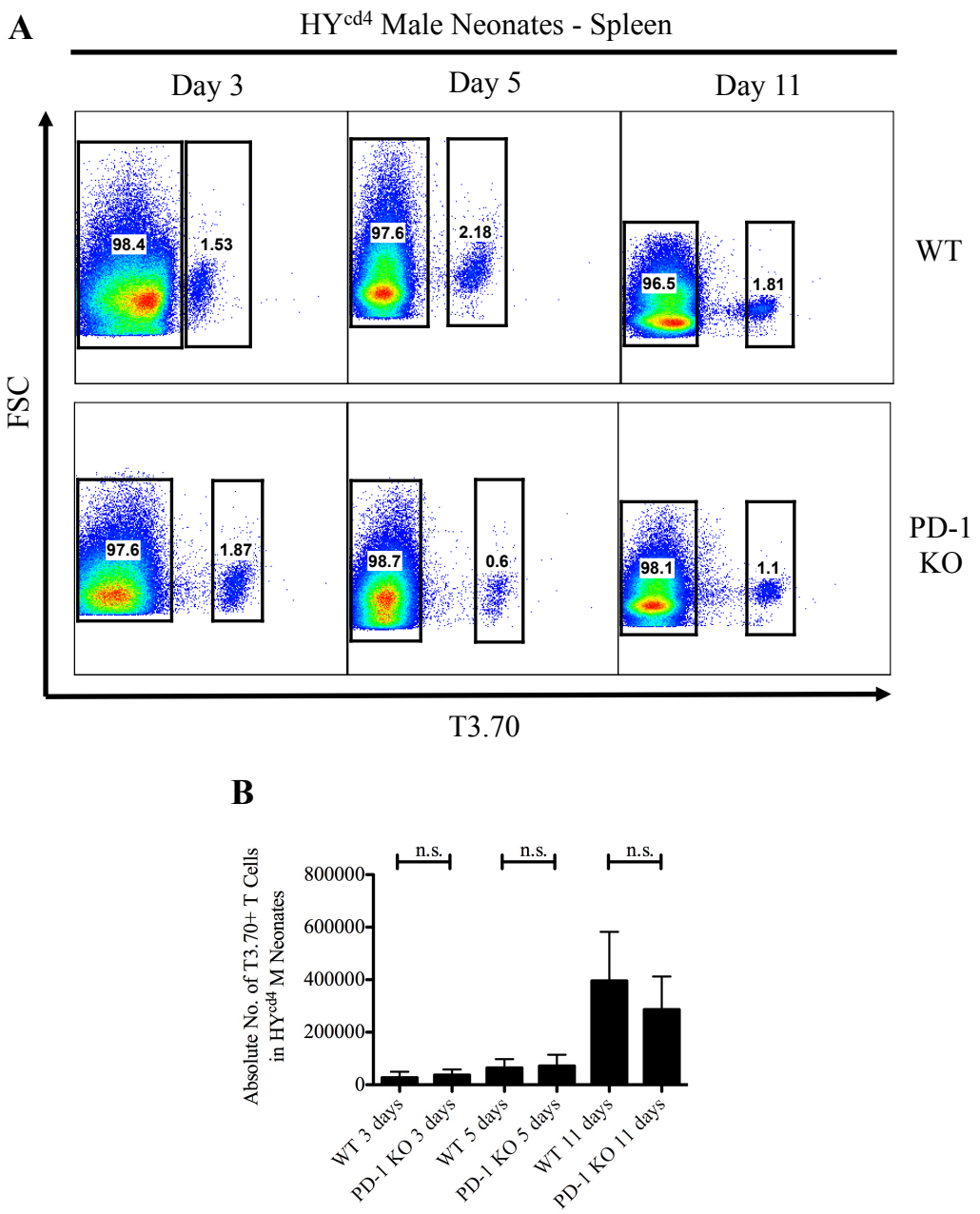


Fig. 3-17. HY^{cd4} wildtype and PD-1 knock-out male neonatal spleens contained similar frequencies and absolute numbers of T3.70+ T cells at Days 3, 5 and 11 post-birth. **A.** Representative flow cytometric plots of T3.70 TCR expression on total splenocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates. **B.** Graphical analysis of the absolute number of T3.70+ splenocytes from HY^{cd4} wildtype and PD-1 knock-out male neonates. n.s.: not significant. Representative of HY^{cd4} wildtype male neonates: Day 3 n = 10; Day 5 n = 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 knock-out male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

the spleen and that the DP T cells likely have escaped clonal deletion in both the HY^{cd4} wildtype and PD-1 knock-out male neonates.

In order to determine if the peripheral T3.70+ DP T cells decreased in number and the CD8+ T cells increased in number, as the flow cytometry plots suggested, the absolute number of cells were calculated and compared between HY^{cd4} wildtype and PD-1 knock-out male neonates. At Days 3 and 5 post-birth, there was a statistically significant increase in DP T cells number in HY^{cd4} PD-1 knock-out male neonates compared to wildtype (**Fig. 3-18 B**). However, at Day 11 post-birth, HY^{cd4} PD-1 deficient male neonates had a two-fold reduction in DP T cell number compared to HY^{cd4} wildtype male neonates; this difference was statistically significant. Therefore, it appeared that PD-1 deficiency allowed for higher numbers of DP T cells within the spleen at Days 3 and 5 post-birth.

Within the CD8+ T cell compartment, the number of cells remained well below 50 000 at Days 3 and 5, with no statistically significant difference between HY^{cd4} wildtype and PD-1 deficient male neonates (**Fig. 3-18 C**). At Day 11, there was a 3-4 fold increase to approximately 150 000 CD8+ T cells within both HY^{cd4} wildtype and PD-1 knock-out male neonates. However, similar to the observations at Day 3 and 5, there was no statistically significant difference in the absence of PD-1. Therefore, within the spleen of HY^{cd4} male neonates, it appeared that the DP T cells marginally increased between Days 3 and 11, except in HY^{cd4} PD-1 knock-out male neonates, which had a decrease in number at Day 11. The CD8+ T cell subsets increased in number between Day 3 and Day 11 post-birth. These changes in splenocyte number were not PD-1 dependent. Based on this evidence, two substantial populations of T3.70+ T cells are present within the periphery of HY^{cd4} male neonatal mice. It is possible that these cells originate from imperfect central tolerance mechanisms in a PD-1 independent manner, however the data presented here does not directly show that. Alternatively, it is possible that these cells may be extrathymic in origin.

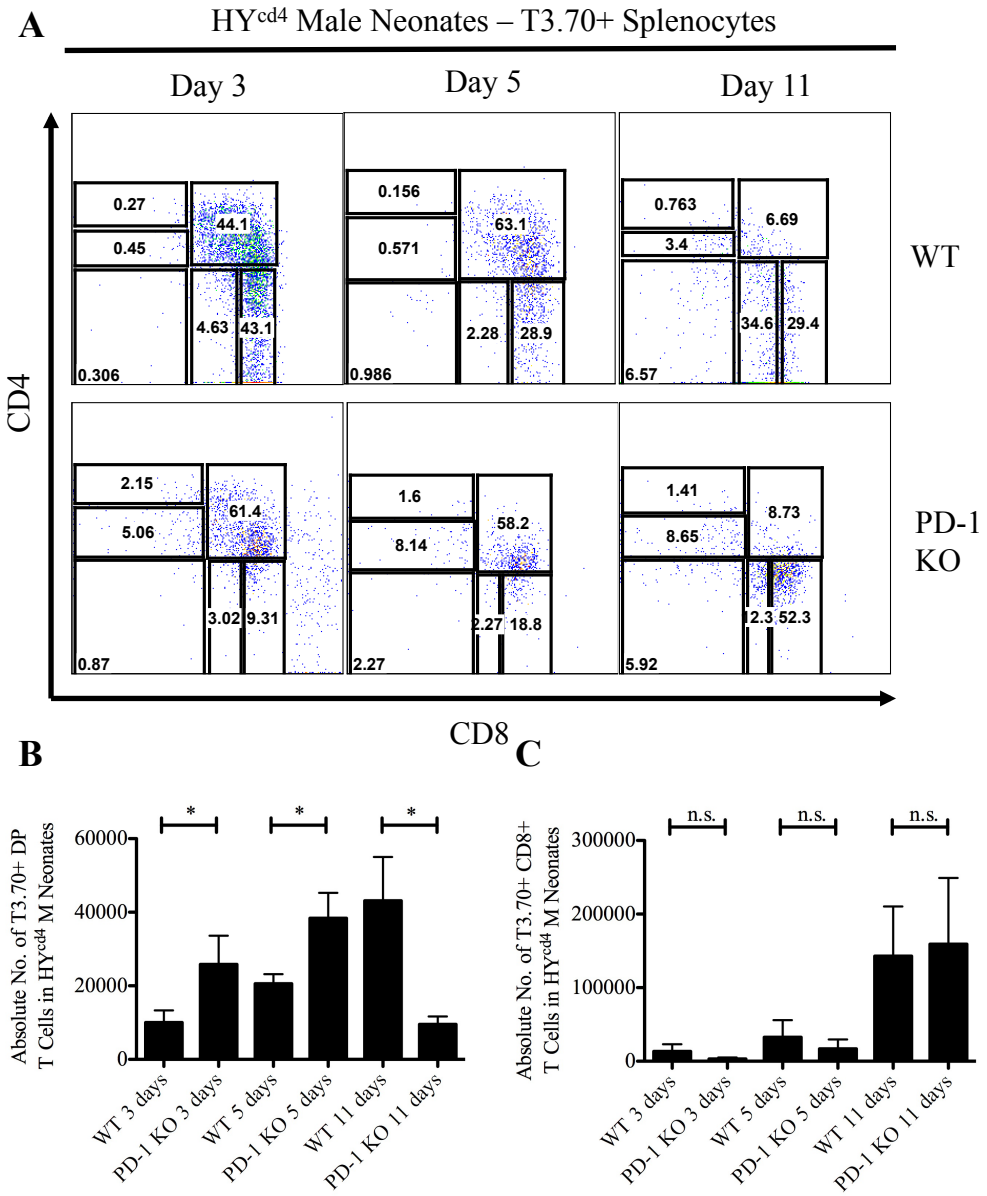


Fig. 3-18. The spleens of both HY^{cd4} wildtype and PD-1 knock-out male neonates contained substantial T3.70+ DP and CD8+ T cell populations at Day 3 post-birth. **A.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression on T3.70+ splenocytes of HY^{cd4} wildtype and PD-1 knock-out male neonates. **B.** Graphical analysis of the absolute number of T3.70+ DP T cells from the spleens of HY^{cd4} wildtype and PD-1 knock-out male neonates. Day 3 HY^{cd4} wildtype to PD-1 KO: $p = 0.0473$. Day 5 HY^{cd4} wildtype to PD-1 KO: $p = 0.0111$. Day 11 HY^{cd4} wildtype to PD-1 KO: $p = 0.0334$. **C.** Graphical analysis of the absolute number of T3.70+ CD8+ T cells from the spleens of HY^{cd4} wildtype and PD-1 knock-out male neonates. n.s.: not significant. Representative HY^{cd4} WT M neonates Day 3 n = 10; Day 5 n = 10; Day 11 n = 8; HY^{cd4} PD-1 KO M neonates Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

Antigen-specific T3.70+ DP T cells from the spleens of HY^{cd4} neonatal mice do not undergo selection events within the periphery

T3.70+ DP T cells from HY^{cd4} male neonatal mice were not overtly activated within the periphery and undergoing apoptosis, but expressed high levels of PD-1

By convention, DP thymocytes undergo selection processes and mature into SP thymocytes within the thymus and then enter the periphery. However, I found that the spleen of HY^{cd4} male neonates had a surprisingly high proportion of DP T cells. It was considered that the peripheral DP T cells might be undergoing further selection and maturation events within the spleen. Therefore expression of various molecules associated with negative selection events were assessed on the T3.70+ DP T cells of HY^{cd4} male neonates. As previously analyzed within the thymus, CD69 and PD-1 surface expression were assessed as markers of TCR signaling and activation and active caspase 3 expression was assessed for apoptosis induction during clonal deletion. Due to the unique nature of this T cell population, there were no adult mouse control populations available for direct comparison of CD69 and PD-1 surface expression. Therefore, CD69 and PD-1 surface expression levels of DP T cells from HY^{cd4} male neonates were compared to CD8+ T cells of HY^{cd4} adult male mice, a population not readily activated within the periphery (*unpublished data*), and B6 adult female mice.

The DP T cell population was initially analyzed for surface expression of CD69 and PD-1, molecules whose expression is associated with T cell activation following downstream TCR signaling (151). HY^{cd4} wildtype and PD-1 knock-out male neonate DP T cells displayed a very slight up regulation in CD69 surface expression at Days 3 to 11 compared to CD8+ T cells from HY^{cd4} adult male and B6 adult female controls (**Fig. 3-19 A**). Generally, CD69 surface expression is coupled with PD-1, therefore when I examined PD-1 expression on DP T cells I expected to see only slight upregulation on DP T cells from HY^{cd4} male neonates in comparison to the adult controls, similar to the CD69 profiles. However, PD-1

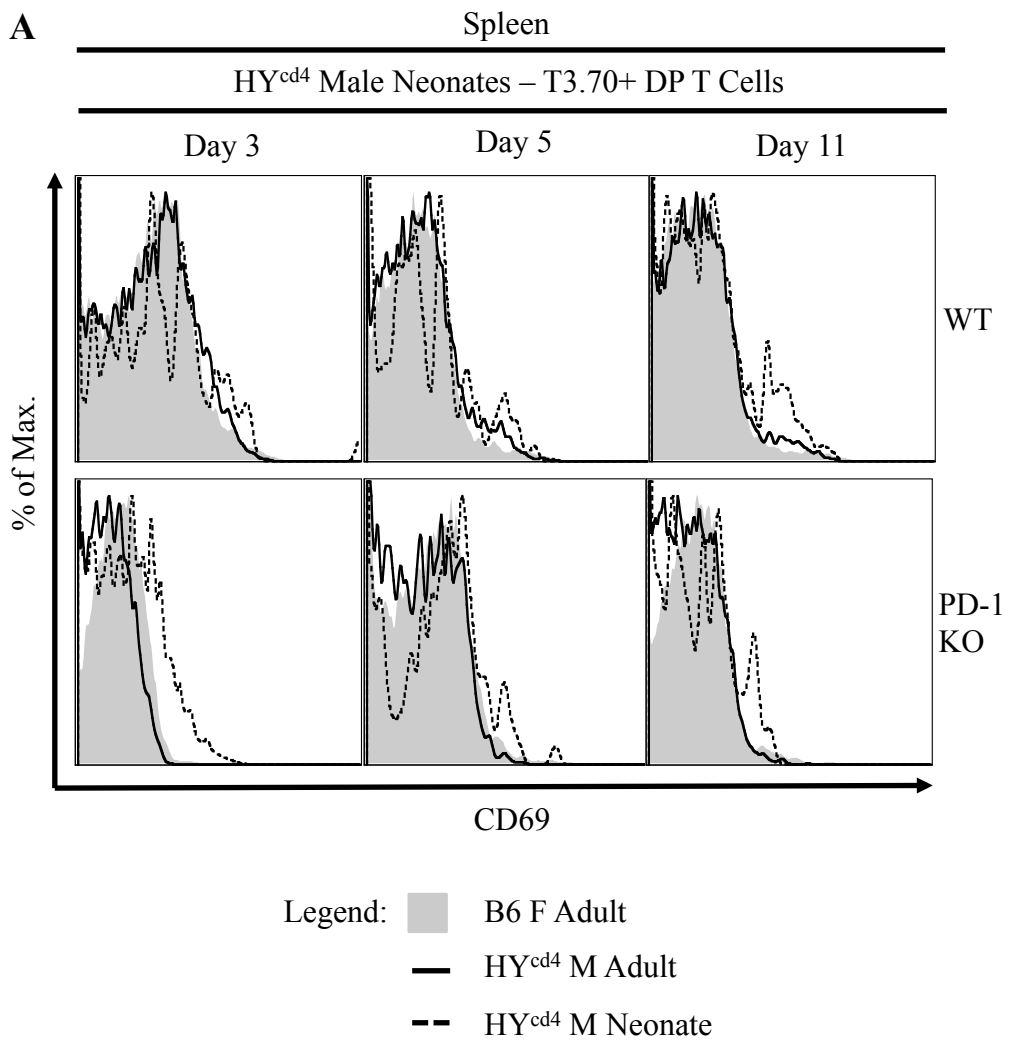


Fig. 3-19. Splenic T3.70+ DP T cells from HY^{cd4} male neonates had slightly up regulated levels of CD69 activation marker compared to T3.70+ CD8+ T cells from HY^{cd4} adult male mice and TCRβ+ CD8+ T cells from B6 adult female mice. **A.** Histogram overlays of CD69 surface expression on T3.70+ DP T cells from HY^{cd4} wildtype and PD-1 knock-out male neonates compared to T3.70+ CD8+ T cells from HY^{cd4} adult male controls and TCRβ+ CD8+ T cells from B6 adult female controls. The solid grey represents the B6 adult female control, the solid line represents the HY^{cd4} adult male control and the dashed line represents the HY^{cd4} male neonate at the respective day post-birth. Representative of HY^{cd4} WT male neonates: Day 3 n = 10; Day 5 n= 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 KO male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

expression was drastically higher on the surface of DP T cells from HY^{cd4} male neonates compared to HY^{cd4} adult male and B6 adult female CD8+ T cells (**Fig. 3-20 A**). The differential expression of CD69 and PD-1 suggested that these receptors were not coupled in their expression between Days 3 and 11 post-birth. It was interesting to find PD-1 expression without CD69 surface expression on the DP T cells from HY^{cd4} male neonates since I did not see this difference within the thymus.

Although the DP T cells from HY^{cd4} male neonates were not overtly activated, based on minimal CD69 surface expression, we still considered that these cells might be undergoing further development within the spleen. I assessed intracellular active caspase 3 to determine if the DP T cells were undergoing clonal deletion by apoptosis within the spleen. The frequency of active caspase 3 expressing DP T cells was variable among the HY^{cd4} wildtype male neonates of different days post-birth. At Day 3, the proportion of cells was approximately 0.1% (**Fig. 3-21 A**). The proportion of active caspase 3+ DP T cells increased to about 0.8-1.3% at Day 5 and sustained at that frequency at Day 11 (**Fig. 3-21 A**). The changing frequency of active caspase 3 positive DP T cells within the spleen of HY^{cd4} wildtype male neonates suggested these cells were undergoing apoptosis to varying degrees. Further conclusions about apoptosis induction within this unique DP T cell population from the spleens of HY^{cd4} male neonates were impossible due to the lack of a suitable control population. However interestingly, the spleen DP T cells did not show a separate active caspase 3+ population as was seen within the thymus (**Fig. 3-14 A**).

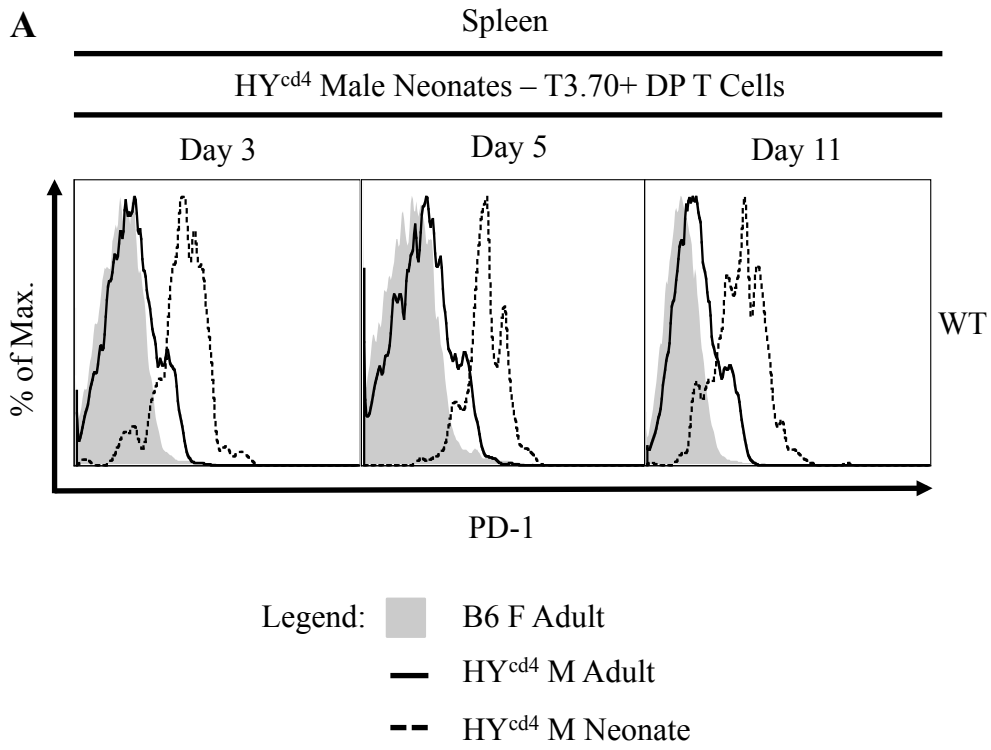


Fig. 3-20. Splenic T3.70+ DP T cells from HY^{cd4} wildtype male neonates expressed drastically higher levels of the inhibitory receptor PD-1 compared to control T cells from HY^{cd4} adult male mice and B6 adult female mice. **A.** Histogram overlays of PD-1 surface expression on T3.70+ DP T cells from HY^{cd4} wildtype male neonates compared to T3.70+ CD8+ T cells from HY^{cd4} adult male controls and TCRβ+ CD8+ T cells from B6 adult female controls. The solid grey represents the B6 adult female control, the solid line represents the HY^{cd4} adult male control and the dashed line represents the HY^{cd4} male neonate at the respective day post-birth. Representative of HY^{cd4} WT male neonates: Day 3 n = 10; Day 5 n= 10; Day 11 n = 8.

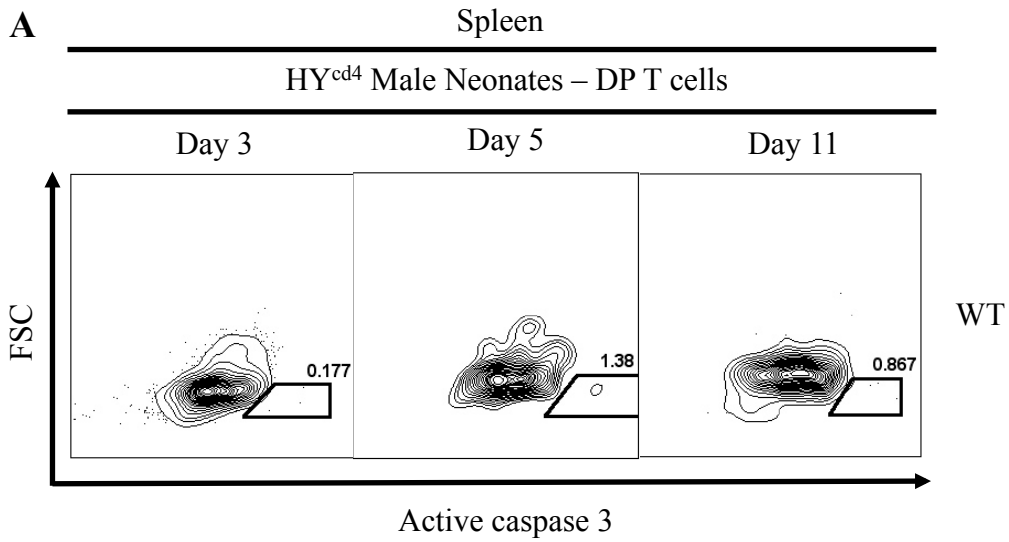


Fig. 3-21. Variable frequencies of active caspase 3 expressing T3.70+ DP T cells from HY^{cd4} male neonatal spleens may suggest that these cells were undergoing apoptosis to varying degrees. **A.** Representative flow cytometric plots of active caspase 3 expressing T3.70+ DP T cells from the spleens of HY^{cd4} wildtype male neonates at Day 3, 5 and 11 post-birth. Representative of HY^{cd4} WT male neonates: Day 3 n = 10; Day 5 n= 10;

Antigen-specific T3.70+ CD8+ T cells within the spleen of HY^{cd4} male neonatal mice have similar characteristics to HY^{cd4} adult male CD8+ T cells

Antigen-specific T3.70+ CD8+ T cells from the spleen of HY^{cd4} male neonatal mice had a central memory phenotype as early as Day 3 post-birth

As previously shown within this thesis, the spleens from HY^{cd4} male neonatal mice contained a substantial proportion of CD8+ T cells. Once in the periphery, naïve CD8+ T cells can adopt different memory phenotypes following antigen encounter, such as central memory or effector memory and these populations are identified by differential expression of CD44 and CD62L. The central memory and effector memory phenotypes are acquired within the periphery as CD8+ T cells are not exported from the thymus with these phenotypes. Therefore, we wanted to examine the surface expression of CD62L and CD44 within the HY^{cd4} male neonatal CD8+ T cell population in the spleen to determine when these cells transition from a naïve phenotype to a memory phenotype.

Prior to analyzing the HY^{cd4} male neonatal CD8+ T cell population, the CD8+ T cells of the B6 adult female and HY^{cd4} adult female and male mice were examined for their memory phenotype. This analysis provided a baseline of the peripheral memory CD8+ T cell populations within the adult controls that could then be compared to the HY^{cd4} male neonates. The memory profiles of the polyclonal CD8+ T cell population of the B6 adult female differed from the monoclonal T3.70+ CD8+ T cells of HY^{cd4} adult female and male mice. Within the CD8+ T cell population of the B6 adult female spleen, the majority of cells were defined as naïve with a small proportion of cells being central memory T cells (**Fig. 3-22 A**). HY^{cd4} adult female mice also had a large proportion of naïve CD8+ T cells but had much fewer central memory T cells compared to the B6 adult female control. In comparison, the majority of CD8+ T cells from the HY^{cd4} adult male spleen were central memory T cells and the remainder had a naïve phenotype (**Fig. 3-22 A**). The large frequency of central memory CD8+ T cells is

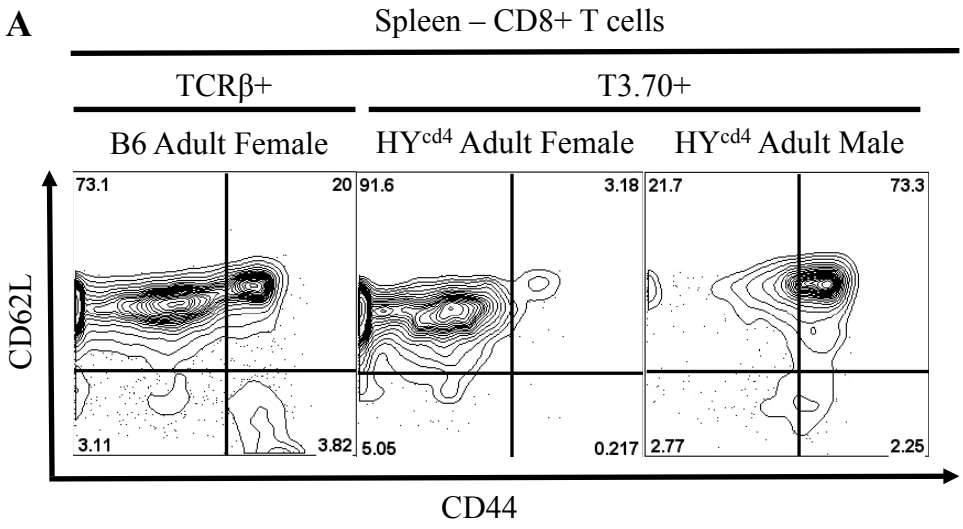


Fig. 3-22. B6 and HY^{cd4} adult female mice have a small proportion of CD62L+ CD44+ central memory CD8+ T cells whereas the majority of T3.70+ CD8+ T cells from HY^{cd4} adult male mice are CD62L+ CD44+ central memory T cells. **A.** Representative flow cytometric plots of the surface expression CD62L and CD44 on CD8+ T cells from the TCRβ+ T cell population in the spleen of a B6 adult female control and from the T3.70+ T cell populations in the spleens of HY^{cd4} adult female and male mice. Representative of B6 n = 10; HY^{cd4} F n = 9; HY^{cd4} M n = 9.

characteristic of the HY^{cd4} male mouse model (*unpublished data*). Therefore, the HY^{cd4} adult male CD8+ T cells had adopted a central memory phenotype within the spleen whereas the majority of cells from the HY^{cd4} adult female were naïve.

The proportion of central memory CD8+ T cells from HY^{cd4} wildtype and PD-1 knock-out male neonates was drastically reduced compared to the HY^{cd4} adult male control. The HY^{cd4} male neonates had approximately 30-40% less central memory CD8+ T cells compared to the HY^{cd4} adult male control. Within the spleen of HY^{cd4} male neonates, CD8+ T cells had an increased proportion of CD62L+ CD44+ central memory T cells between Day 3 to Day 5, which then was sustained to Day 11 (**Fig. 3-23 A**). The absence of PD-1 did not appear to alter the overall frequencies of T3.70+ CD8+ central memory T cells within the HY^{cd4} male neonates (**Fig. 3-23 A**). Therefore this data suggested that peripheral CD8+ T cells from HY^{cd4} male neonates could adopt a central memory phenotype at Day 3 post-birth and this process did not appear to be PD-1 dependent.

Splenic T3.70+ CD8+ T cells of HY^{cd4} male neonatal mice have gradually down regulated CD8 coreceptor and but have high expression of PD-1

Both the HY^{cd4} male neonates and adult male controls have an antigen-specific T3.70+ CD8+ T cell population within the spleen, characteristic of the HY^{cd4} male mouse model. Despite the presence of this antigen-specific CD8+ T cell population within the periphery, these cells do not cause overt autoimmunity (*unpublished data*). Therefore, peripheral tolerance mechanisms must be enforced on these cells to prevent activation. One proposed method of altering TCR sensitivity to antigen stimulation within the periphery is through CD8 coreceptor modulation by IL-7R signaling (149). Within the HY^{cd4} adult male mouse, peripheral CD8+ T cells displayed CD8 coreceptor down regulation. In order to determine if HY^{cd4} male neonates displayed similar characteristics, CD8+ T cells were examined for CD8 coreceptor expression and compared to HY^{cd4} adult male and B6 adult female controls.

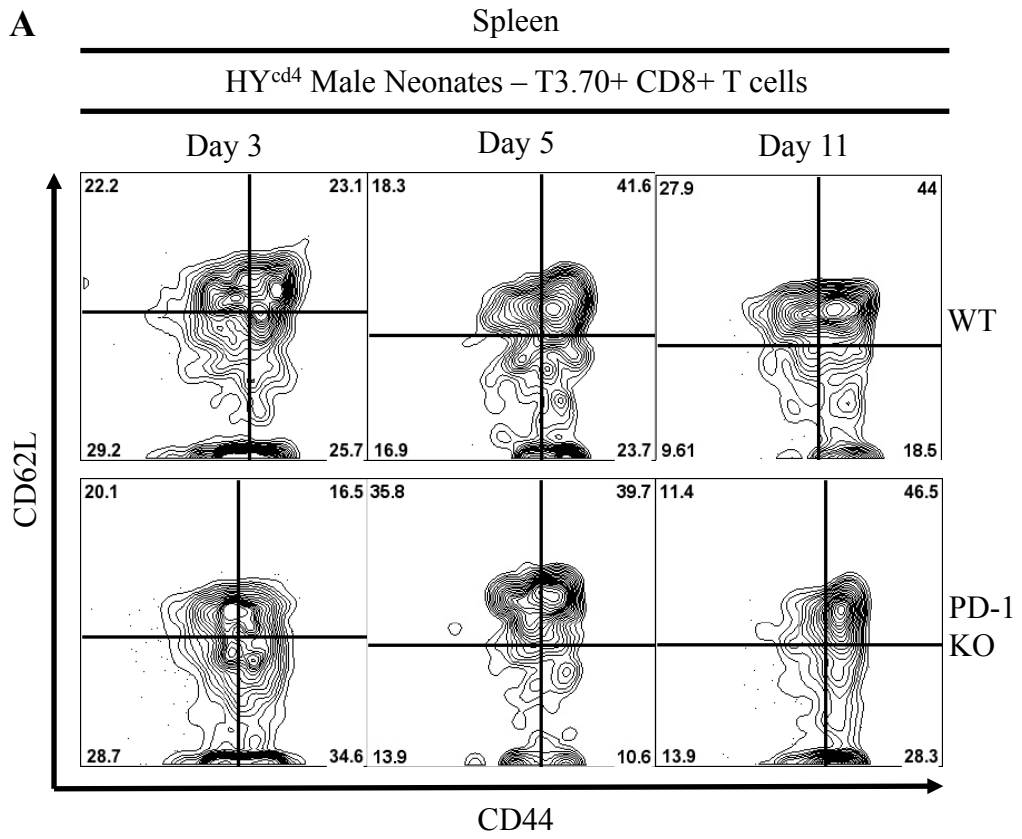


Fig. 3-23. No apparent differences were found in the frequency of CD62L+ CD44+ central memory T3.70+ CD8+ splenocytes from HY^{cd4} wildtype and PD-1 knock-out male neonatal mice at Days 3, 5 and 11 post-birth. **A.** Representative flow cytometric plots of the surface expression CD62L and CD44 on CD8+ T cells from the T3.70+ T cell populations in the spleens of HY^{cd4} wildtype and PD-1 knock-out male neonates at the respective days post-birth. Representative of HY^{cd4} WT male neonates: Day 3 n = 10; Day 5 n= 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 KO male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

The histogram overlays of CD8 coreceptor expression showed a very interesting change between Day 3, 5 and 11 HY^{cd4} male neonates. At Day 3, CD8 coreceptor expression on CD8+ T cells from HY^{cd4} male neonates was identical to CD8+ T cells from the B6 adult female control (**Fig. 3-24 A**). However, at Day 5 and Day 11, the CD8 coreceptor levels gradually reduced to a level below the B6 adult female control but identical to the HY^{cd4} adult male control CD8+ T cells. This change was evident in the HY^{cd4} PD-1 knock-out male neonates as well, in comparison to the respective controls (**Fig. 3-24 A**). Therefore, this data suggested that HY^{cd4} male neonatal CD8+ T cells gradually reduced CD8 coreceptor expression levels after birth and established a similar CD8 expression pattern to HY^{cd4} adult male CD8+ T cells at Day 11 post-birth. Although this evidence with HY^{cd4} male neonates did support CD8 coreceptor down regulation as a characteristic of the HY^{cd4} male mouse at both adult and neonatal stages, it did not conclusively define CD8 down regulation as a form of peripheral tolerance. Further analysis shown within the Appendix of this thesis presents evidence of CD8 coreceptor modulation as a form of peripheral tolerance within the HY^{cd4} male mouse model.

Finally, we were interested in examining the expression of PD-1 on the CD8+ T cell population within HY^{cd4} male neonates. As shown above, the DP T cells within the spleen of HY^{cd4} male neonates showed increased levels of PD-1 surface expression compared to the DP thymocytes within the thymus (**Fig. 3-20 A**, **3-12 A**). This high expression level appeared to be restricted to the HY^{cd4} neonatal mice, as HY^{cd4} adult male CD8+ T cells, used as controls, had less peripheral expression of PD-1. Therefore, we considered that PD-1 may also be expressed on the surface of HY^{cd4} male neonatal CD8+ T cells in the spleen and could potentially have a role in tolerance induction of this self-reactive population within the periphery. PD-1 surface expression was assessed on splenic CD8+ T cells from HY^{cd4} male neonates and then compared to CD8+ T cells from HY^{cd4} adult male and B6 adult female controls. Within the T3.70+ CD8+ T cells of the HY^{cd4} male neonatal spleen, there was a shift in PD-1 surface expression at

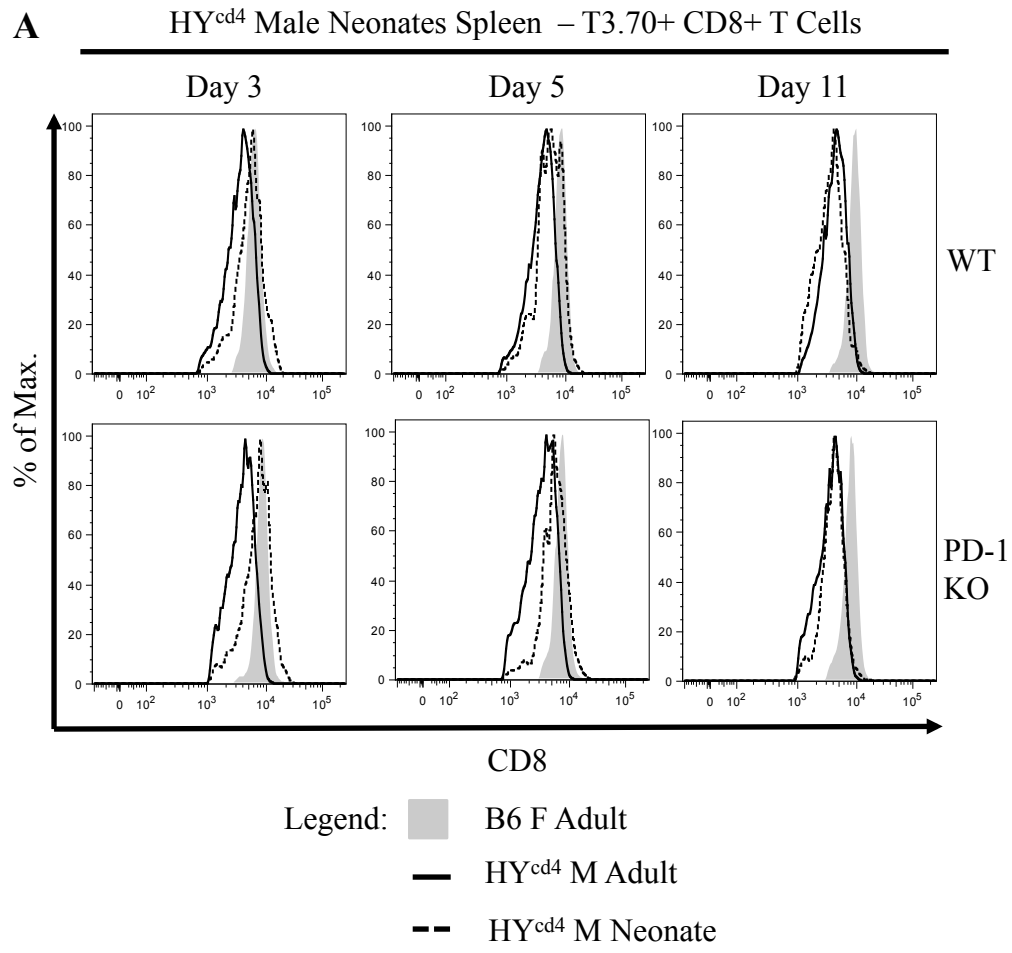


Fig. 3-24. Surface expression levels of CD8 coreceptor on HY^{cd4} male neonatal CD8+ T cells gradually reduced from wildtype levels at Day 3 post-birth to levels identical to HY^{cd4} adult male CD8+ T cells at Day 11 post-birth. **A.** Histogram overlays of CD8α coreceptor surface expression on T3.70+ CD8S+ T cells from the spleens HY^{cd4} wildtype and PD-1 knock-out male neonates compared to T3.70+ CD8+ T cells from HY^{cd4} adult male controls and TCRβ+ CD8+ T cells from B6 adult female controls. The solid grey represents the B6 adult female control, the solid line represents the HY^{cd4} adult male control and the dashed line represents the HY^{cd4} male neonate at the respective day post-birth. Representative of HY^{cd4} WT male neonates: Day 3 n = 10; Day 5 n= 10; Day 11 n = 8. Representative of HY^{cd4} PD-1 KO male neonates: Day 3 n = 6; Day 5 n = 5; Day 11 n = 7.

Days 3, 5 and 11 post-birth compared to the adult controls. Interestingly, PD-1 expression appeared to start to coming down on T3.70+ CD8+ T cells from Day 11 HY^{cd4} male neonates (**Fig. 3-25 A**). This observation coincided with the reduced CD8 coreceptor expression also seen within Day 11 HY^{cd4} male neonates (**Fig. 3-24 A**). Based on the high levels of PD-1 surface expression on peripheral CD8+ T cells from HY^{cd4} male neonates, it is possible that this may be a form of peripheral T cell regulation. The T3.70+ CD8+ T cells from HY^{cd4} adult male had reduced levels of PD-1 surface expression, therefore it was considered at what stage of development does PD-1 surface expression get turned off. In order to address this question, an experiment analyzing Day 15 and Day 21 HY^{cd4} male neonates may demonstrate the shift in PD-1 surface expression on T3.70+ CD8+ T cells. I can speculate, based on this data, that PD-1 may be functioning in a tolerating manner to prevent the activation of the antigen-specific CD8+ T cells within HY^{cd4} male neonates until another mechanism is established, allowing for PD-1 down regulation as seen on CD8+ T cells from HY^{cd4} adult male spleens.

The directly *ex vivo* studies of HY^{cd4} wildtype and PD-1 knock-out male neonates have shown the presence of antigen-specific T3.70+ DP and CD8+ T cells within the spleen. Despite the process of negative selection enforcing central tolerance, T3.70+ T cells have somehow escaped the thymus and entered the periphery. Furthermore, the inhibitory receptor PD-1 was highly expressed on both DP T cells and CD8+ T cells from HY^{cd4} male neonates suggesting that PD-1 may have a functional role in the neonatal T cell repertoire.

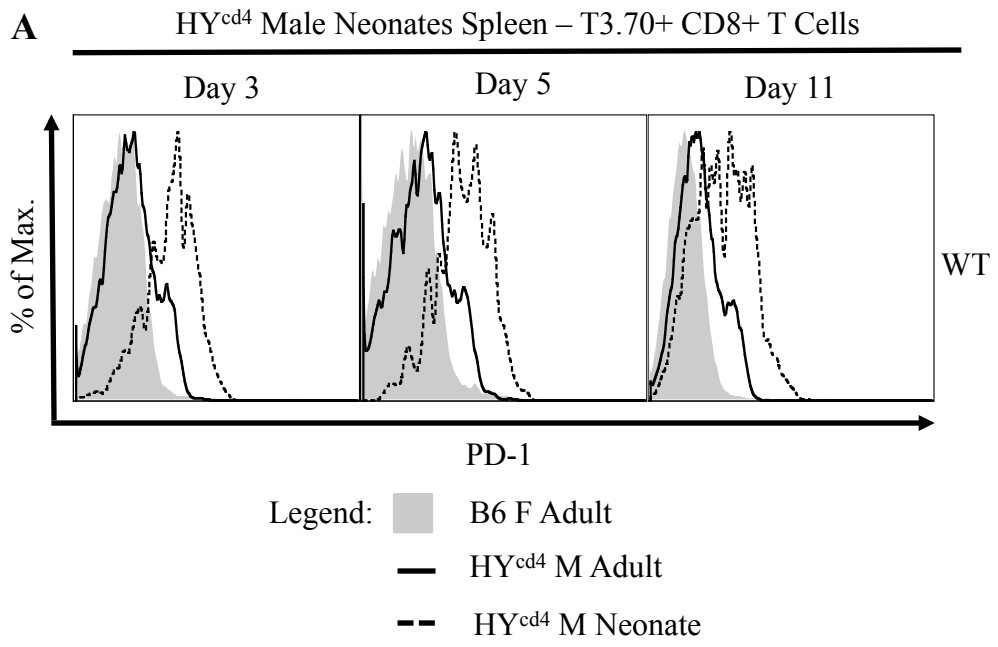


Fig. 3-25. T3.70+ CD8+ splenocytes from HYcd4 wildtype male neonates had high surface expression of the inhibitory receptor PD-1 at Days 3, 5 and 11 post-birth compared to both the HYcd4 adult male and B6 adult female controls. **A.** Histogram overlays of PD-1 surface expression on T3.70+ CD8+ T cells from the spleens HY^{cd4} wildtype male neonates compared to T3.70+ CD8+ T cells from HY^{cd4} adult male controls and TCRβ+ CD8+ T cells from B6 adult female controls. The solid grey represents the B6 adult female control, the solid line represents the HY^{cd4} adult male control and the dashed line represents the HY^{cd4} male neonate at the respective day post-birth. Representative of HY^{cd4} WT male neonates: Day 3 n = 10; Day 5 n= 10; Day 11 n = 8.

T3.70+ T cell escape from the thymus is not due to the immature HY^{cd4} neonatal thymic tissue

HY^{cd4} wildtype and PD-1 knock-out male mixed bone marrow chimeras demonstrate that negative selection is not intact within an adult model

It was considered that the presence of the antigen-specific T3.70+ T cell population within the periphery of HY^{cd4} male neonates might be a result of neonatal thymic development. It is known that the thymic epithelium lacks maturity within neonatal mice, based on the analysis of neonatal thymic epithelium showing both a cTEC and mTEC fate potential in the early days post-birth (152). Therefore it is possible that the T3.70+ T cells escaped negative selection strictly within the HY^{cd4} male neonates and then persisted within the periphery to adult stages. In order to address this question, HY^{cd4} wildtype and PD-1 knock-out male mixed bone marrow chimeras were generated. HY^{cd4} PD-1 knock-outs were included within this assay to determine if PD-1 had a functional role within T cell development that could not be observed directly *ex vivo* from a knock-out mouse. Therefore, the competitive environment of the mixed bone marrow chimera would demonstrate the contribution of PD-1 to T cell development.

The thymocytes generated from the HY^{cd4} wildtype and PD-1 knock-out male bone marrow were analyzed at 10 weeks directly *ex vivo*. Within the male mixed bone marrow chimeras, the relative proportion of antigen-specific T3.70+ thymocytes present within the thymus was 10-20% (**Fig. 3-26 A**). In order to determine the relative chimerism of the T3.70+ T cells, the HY^{cd4} bone marrow was distinguished by CD45.1 congenic marker expression. HY^{cd4} wildtype bone marrow-derived thymocytes were CD45.1+ and HY^{cd4} PD-1 knock-out bone marrow-derived thymocytes were CD45.1-. The majority of T3.70+ thymocytes were CD45.1- (**Fig. 3-26 B**); therefore the HY^{cd4} PD-1 knock-out bone marrow appeared to be outcompeting the wildtype bone marrow.

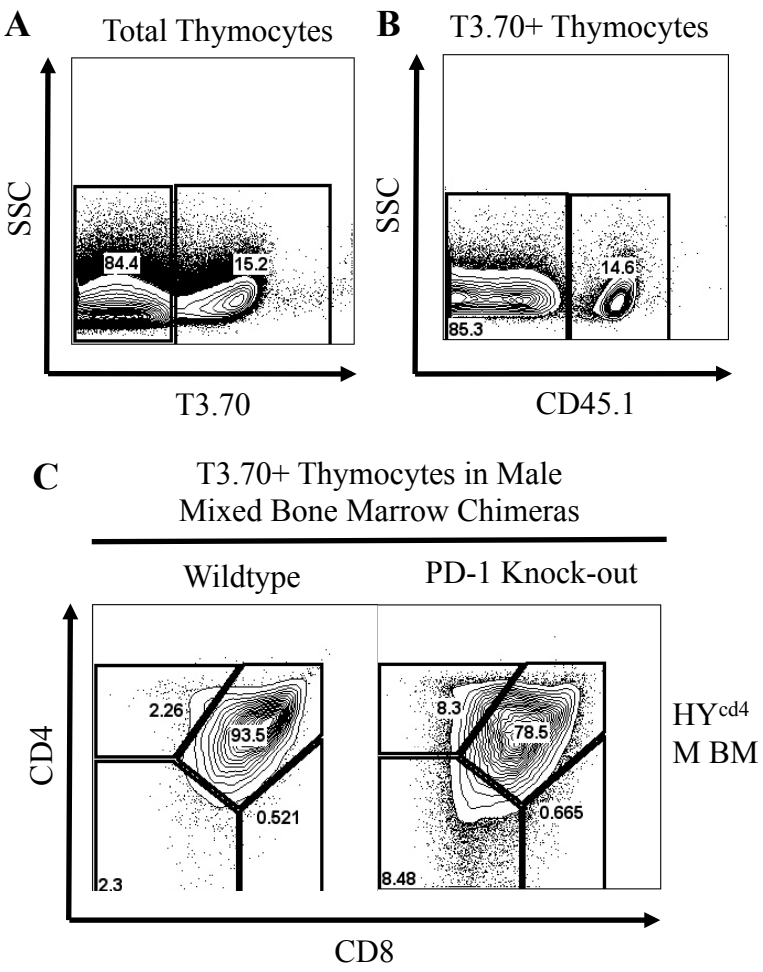


Fig. 3-26. Within the thymus of male mixed bone marrow chimeras, HY^{cd4} PD-1 knock-out male-derived thymocytes contributed to the majority of T3.70+ thymocytes. **A.** Representative flow cytometric plot of T3.70 expression of thymocytes within male mixed bone marrow chimeras. **B.** Representative flow cytometric plot of CD45.1 expression showing chimerism within the T3.70+ thymocyte population of the male mixed bone marrow chimeras. CD45.1+: HY^{cd4} wildtype male thymocytes; CD45.1-: HY^{cd4} PD-1 knock-out male thymocytes. **C.** Representative flow cytometric plots of the T3.70+ thymocyte subsets derived from HY^{cd4} wildtype and PD-1 knock-out male bone marrow based on CD4 and CD8 coreceptor expression. Representative of n = 1 at 24 hours, n = 3 at 48 hours and n = 3 at 72 hours.

When the thymic T cell subsets were further analyzed for CD4 and CD8 coreceptor expression, there were no gross differences in the thymocytes derived from HY^{cd4} wildtype or PD-1 knock-out bone marrow. The broad population of DP thymocytes had marginal down regulation of both CD4 and CD8 coreceptors, which appeared to be more drastic in the HY^{cd4} PD-1 knock-out bone marrow subset (**Fig. 3-26 C**). The absence of a T3.70+ CD8SP thymocyte population from both HY^{cd4} bone marrow-derived populations suggested that negative selection was intact within the male mixed bone marrow chimeras.

The peripheral compartment of the male mixed bone marrow chimeras was analyzed to determine if an antigen-specific T3.70+ T cell population was present. We reasoned that if these T cells were present within the periphery, it would support the conclusion that negative selection is imperfect and that T3.70+ T cell escape was not due to the immature thymus within the HY^{cd4} male neonates. Within the spleen of the male mixed bone marrow chimera, T3.70+ T cells accounted for a small but distinguishable proportion of total cells (**Fig. 3-27 A**), suggesting that negative selection was leaky and some T3.70+ T cells have escaped selection. The relative chimerism of the T3.70+ was again determined by CD45.1 expression, therefore T cells derived from HY^{cd4} wildtype bone marrow were CD45.1+ and T cells derived from HY^{cd4} PD-1 knock-out bone marrow were CD45.1-. The majority of the peripheral T cells was CD45.1- and therefore derived from the HY^{cd4} PD-1 knock-out bone marrow (**Fig. 3-27 B**), suggesting that more PD-1 deficient T cells contributed to the overall T cell population compared to wildtype.

We then analyzed the T3.70+ T cells for CD4 and CD8 coreceptor expression in the spleen of male mixed bone marrow chimeras to determine if the T3.70+ T cell population was composed of CD8+ T cells. The majority of T3.70+ T cells were CD8+ T cells, ranging in frequency from 55-75% and had reduced levels of CD8 coreceptor (**Fig. 3-27 C**). Therefore, the presence of the T3.70+ CD8+ T cells within the spleen of the male mixed bone marrow chimeras suggested that

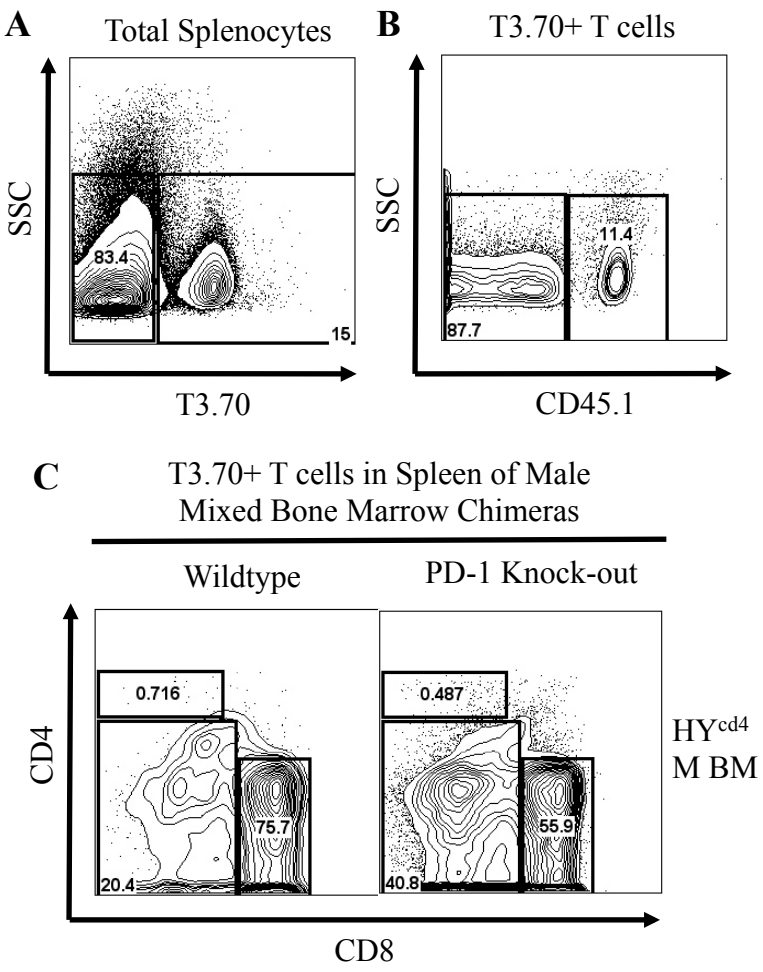


Fig. 3-27. Male mixed bone marrow chimeras contained a sizeable proportion of splenic T3.70+ T cells that were majorly derived from HY^{cd4} PD-1 knock-out male bone marrow, suggesting that thymic negative selection is leaky and some antigen-specific T cells have escaped. **A.** Representative flow cytometric plot of T3.70 expression of splenocytes within male mixed bone marrow chimeras. **B.** Representative flow cytometric plot of CD45.1 expression showing chimerism within the T3.70+ T cell population of the spleen from male mixed bone marrow chimeras. CD45.1+: HY^{cd4} wildtype male T cells; CD45.1-: HY^{cd4} PD-1 knock-out male T cells. **C.** Representative flow cytometric plots of the splenic T3.70+ T cell subsets derived from HY^{cd4} wildtype and PD-1 knock-out male bone marrow based on CD4 and CD8 coreceptor expression. Representative of n = 1 at 24 hours, n = 3 at 48 hours and n = 3 at 72 hours.

negative selection is imperfect within the thymus and the escape of T3.70+ T cells from the thymus was not due to the immature thymic environment of the HY^{cd4} male neonates. The escape of T3.70+ T cells from the thymus may be a process that naturally occurs within the HY^{cd4} male model system and consequently central tolerance is imperfect.

Antigen-specific T3.70+ T cells are present within the periphery of HY^{cd4} male neonates

The goal of the HY^{cd4} neonatal studies was to analyze the ontogeny of the T3.70+ CD8+ T cells and determine when these T cells are present within the periphery. Our studies have shown that a small proportion of T3.70+ T cells are found within the spleen as early as Day 3 post-birth and are composed of DP and CD8+ T cells.

Between Day 3 and Day 11, the frequency of DP T cells gradually decreased while the CD8+ T cell frequency increased, as well as gradually reduced surface levels of CD8 coreceptor. We examined whether or not the spleen was the environment for further T cell development of the DP T cells and found that they may be undergoing negative selection within the periphery. Furthermore, the peripheral T3.70+ T cells expressed high levels of the inhibitory receptor PD-1 compared to HY^{cd4} adult male controls, suggesting that PD-1 may have a role in peripheral tolerance during the neonatal T cell stage. Therefore the evidence presented suggests that the T3.70+ T cells are present within the periphery in the early days following birth. They may develop into an alternative fate within the periphery or they may be tolerated in a PD-1 dependent manner.

Chapter 4: Results

Analyzing the Role of Gadd45β in Negative Selection

The physiological HY^{cd4} mouse model system allows for examination of T cell selection events. In order to determine what genes may be involved in negative selection, a microarray was performed with HY^{cd4} male DP thymocytes. Gadd45β was identified as one of 388 genes up regulated during negative selection (128). Therefore, in hopes of understanding the contribution of Gadd45β to T cell development, B6 and Gadd45β knock-out mice were characterized. Furthermore, the contribution of Gadd45β to negative selection was analyzed within HY^{cd4} and HY^{cd4} Gadd45β knock-out mice.

Characterization of Gadd45β-deficient mice on the C57BL/6 background

Conventional αβ T cell receptor thymocyte development is not dependent on Gadd45β expression

The thymus of C57BL/6 (B6) wildtype mice contains the four different subsets of thymocytes – $CD4^-$ $CD8^-$ (DN), $CD4^+$ $CD8^+$ (DP), $CD4^+$ $CD8^-$ (CD4SP) and $CD4^-$ $CD8^+$ (CD8SP). T cell progenitors migrate to the thymus from the bone marrow and develop into DN thymocytes, then progressing to the DP T cell stage. The DP compartment is the largest in proportion and number within the B6 wildtype thymus, whereas fewer cells survive selection events to develop into CD4SP and CD8SP T cells (**Fig. 4-1 A**). In comparison to wildtype, Gadd45β knock-out mice on a B6 background have a slightly higher frequency of precursor DN thymocytes and a lower proportion of DP thymocytes. In addition, the proportion of CD4SP and CD8SP T cells that develop within the thymus was not significantly different in the absence of Gadd45β (**Fig. 4-1 A**). The lack of a reduction in cell frequency between wildtype and Gadd45β knock-out mice may suggest that Gadd45β deficiency does not impact thymocyte development.

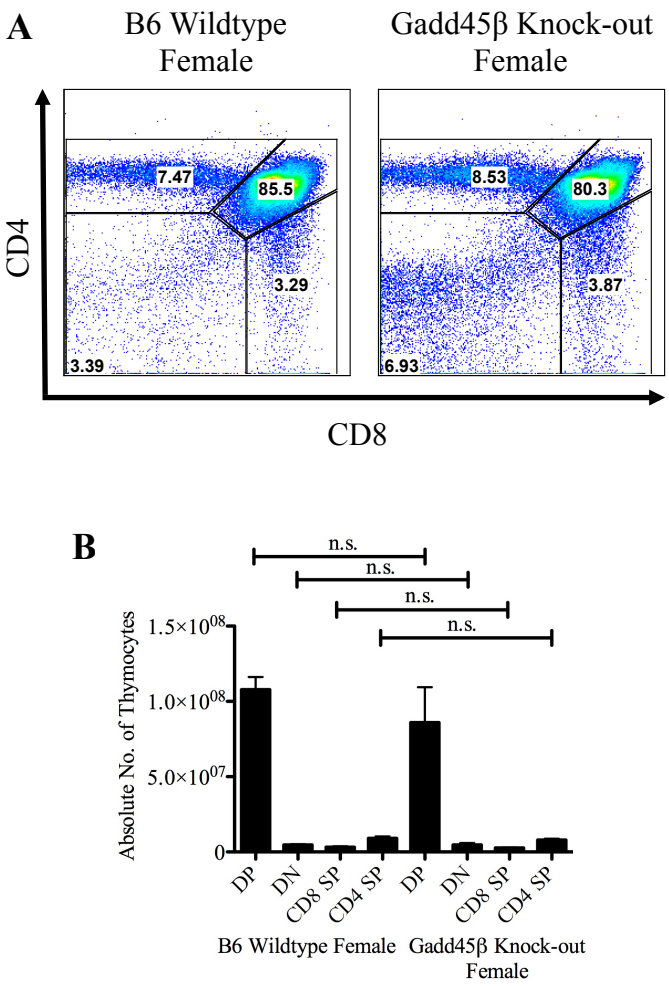


Fig. 4-1. B6 wildtype and Gadd45β knock-out female mice have similar frequencies and absolute numbers of cells within the specific thymocyte subsets. **A.** Representative flow cytometric plots of CD4 by CD8 coreceptor expression on thymocytes. **B.** Graphical analysis of the absolute number of cells of the following subsets: double positive (DP), double negative (DN), CD8 SP (CD8 single positive) and CD4SP (CD4 single positive) in B6 wildtype and Gadd45β knock-out female mice. n.s.: not significant. n =4.

Comparison of the wildtype and Gadd45β knock-out T cell population numbers showed no significant difference in numbers of each individual subpopulation (**Fig. 4-1 B**), further supporting grossly normal T cell development in the absence of Gadd45β. Therefore, Gadd45β deficiency does not alter the development of the CD4SP and CD8SP compartments within the thymus.

While the frequency and number of DN thymocytes was not significantly different in Gadd45β $^{-/-}$ versus WT thymi, I wanted to specifically examine the individual DN subsets to determine if Gadd45β deficiency impacted early T cell development events. In the wildtype thymus, the DN compartment is broken down into four compartments – $CD44^{+}$ $CD25^{-}$ (DN1), $CD44^{+}$ $CD25^{+}$ (DN2), $CD44^{-}$ $CD25^{+}$ (DN3) and $CD44^{-}$ $CD25^{-}$ (DN4). The overall frequency of DN thymocytes increased in the DN3 and DN4 compartments, due to the proliferative burst following β-selection in the DN3 stage (**Fig. 4-2 A**). In the Gadd45β deficient thymus, all the DN thymocyte subpopulations were present and the frequencies of DN3 and DN4 thymocytes increased in a similar fashion to wildtype. However, the frequency of DN4 thymocytes appeared to be slightly elevated in the Gadd45β deficient thymus (**Fig. 4-2 A**). An increase in the overall proportion of DN4 thymocytes may suggest a developmental block between DN4 and DP T cell stages in Gadd45β knock-out mice. Therefore, CD3 expression on DN4 thymocytes was used as a marker for maturation to DP thymocytes. Comparison of the CD3+ populations of DN4 thymocytes showed similar frequencies of cells between wildtype and Gadd45β knock-out mice, suggesting Gadd45β does not have a role in the maturation of DN4 thymocytes (**Fig. 4-2 B**). Absolute numbers of both DN4 thymocytes and CD3+ DN4 thymocytes were calculated to ensure that a phenotype due to Gadd45β deficiency was not masked within frequencies of cells. Although the frequency of DN4 thymocytes was slightly increased, the cell numbers of DN4 and CD3+ DN4 thymocytes from wildtype and Gadd45β knock-out mice were not significantly different (**Fig. 4-2 C**). Therefore, Gadd45β does not appear to have a role in the maturation of DN4 thymocytes to DP thymocytes.

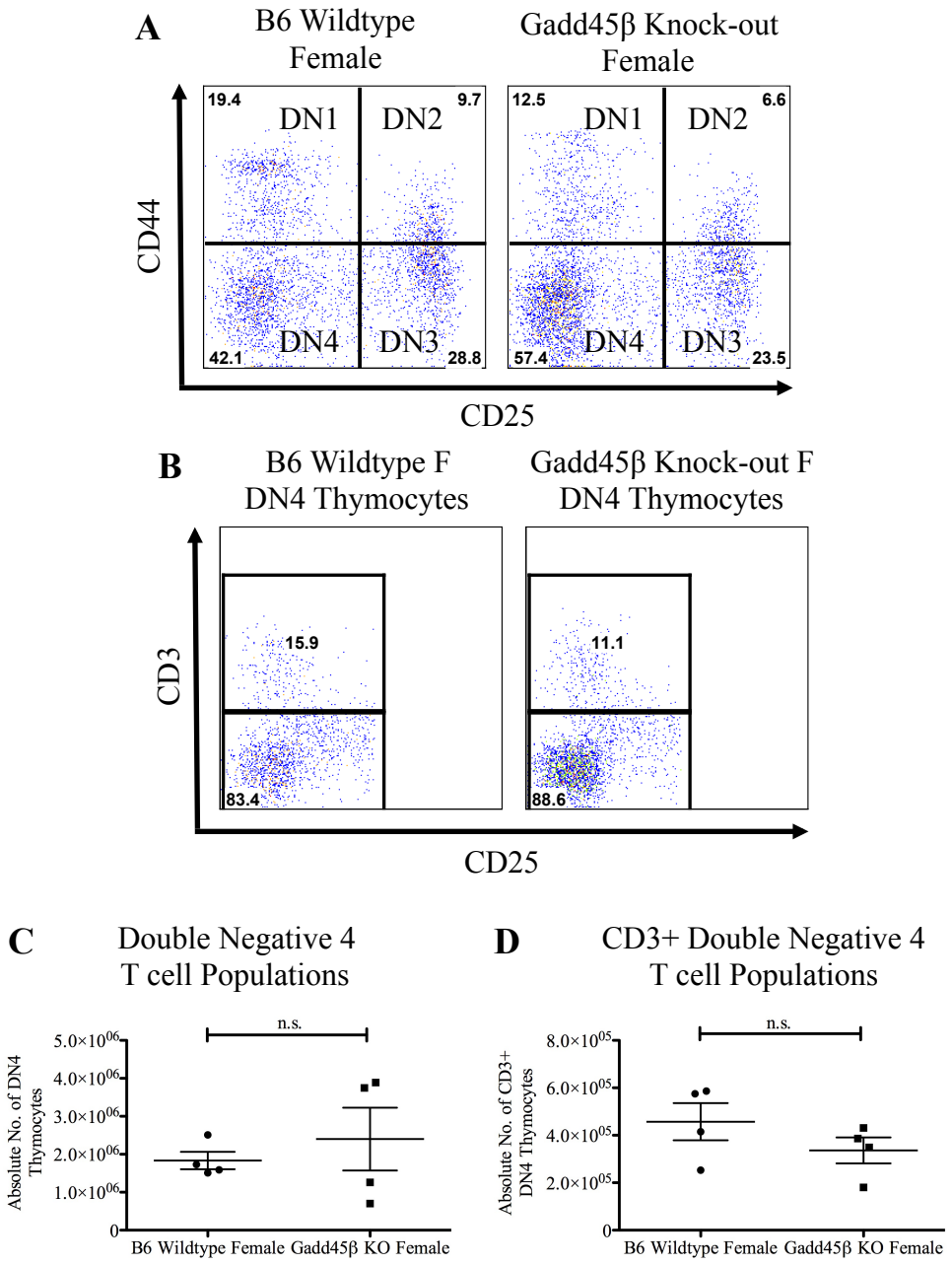


Fig. 4-2. Gadd45β deficiency did not impact the development and progression of CD3+ double negative 4 (DN4) thymocytes. **A.** Representative flow cytometric plots of DN thymocyte populations by CD44 and CD25 surface expression. **B.** Examination of mature DN4 thymocyte populations in B6 wildtype and Gadd45β knock-out thymi by CD3 expression. **C, D.** Graphical analysis for the absolute number of DN4 and CD3+ DN4 thymocytes in B6 wildtype and Gadd45β knock-out female mice. n.s.; not significant. n = 4.

Cell surface expression of CD69 is indicative of antigen encounter for DP thymocytes. Therefore, changes in CD69 expression may indicate alterations in antigen encounter or in TCR downstream signaling. Comparison of CD69 expression on DP thymocytes from wildtype and Gadd45β knock-out mice suggested there was no difference between the two populations. Furthermore, compilation of the overall frequency of DP thymocytes expressing CD69 demonstrated no significant difference between wildtype and Gadd45β knock-out cells (**Fig. 4-3 A**). Therefore, Gadd45β may not be crucial for selection events or downstream TCR signaling following antigen encounter.

SP thymocytes are the final T cell subset requiring thymic environmental cues for maturation prior to export into the periphery. In the absence of Gadd45β, no difference in frequency or number of CD4SP and CD8SP populations was observed. However, if Gadd45β contributed to maturation of the thymic populations prior to export, that defect may not be reflected in frequency. Therefore, maturation of both CD4SP and CD8SP thymocytes was assessed by down regulation of CD24 surface expression. Histogram overlays of CD4SP thymocytes showed gradual CD24 down regulation to a similar extent between B6 wildtype and Gadd45β knock-out females (**Fig. 4-4 A**). A similar trend was observed within the CD8SP thymocyte compartment of B6 wildtype and Gadd45β knock-out female thymi. However, both B6 wildtype and Gadd45β knock-out female mice had a population of CD24hi expressing CD8SP thymocytes (**Fig. 4-4 B**). These cells represent the immature single positive (ISP) thymocyte population that are CD4- CD8+ and known for expressing high levels of CD24 (153). The overall trends suggested that the SP thymocyte compartments were maturing within the thymus prior to export even in the absence of Gadd45β. Therefore, Gadd45β does not appear to contribute to SP thymocyte maturation.

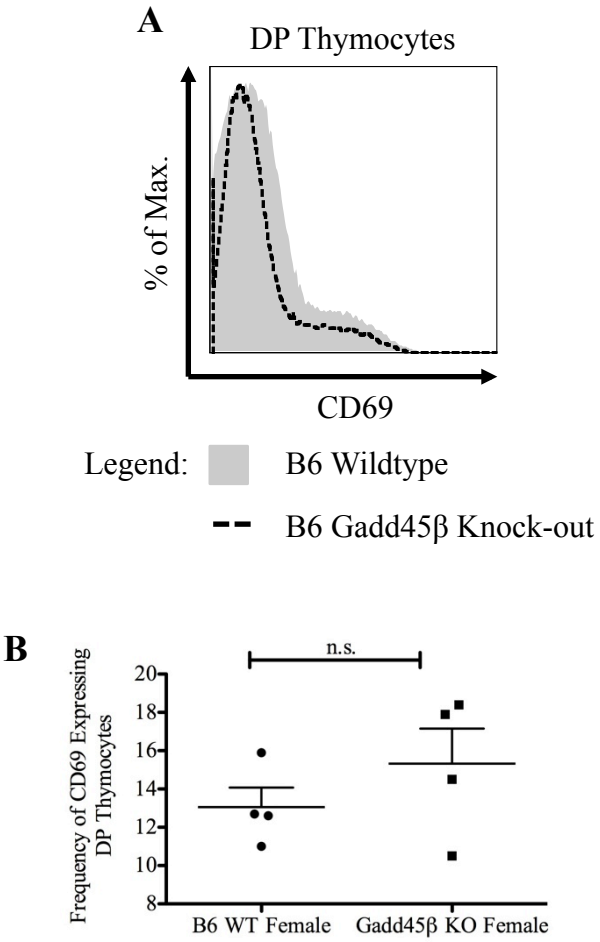


Fig. 4-3. Gadd45β does not appear to have a role in downstream T cell receptor signaling based on CD69 surface expression on DP thymocytes. **A.** CD69 expression on the cell surface of DP thymocytes from B6 wildtype and Gadd45β knock-out mice. **B.** Graphical analysis of the frequency of CD69 expressing DP thymocytes from B6 wildtype and Gadd45β knock-out female mice. The CD69+ population is based on the B6 wildtype female control. n.s.: not significant. Representative of n = 4.

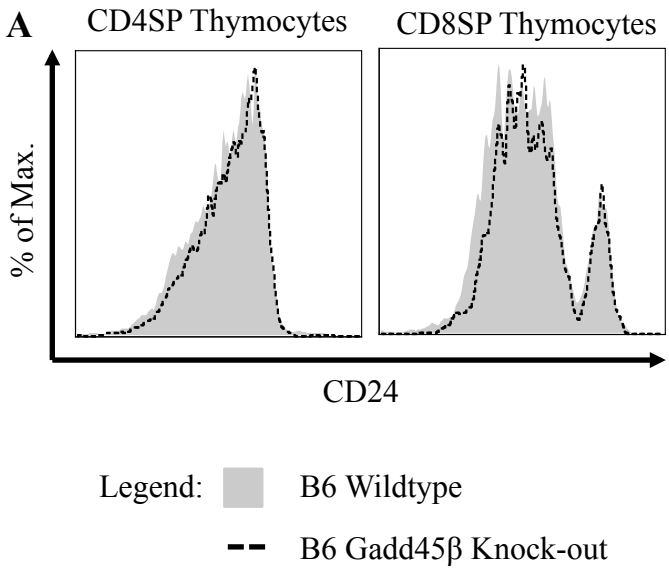


Fig. 4-4. Gadd45β deficiency did not have an impact on the maturation of CD4SP and CD8SP thymocytes. **A.** Histogram overlays of CD24 surface expression on CD4SP and CD8SP thymocytes from B6 wildtype and Gadd45β knock-out female mice. The solid grey represents the B6 wildtype control and the dashed line represents the Gadd45β knock-out. Representative of n = 4.

Development of the NK-T cell population may be dependent on Gadd45β and the lack of Gadd45β does not impair activation and apoptosis of DP thymocytes

Phenotypic analysis of Gadd45β knock-out mice did not reveal any defects within the development of conventional T cell populations within the thymus. In order to expand the phenotypic analysis of Gadd45β knock-out mice, the development of unconventional T cell populations in the thymus were analyzed. These populations included T regulatory cells, γδ T cells and NK-T cells. Within a wildtype thymus, approximately 5% of CD4SP thymocytes make up the T regulatory cell population, based on CD25 surface expression (**Fig. 4-5 A**). The overall majority of CD4SP thymocytes were conventional CD4 T cells. Comparison of the B6 wildtype thymus to the Gadd45β knock-out showed a similar frequency of $CD4^+$ $CD25^+$ SP thymocytes and there was no significant difference in frequency within replicate thymi (**Fig. 4-5 B**). This would suggest that Gadd45β is not required for the generation of the T regulatory cell population.

In addition to αβ-TCR expressing thymocytes, thymic development also gives rise to γδ-expressing T cells. Within the bulk population of thymocytes, γδ thymocytes accounted for approximately 1-2% of cells in wildtype mice and a similar frequency in Gadd45β knock-out mice (**Fig. 4-6 A, B**). Therefore, Gadd45β deficiency did not appear to impact the development of the alternative TCR-lineage population of γδ-thymocytes.

Finally, the NK-T cell population was analyzed by coexpression of the αβ TCR along with NK cell marker NK1.1. This cell subpopulation is unique from conventional T cells and is typically small in frequency within the thymus (154). Within both B6 wildtype and Gadd45β knock-out mice, less than 1% of cells were identified as NK-T cells (**Fig. 4-7 A**). However, the Gadd45β knock-out thymus appeared to have a smaller frequency of NK-T cells compared to the B6 wildtype, but this was not statistically significant (**Fig. 4-7 B**). Calculation of absolute numbers from multiple replicates found a statistically significant

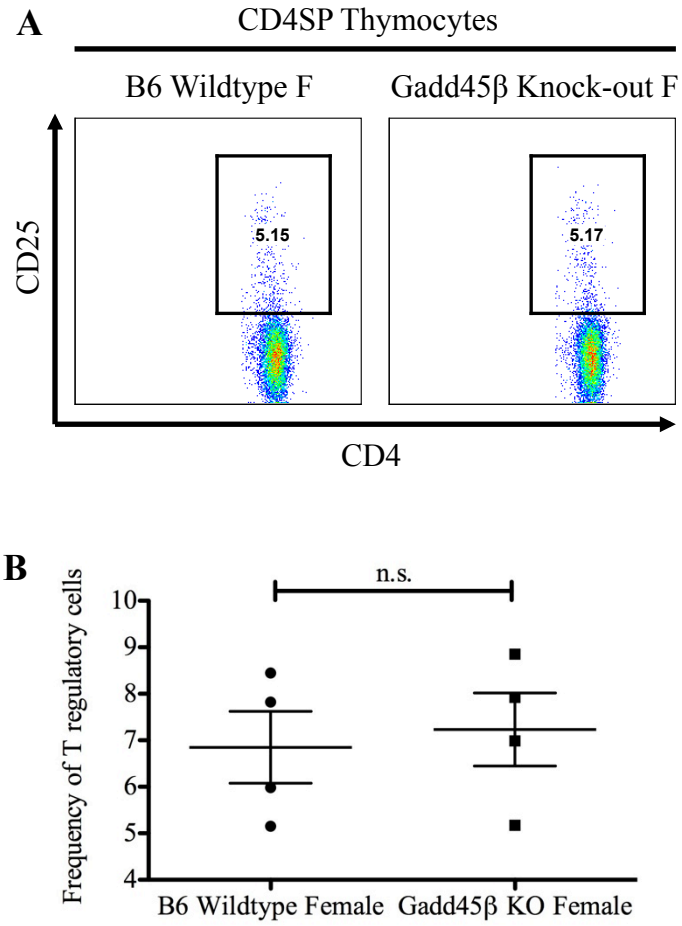


Fig. 4-5. Development of the thymic regulatory T cell population does not require Gadd45β. **A.** Representative flow cytometric plots of T regulatory cells within the thymus. T regulatory cells were identified by coexpression of CD25 and CD4 in the CD4SP thymocyte compartment. **B.** Graphical analysis of the frequency of T regulatory cells within the thymus of multiple B6 wildtype and Gadd45β knockout mice; n.s.: not significant. n = 4.

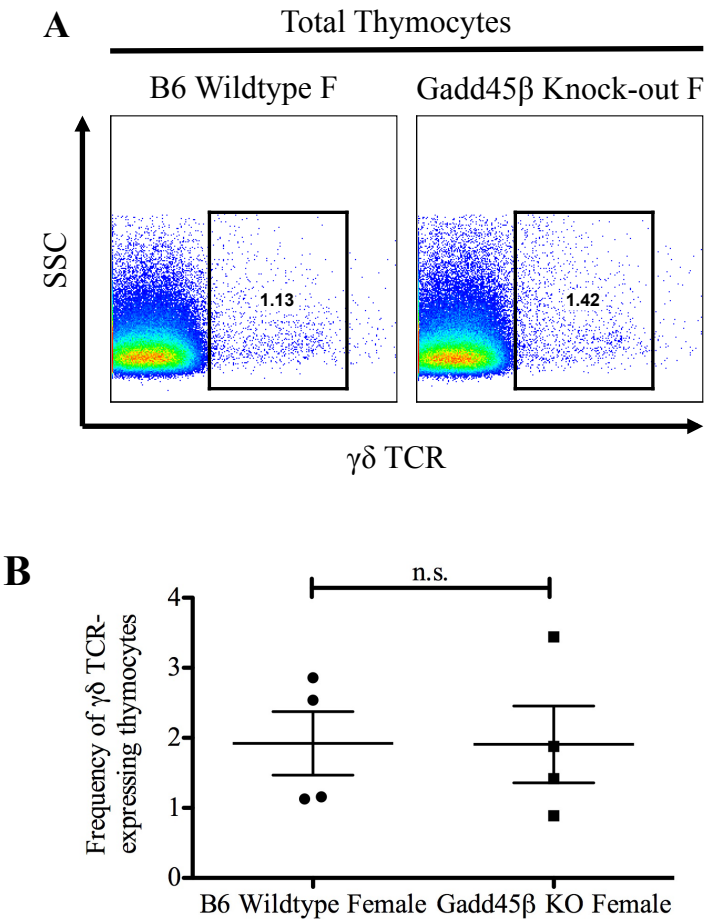


Fig. 4-6. Development of the gamma-delta lineage T cell population was not dependent on Gadd45β. **A.** Representative flow cytometric plots of γδ TCR thymocytes in B6 wildtype and Gadd45β knockout thymi. γδ T cells were analyzed by expression of the γδ T cell receptor (TCR). **B.** Graphical analysis of the frequency of γδ thymocytes within the thymus of multiple B6 wildtype and Gadd45β knock-out mice; n.s.: not significant. n = 4.

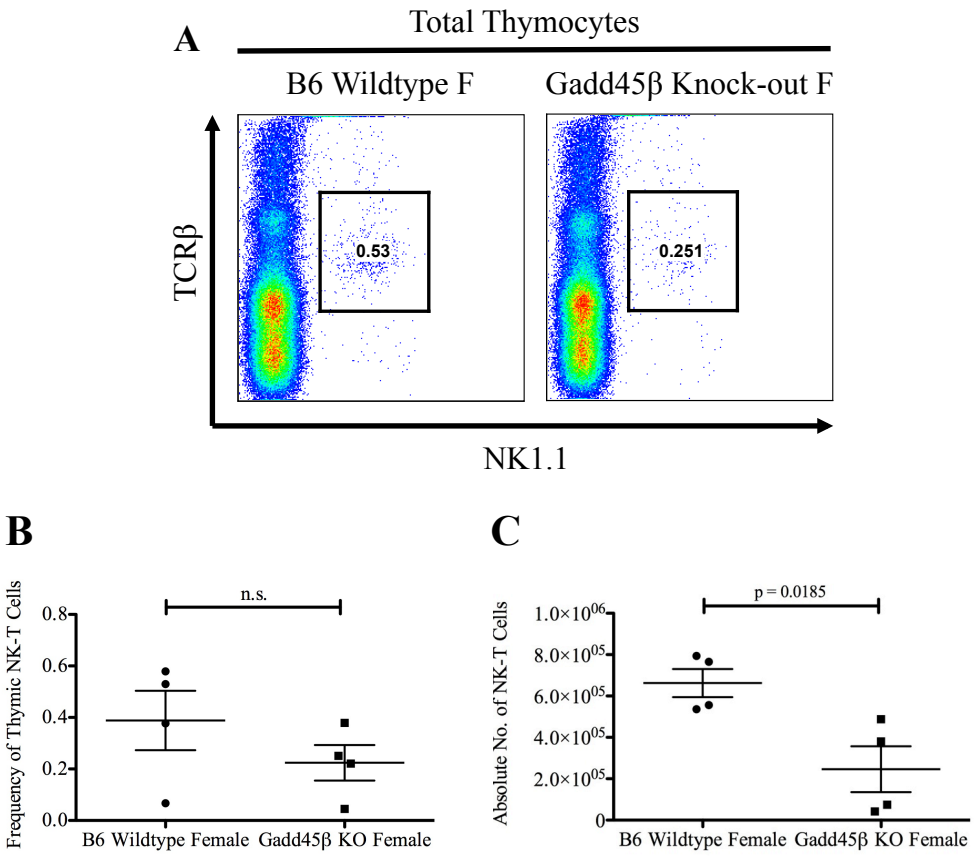


Fig. 4-7. Thymic NK-T cell development may be dependent on Gadd45β. **A.** Representative flow cytometric plots of TCRβ and NK1.1 expression to identify NK-T cells within the thymus of B6 wildtype and Gadd45β knock-out mice. **B.** Graphical analysis of the frequency of NK-T cells within the thymus of B6 wildtype and Gadd45β knock-out mice. n.s.: not significant. **C.** Graphical analysis of the absolute number of NK-T cells within the thymus of B6 wildtype and Gadd45β knock-out mice. Statistically significant, $p = 0.0185$. $n = 4$.

reduction in NK-T cells in Gadd45β knock-out mice compared to B6 wildtype mice (**Fig. 4-7 C**). This suggested that Gadd45β may be important for NK-T cell development and may have a yet unidentified role in their thymic development. However, the contribution of Gadd45β to NK-T cell development has not been further analyzed in this thesis.

Gadd45β does not appear to be required for the development of most thymic-derived T cell populations except NK-T cells. However, since it has been shown to be induced during negative selection (128), the efficiency of T cell responses was examined in the presence or absence of Gadd45β. Therefore, apoptosis induction was assessed in a 16-18 hour *in vitro* stimulation assay with varying concentrations of anti-CD3. B6 Ly5.2 splenocytes served as the antigen-presenting cells and were co-cultured with B6 wildtype or Gadd45β knock-out thymocytes in a 4:1 ratio. Prior to characterizing the induction of cell death of the Gadd45β deficient cells, both experimental populations were segregated from the stimulatory B6 Ly5.2 splenocytes by congenic markers. The wildtype and Gadd45β knock-out thymocytes were identified as $CD45.2^+$ and were easily distinguished from the stimulatory cells (**Fig. 4-8 A**). The congenic marker staining strategy allowed for simplified identification and segregation of the cell populations of interest. Once segregated, DP thymocytes of each genotype could then be analyzed for activation induced cell death.

Surface expression of CD69 was assessed for activation of wildtype and Gadd45β DP thymocytes following a 16-18 hour *in vitro* stimulation with varying concentrations of anti-CD3. Wildtype DP thymocytes did not readily respond to the lowest antibody concentration tested, 0.01 μg/mL anti-CD3. However, CD69 surface expression increased with 30% of T cells responding to stimulation with 0.1 μg/mL of anti-CD3; CD69 expression did not change with a further increase of anti-CD3 to 1 μg/mL (**Fig. 4-8 B**). Gadd45β knock-out DP thymocytes showed a similar phenotype with no increase in CD69 surface expression at 0.01 μg/mL anti-CD3 and an increase to 32% of DP thymocytes expressing CD69 at

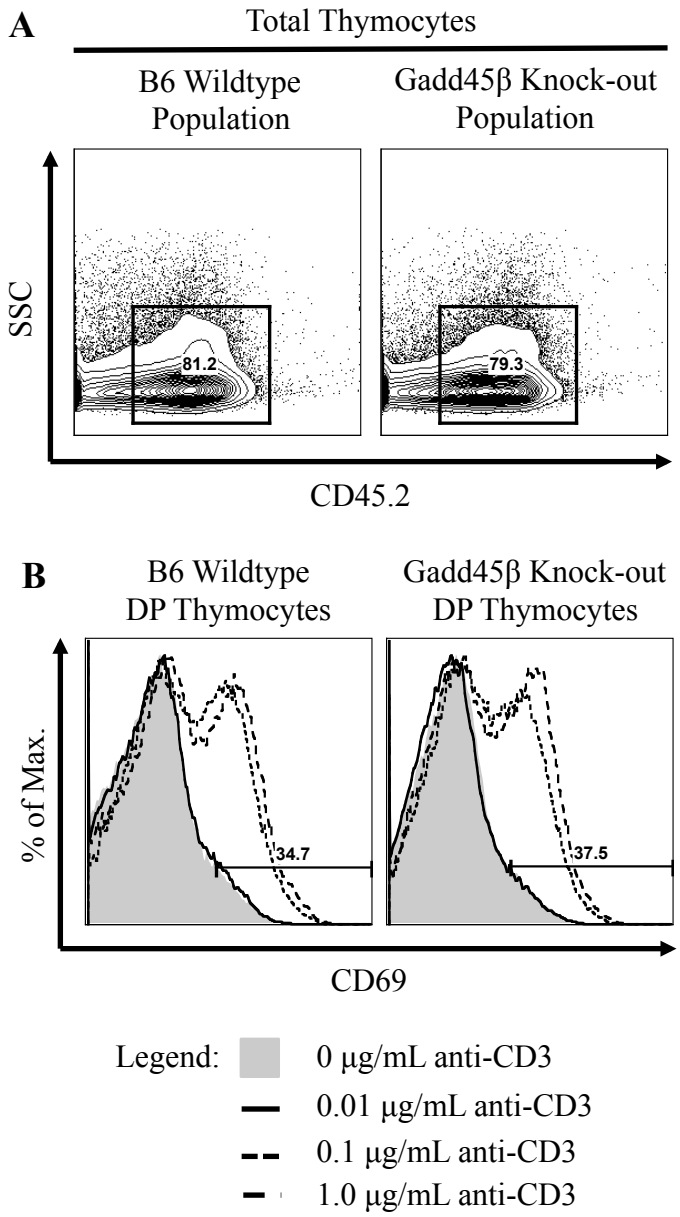


Fig. 4-8. Gadd45β deficiency did not impact the activation of DP thymocytes following *in vitro* stimulation with varying concentrations of anti-CD3. **A.** Surface expression of CD45.2 to separate the total B6 wildtype and Gadd45β knock-out thymocytes from the B6 Ly5.2 stimulatory splenocytes. Representative of n = 2. **B.** Histogram overlays of CD69 surface expression on DP thymocytes following *in vitro* stimulation. Cells were initially gated on live, then separated by CD4 and CD8 coreceptor expression. B6 wildtype and Gadd45β knock-out DP thymocytes were stimulated with 0.01μg/mL(solid), 0.1μg/mL (dashes) and 1.0μg/mL (long dashes) of anti-CD3 loaded splenocytes and assessed for CD69 upregulation against an unstimulated control (grey solid). Representative of n = 4.

1 μg/mL (**Fig. 4-8 B**). As the overall frequency of DP thymocytes expressing CD69 appeared to be similar between wildtype and Gadd45β knock-out mice, Gadd45β deficiency did not appear to impact the activation of DP thymocytes by *in vitro* anti-CD3 stimulation.

Although Gadd45β deficiency may not have a role in T cell activation resulting in CD69 surface expression, CD69 does not strictly define those cells undergoing the process of apoptosis. Signaling downstream of the TCR causes the initiation of the mitochondrial apoptosis pathway, resulting in the exposure of phosphatidylserine and intracellular cleavage of caspase 3 (155, 156). These two hallmarks can be identified by Annexin V staining on the cell surface, identifying phosphatidylserine exposure, and staining for the cleaved form of active caspase 3, at aspartic acid 175, by flow cytometry. These characteristics were studied to determine the role of Gadd45β in the cell death pathway. Similar to the CD69 surface expression phenotype (**Fig. 4-8 B**), Annexin V surface binding was not apparent on wildtype DP thymocytes until stimulated *in vitro* with 0.1 μg/mL and 1.0 μg/mL of anti-CD3, resulting in 17% of cells expressing phosphatidylserine on the cell surface (**Fig. 4-9 A**). The Gadd45β knock-out DP thymocytes showed nearly an identical frequency of Annexin V positive cells at 1.0 μg/mL of anti-CD3 compared to the B6 wildtype (**Fig. 4-9 A**). This suggested that following TCR engagement, the absence of Gadd45β does not regulate the expression of phosphatidylserine on the cell surface of DP thymocytes during cell death. To specifically examine the induction of apoptosis, intracellular staining for caspase 3 cleavage was assessed within DP thymocytes and percent-specific values were determined based on negative controls. Wildtype DP thymocytes showed a slight reduction in percent specific caspase 3 cleavage compared to Gadd45β knock-out DP thymocytes (**Fig. 4-9 B**). However, this difference was not statistically significant when examined in multiple replicates. Therefore, it appeared that Gadd45β deficiency did not impact the intrinsic pathway of apoptosis and therefore does not have a role in the apoptosis of DP thymocytes.

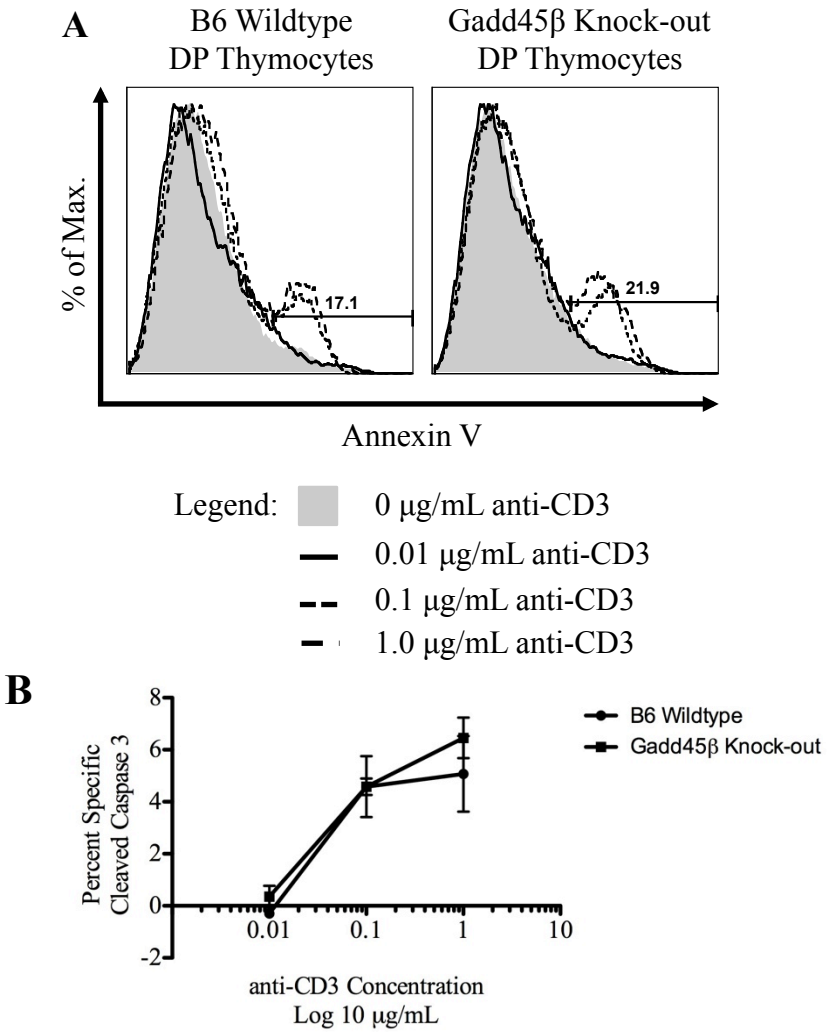


Fig. 4-9. Following T cell receptor stimulation *in vitro*, B6 wildtype and Gadd45β knock-out DP thymocytes undergo apoptosis to similar extents based on Annexin V binding and intracellular active caspase 3 levels. **A.** Histogram overlays of AnnexinV binding to surface expressed phosphatidylserine on DP thymocytes from B6 wildtype and Gadd45β knock-out mice. The surface binding of Annexin V of DP thymocytes stimulated with 0.01μg/mL (solid), 0.1μg/mL (dashes) and 1.0μg/mL (long dashes) was compared to the unstimulated control (grey solid). B6 n = 2; Gadd45β KO n = 3. **B.** Graphical analysis of the percent specific cleaved caspase 3 in DP thymocytes from B6 wildtype and Gadd45β knock-out mice. B6 n = 2; Gadd45β KO n = 3.

The absence of Gadd45β expression has no impact on the frequency and number of conventional αβ T cell receptor T cells within the spleen and lymph nodes

Mature CD4SP and CD8SP thymocytes are exported from the thymus into the blood and can then traffic to the secondary lymphoid tissue. The spleen is composed of a small frequency of DN T cells, a modest frequency CD8+ T cells and a larger population of CD4+ T cells. The ratio between the CD4+ to CD8+ T cell population is approximately 1.5:1. To determine if Gadd45β deficiency influenced the peripheral T cell compartment, splenocytes from B6 wildtype and Gadd45β knock-out mice were examined. The splenic compartment of Gadd45β knock-out mice had similar frequencies of DN, CD8+ and CD4+ T cells to B6 wildtype mice (**Fig. 4-10 A**). A calculation of the absolute numbers of splenic T cell subsets demonstrated no differences between B6 wildtype and Gadd45β knock-out mice (**Fig. 4-10 B**). Additional examination of lymph nodes was done to ensure there were no major differences between the two peripheral organs. Similar to the splenic compartment, the lymph nodes showed similar frequencies of the DN, CD8+ and CD4+ T cell subsets between wildtype and Gadd45β knock-out mice (**Fig. 4-11 A**). Furthermore, no significant difference in the number of CD4 or CD8 T cells from the lymph nodes of B6 wildtype and Gadd45β knock-out mice was observed (**Fig. 4-11 B**).

Within the periphery, CD8+ and CD4+ T cell populations can be characterized as naïve, central memory or effector memory based upon the expression of CD44 and CD62L. Prior to antigen encounter, T cells remain naïve and are characterized by a $CD44^{-}$ $CD62L^{+}$ phenotype. Following antigen encounter and activation, T cells can transition into effector memory cells characterized by a $CD44^{+}$ $CD62L^{-}$ phenotype. Finally, once the immune function of the expanded T cell population is no longer required, these cells will contract and some will remain as central memory T cells defined by a $CD44^{+}$ $CD62L^{+}$ phenotype (107). Therefore, a deficiency in the proportion or number of memory T cells within Gadd45β deficient mice would be dependent on Gadd45β. Within the CD4+ T

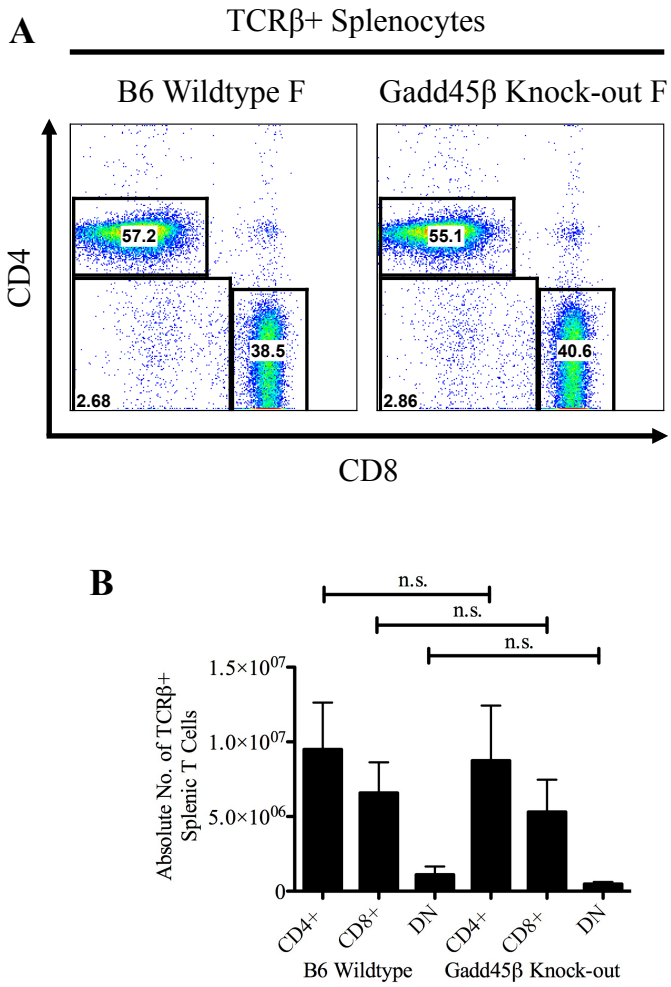


Fig. 4-10. B6 wildtype and Gadd45β knock-out female mice have similar frequencies and absolute numbers of cells within the peripheral T cell subsets of the spleen. **A.** Representative flow cytometric plots of CD4 by CD8 coreceptor expression on splenic T cells from B6 wildtype and Gadd45β knock-out mice. **B.** Graphical analysis of the absolute numbers of splenic TCRβ+ DN, CD4+ and CD8+ T cells within the spleen of B6 wildtype and Gadd45β knock-out mice. n.s.: not significant. n = 4.

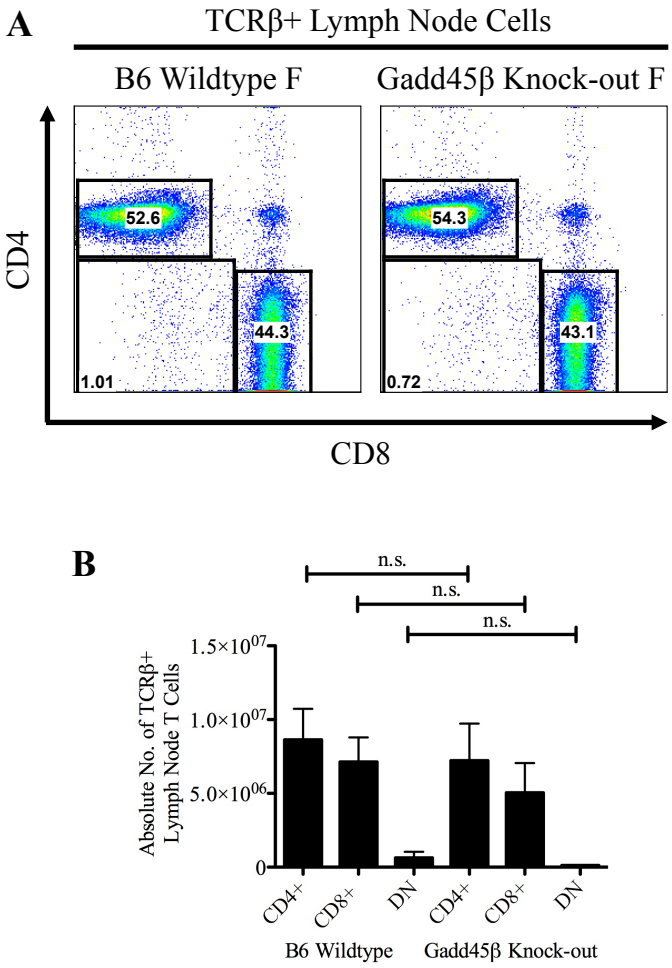


Fig. 4-11. B6 wildtype and Gadd45β knock-out female mice have similar frequencies and absolute numbers of cells within the peripheral T cell subsets of the lymph nodes. **A.** Representative flow cytometric plots of CD4 by CD8 coreceptor expression on lymph node T cells from B6 wildtype and Gadd45β knock-out mice. **B.** Graphical analysis of the absolute numbers of lymph node TCRβ+ DN, CD4+ and CD8+ T cells from B6 wildtype and Gadd45β knock-out female mice. n.s.: not significant. n = 4.

cell compartment of both wildtype and Gadd45β knock-out mice, the majority of the cells were naïve with a small proportion of effector memory cells. Absolute numbers of these subpopulations showed no difference between the genotypes suggesting Gadd45β expression does not affect the CD4+ T cell population (**Fig. 4-12 A, B**). Similarly, analysis of the CD8+ splenocyte compartment demonstrated the largest proportion of cells were naïve, whereas smaller frequencies of cells were central memory and effector memory in both wildtype and Gadd45β knock-out mice. The wildtype spleen had a slightly higher proportion of naïve CD8+ T cells than the Gadd45β knock-out spleen; however, calculation of the absolute numbers demonstrated no significant difference in the number of CD8+ T cell subsets within the spleen (**Fig. 4-13 A, B**). Therefore, the peripheral phenotyping of the CD4+ and CD8+ T cell subsets suggested that Gadd45β deficiency does not grossly alter the composition of the peripheral T cell pool.

The absence of Gadd45β does not impact the regulatory T cell, γδ T cell and NK-T cell populations within the periphery of mice

The composition of the conventional αβ TCR T cells was unchanged in Gadd45β deficient mice. Even so, small populations of T regulatory cells, γδ T cells and NK-T cells are present within the periphery as well. We therefore wanted to analyze the effect of Gadd45β deficiency on these T cell lineages. The T regulatory cell population was first analyzed based on co-expression of CD4 and CD25. Within the CD4+ T cell subcompartment of B6 wildtype mice, about 8% of cells were classified as T regulatory cells based upon expression of CD25 on the cell surface (**Fig. 4-14 A**). Multiple B6 wildtype replicates had a mean T regulatory cell frequency of ~10% (**Fig. 4-14 B**). Similarly, about 8% of CD4+ T cells were classified as T regulatory cells within the splenic compartment of Gadd45β knock-out mice. Therefore, no significant difference was found in the proportion of T regulatory cells within the periphery of Gadd45β knock-out mice compared to wildtype mice.

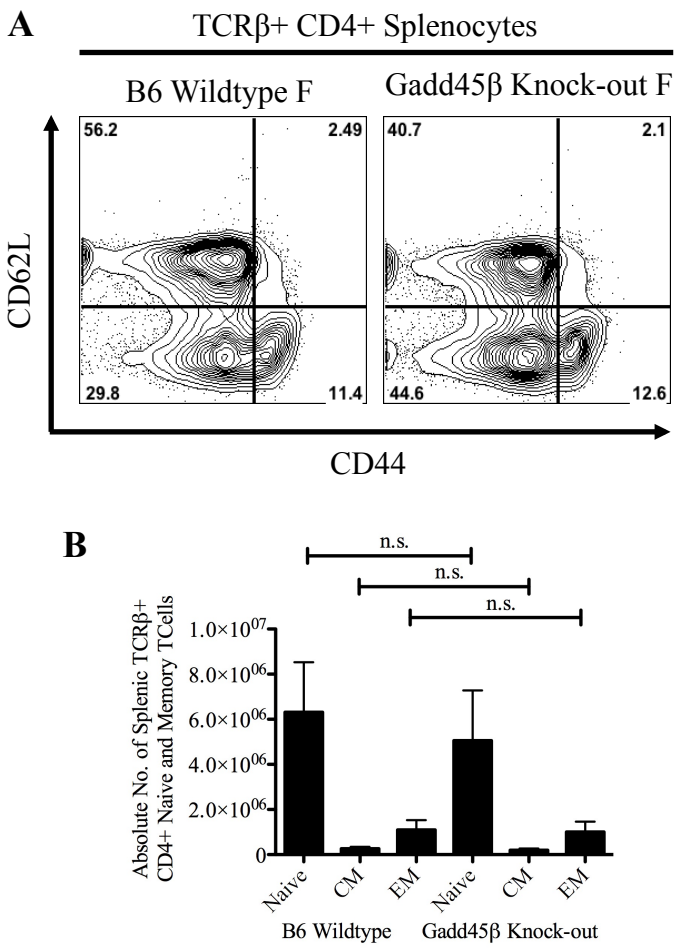


Fig. 4-12. Gadd45β does not have a role in the establishment of central memory and effector memory CD4+ T cells within the spleen. **A.** Representative flow cytometric plots of CD62L and CD44 expression on CD4+ T cells from B6 wildtype and Gadd45β knock-out female spleens. The populations are: naïve (CD44- CD62L+), central memory (CD44+ CD62L+) and effector memory (CD44+ CD62L-). **B.** Graphical analysis of the absolute numbers of naïve and memory CD4+ T cells in the spleen. CM: central memory; EM: effector memory. n.s.: not significant. n = 4.

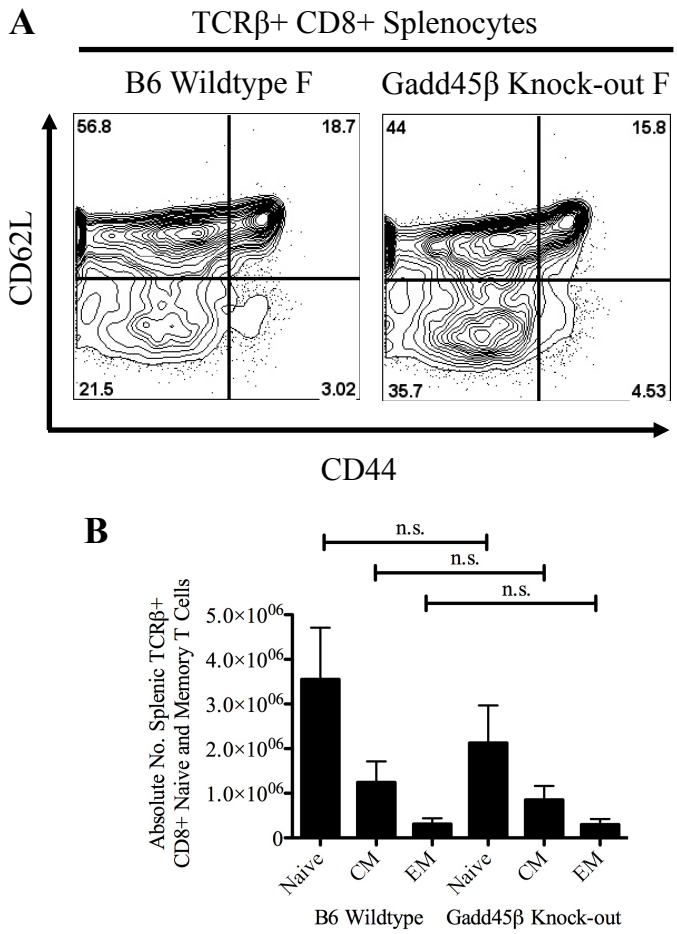


Fig. 4-13. Gadd45β does not have a role in the establishment of central memory and effector memory CD8+ T cells within the spleen. **A.** Representative flow cytometric plots of CD62L and CD44 expression on CD8+ T cells from B6 wildtype and Gadd45β knock-out female spleens. The populations are: naïve (CD44- CD62L+) central memory (CD44+ CD62L+)and effector memory (CD44+ CD62L-). **B.** Graphical analysis of the absolute numbers of naïve and memory CD8+ T cells in the spleen. CM: central memory; EM: effector memory. n.s.: not significant. Representative of n = 4.

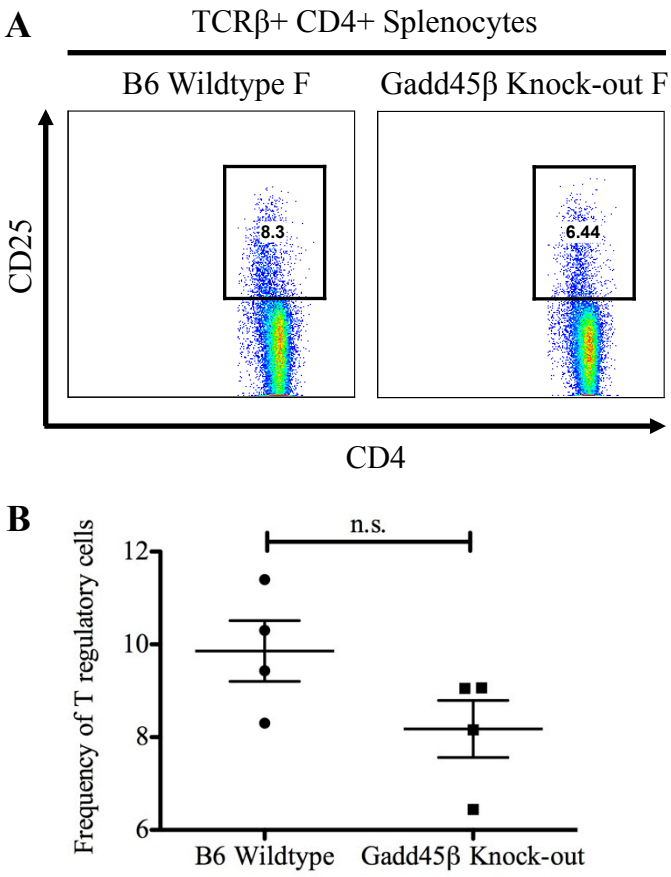


Fig. 4-14. Gadd45β is not required for the development of the splenic regulatory T cell population. **A.** Representative flow cytometric plots of CD25+ CD4+ splenocytes classified as T regulatory cells within the spleen of B6 wildtype and Gadd45β knock-out mice. **B.** Graphical analysis of the frequency of T regulatory cells within the spleen of multiple B6 wildtype and Gadd45β knock-out mice; n.s. : not significant. n = 4.

I then examined the γδ TCR expressing T cell subset within the spleen of B6 wildtype and Gadd45β deficient mice to determine if the absence of Gadd45β had an effect on this T cell lineage. Representative flow cytometric plots of the γδ T cell population showed similar frequencies of cells in both B6 wildtype and Gadd45β knock-out mice (**Fig. 4-15 A**). Analysis of multiple replicates showed similar mean frequency of approximately 1.5-2% of γδ T cells within the spleen of both wildtype and Gadd45β knock-out mice (**Fig. 4-15 B**). This observation was similar to what was seen within the thymic population of B6 wildtype and Gadd45β knock-out mice. Therefore it appeared that Gadd45β deficiency had no impact on the γδ T cell lineage.

Finally, we examined the splenic NK-T cell population. As stated previously, the frequency of NK-T cells within the thymus did not differ between wildtype and Gadd45β knock-out female mice (**Fig. 4-7 A, B**). However, there was a significant reduction in the absolute number of NK-T cells (**Fig. 4-7 C**). Therefore, the splenic NK-T cell population was analyzed to see if they exhibited a similar reduction in number. Analysis of NK-T cells from B6 wildtype and Gadd45β knock-out mice showed no difference in NK-T cell frequency (**Fig. 4-16 A**). Furthermore, when absolute numbers of NK-T cells were calculated, there was no significant difference between B6 wildtype and Gadd45β knock-out mice, although the range was quite large (**Fig. 4-16 B**). Therefore, the peripheral NK-T cell population may be affected by the absence of Gadd45β.

Gadd45β deficiency does not alter the proliferative response of TCRβ+ CD8+ T cells following in vitro stimulation with anti-CD3

Within the peripheral compartment of Gadd45β knock-out mice no apparent deficiencies in frequency or number of the various T cell populations were found. It was then considered that Gadd45β might contribute to a functional role within CD4+ and CD8+ T cells. Therefore, the ability of peripheral T cells to proliferate was assessed by CFSE dilution following *in vitro* stimulation with anti-CD3

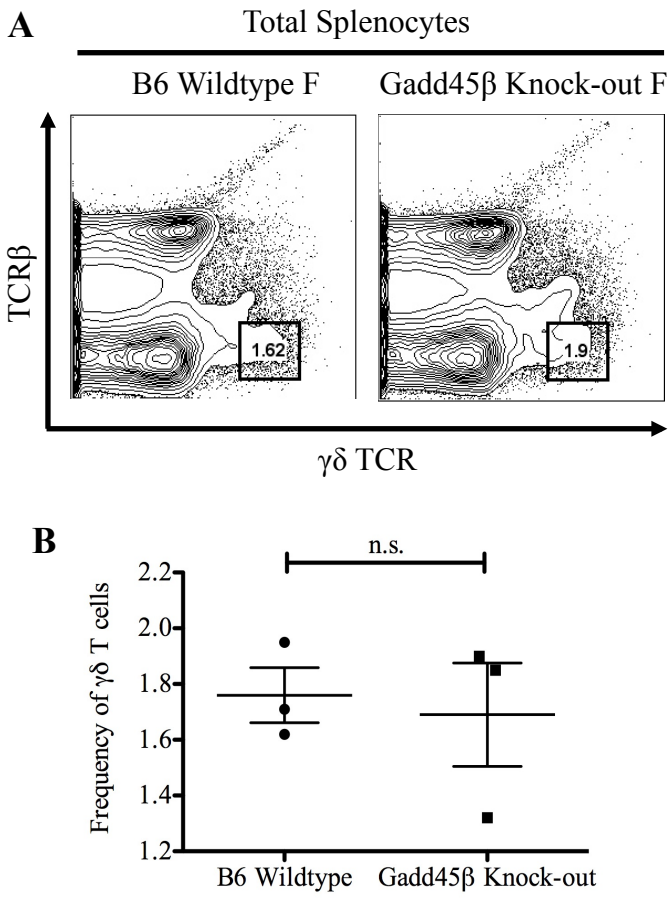


Fig. 4-15. The development of the splenic γδ T cell population is not dependent on Gadd45β. **A.** Representative flow cytometric plots of γδ T cells in B6 wildtype and Gadd45β knock-out spleen. γδ T cells were analyzed by the lack of TCRβ expression and expression of the γδ TCR. **B.** Graphical analysis of the frequency of γδ T cells within the spleen of multiple replicates B6 wildtype and Gadd45β knock-out mice; n.s.: not significant. n = 3.

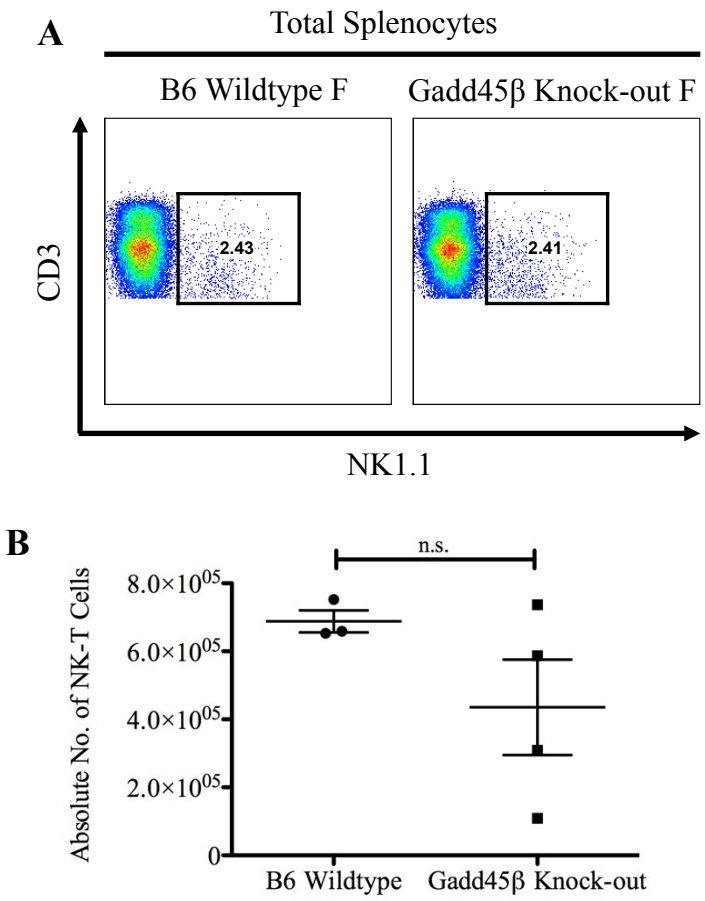


Fig. 4-16. Contrary to what was observed in the thymus, Gadd45β deficiency did not impact the splenic NK-T cell population. **A.** Representative flow cytometric plots of CD3+ NK1.1+ NK-T cells within the spleen of B6 wildtype and Gadd45β knock-out mice. **B.** Graphical analysis of the absolute number of NK-T cells within the spleen of B6 wildtype and Gadd45β knock-out mice; n.s.: not significant. B6 n = 3; Gadd45β KO n = 4.

loaded splenocytes. I mixed B6 wildtype CD45.1+ splenocytes with Gadd45β CD45.2+ splenocytes, CFSE labeled the cells and then stimulated with specified concentrations of anti-CD3 for up to 72 hours. Dilution of CFSE dye is indicative of cell proliferation; therefore comparison between wildtype and Gadd45β knock-out cells would show a proliferative defect in the absence of Gadd45β. The CFSE profile of the unstimulated cells at day 3 showed the undivided fraction of T cells. Following 72-hours of *in vitro* stimulation with 2.0 μg of anti-CD3, the majority of wildtype and Gadd45β knock-out CD4+ T cells have undergone one or two rounds of division (**Fig. 4-17 A**).

Interestingly, a larger proportion of Gadd45β knock-out CD4+ T cells appear to have not divided compared to the B6 wildtype cells. Therefore, Gadd45β deficiency may influence entry into the cell cycle. Analysis of the CD8+ T cell compartment showed that the majority of both B6 wildtype and Gadd45β knock-out cells have undergone two rounds of division with a few remaining in the first round (**Fig. 4-17 A**). There appeared to be no kinetic delay in the CD8+ T cells, as there were no cells present in the undivided fraction of the CD8+ T cell population.

In order to determine if Gadd45β deficiency was contributing to altered proliferation kinetics, the frequency of cells within the undivided CFSE fraction was plotted against the concentration of anti-CD3. Within the CD4+ splenocyte population, Gadd45β knock-out cells were present in the undivided fraction at a higher frequency to wildtype cells at 48 and 72 hours (**Fig. 4-17 B**). Interestingly, the reduced proliferative ability of T cells in Gadd45β knock-out mice appeared to be restricted to CD4+ T cells, as Gadd45β knock-out CD8+ T cells were in similar proportion to wildtype CD8+ T cells following 48 and 72 hours of *in vitro* stimulation (**Fig. 4-17 B**). Therefore, Gadd45β may have a minor role in the kinetics of cellular proliferation of CD4+ T cells following TCR stimulation, as the phenotype became apparent only after 72 hours of *in vitro* culture. However, further analysis into this possible kinetic delay was not done within this thesis.

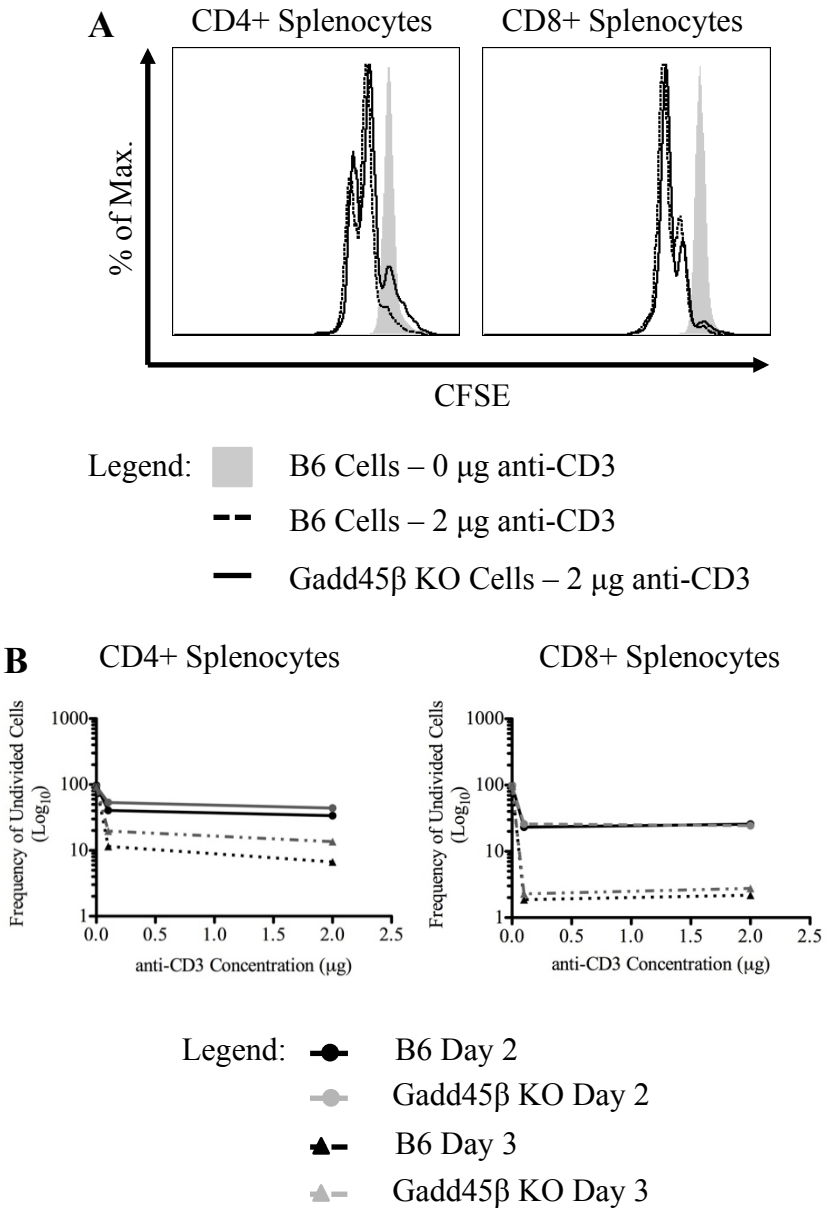


Fig. 4-17. The absence of Gadd45β does not appear to alter the proliferative response of both CD4+ and CD8+ T cells following *in vitro* stimulation with anti-CD3. **A.** Histogram overlays of CFSE dilution of CD4+ T cells and CD8+ T cells from B6 wildtype and Gadd45β knock-out spleens following 72 hours of *in vitro* stimulation with 2.0 μg of anti-CD3. **B.** Graphical analysis of the frequency of CFSE+ undivided CD4+ T cells and CD8+ T cells from B6 wildtype and Gadd45β knock-out mice based on the stimulatory concentration of anti-CD3. Representative of n = 1.

Characterization of Gadd45β-deficient mice on the HY^{cd4} transgenic background of thymocyte selection

Gadd45β deficiency does not change the frequency or absolute number of T3.70+ thymocytes within HY^{cd4} female and male mice

C57BL/6 (B6) Gadd45β knock-out mice were crossed on to the HY^{cd4} transgenic background to determine the contribution of Gadd45β to positive and negative selection. The HY^{cd4} transgenic mouse model is a physiological model of thymocyte selection. The CD4 promoter regulates expression of the Cre recombinase which in turn regulates expression of the TCRα chain; therefore, its expression is restricted to the DP thymocyte stage and allows for physiological timing of thymocyte selection (126). The HY TCR is restricted by the MHC Class I molecule H-2D^{b} and is specific for HY male antigen. Therefore, female mice that lack HY male antigen are a model of positive selection and male mice that express HY male antigen are a model of negative selection (126). HY TCR expressing T cells can be identified by the T3.70 monoclonal antibody (mAb) from HY^{cd4} female and male mice. Using flow cytometry, HY^{cd4} Gadd45β knock-out mice were then analyzed to define the contribution of Gadd45β to both positive and negative selection within a monoclonal T cell repertoire.

Within the thymus of HY^{cd4} female and male mice, T3.70+ (HY TCR+) antigen-specific cells made up approximately 50% of total thymocytes; the other 50% expressed an endogenously rearranged TCR. HY^{cd4} Gadd45β knock-out female and male mice had a similar proportion of antigen-specific cells within the thymus (**Fig. 4-18 A**). In addition, HY^{cd4} Gadd45β wildtype and knock-out female mice had similar numbers of T3.70+ cells, as do the male mice. Comparison of HY^{cd4} wildtype female and male T cell populations showed the characteristic 2 to 3-fold reduction in T3.70+ cell numbers in male mice with statistical significance. Gadd45β did not appear to affect the number of antigen-specific cells in male mice, as that significant 2 to 3-fold reduction was still apparent between the

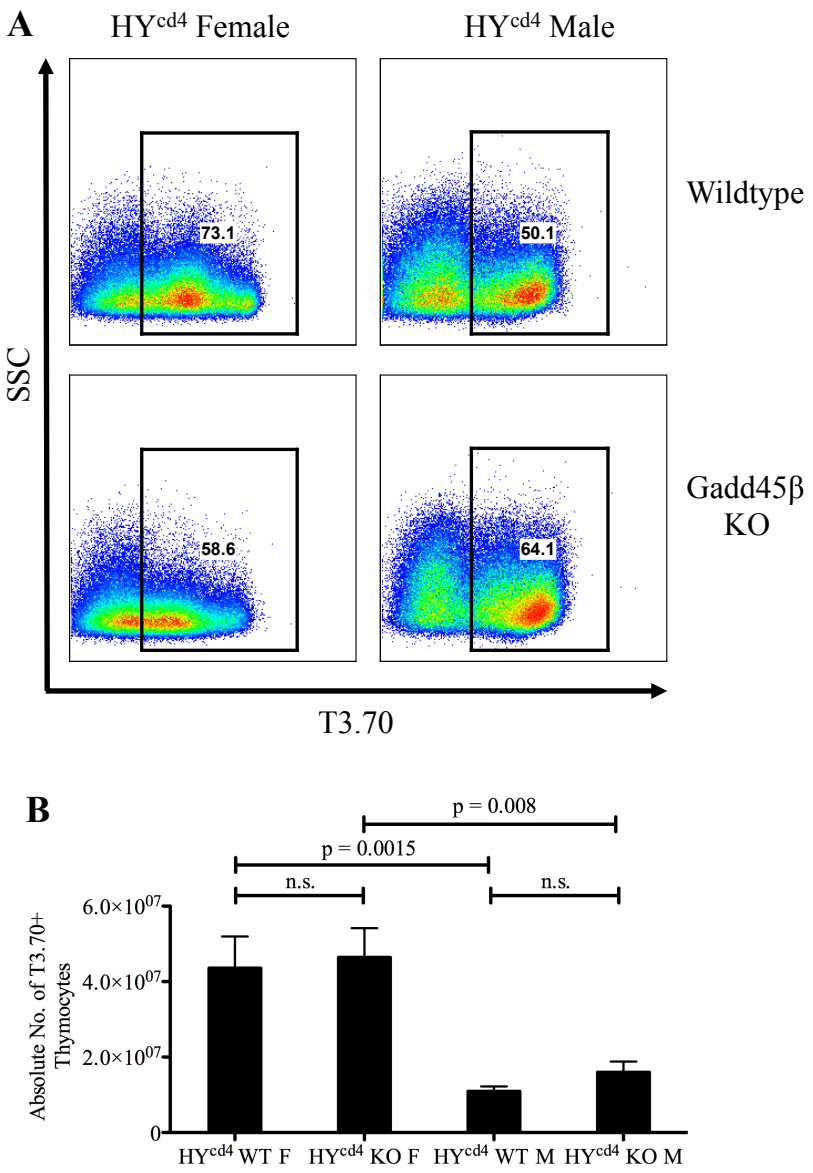


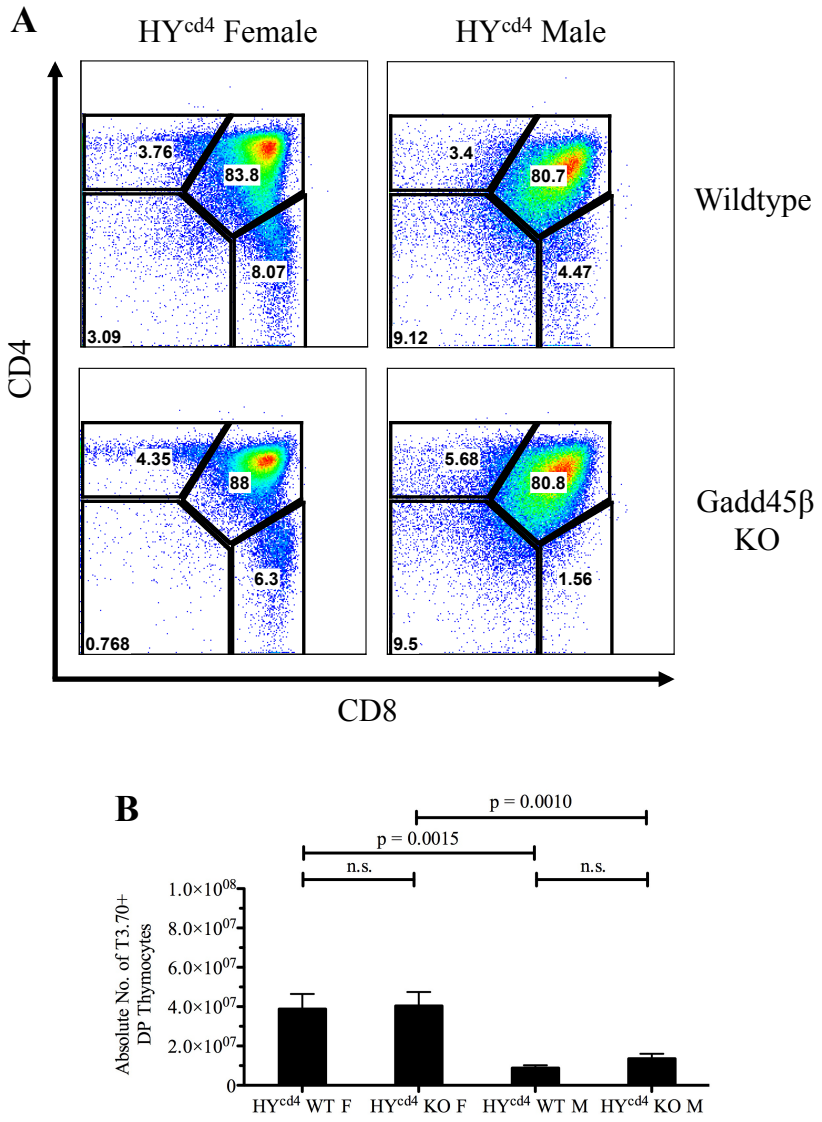
Fig. 4-18. Thymic development of antigen-specific T3.70+ thymocytes within HY^{cd4} female and male mice is not dependent on Gadd45β. **A.** Representative flow cytometric plots of T3.70+ thymocytes from HY^{cd4} wildtype and Gadd45β knock-out female and male mice. **B.** Graphical analysis of the absolute number of T3.70+ thymocytes in HY^{cd4} wildtype and Gad45β knock-out female and male mice. n.s.: not significant. HY^{cd4} WT F n = 9; HY^{cd4} Gadd45β KO F n = 7; HY^{cd4} WT M n = 9; HY^{cd4} Gadd45β KO M n = 10.

HY^{cd4} Gadd45β knock-out female and male mice (**Fig. 4-18 B**).

The thymic T cell subsets present in HY^{cd4} wildtype mice reflect positive selection events in female mice and the negative selection events in male mice. Within the T3.70+ fraction of thymocytes from female mice, a small proportion of thymocytes were DN, whereas the majority of cells formed the DP thymocyte population. These T cells undergo positive selection, as female mice lack HY male antigen, and further progress into CD8SP T cells. A small proportion of CD4SP thymocytes were also seen in the HY^{cd4} female thymus. HY^{cd4} Gadd45β knock-out female mice had similar frequencies of the thymocyte subsets compared to HY^{cd4} mice (**Fig. 4-19 A**).

In contrast, the HY^{cd4} male mice contained a robust and broad DP thymocyte population undergoing negative selection. Some DP thymocytes had reduced levels of CD4 and CD8 coreceptors, causing a small proportion of cells to fall within the DN thymocyte gate. Note the absence of the CD8SP population in HY^{cd4} wildtype male mice compared to female mice (**Fig. 4-19 A**). HY^{cd4} Gadd45β knock-out male mice also had the large, negatively selecting DP T cell population and lacked the CD8SP population. The overall frequencies of each thymic subset were similar between the HY^{cd4} Gadd45β wildtype and knock-out male mice (**Fig. 4-19 A**). Therefore, Gadd45β did not appear to alter T cell frequencies of T3.70+ T cells during thymic development.

Graphical analysis of the absolute numbers of thymocytes showed similar trends in the thymocyte frequencies of HY^{cd4} wildtype and Gadd45β knock-out mice. The number of DP thymocytes was similar between HY^{cd4} Gadd45β wildtype and knock-out female mice with no stastical significance. However, there was a statistically significant reduction in DP thymocyte number in male mice compared to female mice (**Fig. 4-19 B**). Female HY^{cd4} wildtype and Gadd45β knock-out mice also had similar numbers of CD8SP thymocytes. Within HY^{cd4} wildtype and Gadd45β knock-out male mice there was a significant, 1-log reduction in


A
HY^cd4 Female
HY^cd4 Male
3.76
83.8
8.07
3.09
3.4
80.7
4.47
9.12
Wildtype
4.35
88
6.3
0.768
5.68
80.8
1.56
9.5
Gadd45β KO
CD4
CD8
B
p = 0.0010
p = 0.0015
n.s.
n.s.
Absolute No. of T3.70+ DP Thymocytes
1.0×10^08
8.0×10^07
6.0×10^07
4.0×10^07
2.0×10^07
0
HY^cd4 WT F
HY^cd4 KO F
HY^cd4 WT M
HY^cd4 KO M

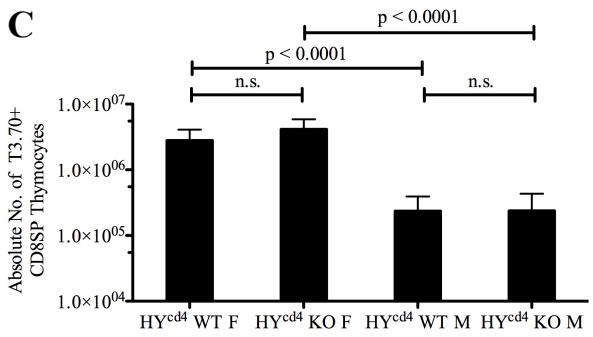


Fig. 4-19. The frequency and absolute number of T3.70+ DP and CD8SP thymocytes does not differ in the absence of Gadd45β. **A.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression of T3.70+ thymocytes from HY^{cd4} wildtype and Gadd45β knock-out female and male mice. **B.** Graphical analysis of the absolute number of T3.70+ DP thymocytes in HY^{cd4} wildtype and Gad45β knock-out female and male mice. n.s.: not significant. **C.** Graphical analysis of the absolute number of T3.70+ CD8SP thymocytes in HY^{cd4} wildtype and Gadd45β knock-out female and male mice. n.s.: not significant. HY^{cd4} WT F n = 9; HY^{cd4} Gadd45β KO F n = 7; HY^{cd4} WT M n = 9; HY^{cd4} Gadd45β KO M n = 10.

CD8SP thymocyte numbers when compared to female mice (**Fig. 4-19 C**). Based upon similar frequencies and numbers of DP and CD8SP thymocytes from HY^{cd4}wildtype and Gadd45β knock-out females and males, respectively, Gadd45β did not grossly affect the development of the individual thymic subsets.

The absence of Gadd45β does not alter the expression of molecular mediators correlated with thymocyte selection in HY^{cd4} female and male mice

Although Gadd45β deficiency did not have an impact on overall T3.70+ thymocyte count in HY^{cd4} mice, it may have a role in the selection events imposed on DP thymocytes. To determine the impact of Gadd45β deficiency on selection, I examined the expression of CD69 and PD-1, two molecules whose expression is regulated during selection. Within the HY^{cd4} Gadd45β wildtype and knock-out female DP thymocytes, CD69 was up regulated to a similar extent in both populations compared to non-transgenic, B6 DP thymocytes. This was reflective of antigen encounter and presumably positive selection. In contrast, HY^{cd4} Gadd45β wildtype and knock-out male DP thymocytes drastically up regulated CD69, compared to non-transgenic, B6 DP thymocytes, in response to high affinity antigen encounter during negative selection (**Fig. 4-20 A**). Surface expression of PD-1 shortly follows TCR stimulation and is coupled with CD69 expression. HY^{cd4} wildtype and Gadd45β knock-out female DP thymocytes showed similar expression levels of PD-1, which were slightly elevated compared to non-transgenic B6 thymocytes (**Fig. 4-20 B**). Both the HY^{cd4} wildtype and Gadd45β knock-out male DP thymocytes had higher surface levels of PD-1 compared to non-transgenic, B6 DP thymocytes. However, Gadd45β deficiency did not alter PD-1 surface expression on DP thymocytes from HY^{cd4} male mice (**Fig. 4-20 B**). The increased levels of PD-1 on HY^{cd4} male DP thymocytes coincided with CD69 surface expression. Therefore, as HY^{cd4} Gadd45β knock-out DP thymocytes had similar surface expression profiles of CD69 and PD-1 as their wildtype counterparts, this suggested that Gadd45β does not modulate downstream TCR signaling within DP thymocytes.

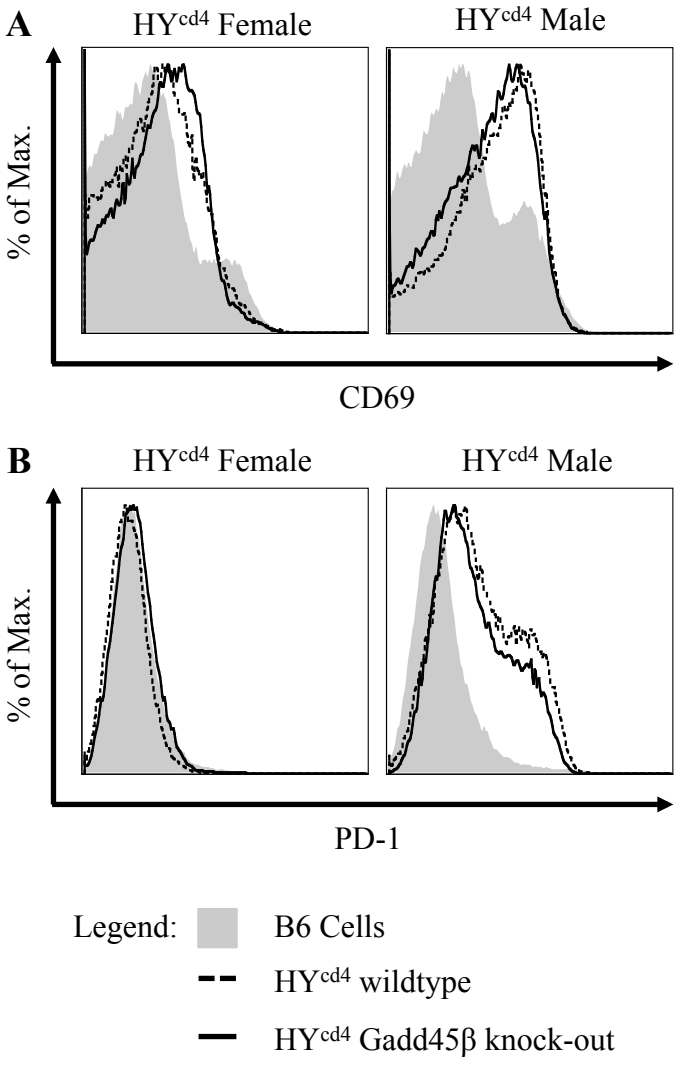


Fig. 4-20. Surface expression of CD69 and PD-1 on T3.70+ DP thymocytes directly *ex vivo* is not dependent on Gadd45β. **A.** Histogram overlays of CD69 expression on DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out female and male mice compared to non-transgenic B6 DP thymocytes. **B.** Histogram overlays of PD-1 expression on DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out female and male mice compared to non-transgenic B6 DP thymocytes. The solid grey represents the non-transgenic B6 control, the dotted line represents the HY^{cd4} wildtype thymocytes, and the solid line represents the HY^{cd4} Gadd45β knock-out thymocytes. HY^{cd4} WT F n = 9; HY^{cd4} Gadd45β KO F n = 7; HY^{cd4} WT M n = 9; HY^{cd4} Gadd45β KO M n = 10.

There are known modulators of cell death that can operate during clonal deletion. Three individual proteins of interest that have been shown to regulate apoptosis are Bim, Nur77 and Bcl-2. Therefore, it was considered that Gadd45β might affect the apoptotic pathway by regulating the expression of one of these proteins. The expression of Bim was first assessed in HY^{cd4} wildtype and Gadd45β knock-out thymocytes. The intracellular expression of Bim, the BH-3 only protein, did not change in HY^{cd4} Gadd45β wildtype or knock-out female mice compared to B6 wildtype levels. However within HY^{cd4} male mice there was a drastic shift in intracellular Bim expression level above B6 wildtype levels, which was not altered by Gadd45β deficiency (**Fig. 4-21 A**). Based on these profiles Gadd45β deficiency did not alter high affinity antigen induced Bim expression. Next, we examined the expression of Nur77 in HY^{cd4} wildtype and Gadd45β knock-out DP thymocytes. HY^{cd4} Gadd45β wildtype and knock-out female thymi had $\leq$ 1% of DP thymocytes expressing Nur77 (**Fig. 4-21 B**). As these cells are not actively undergoing negative selection, finding few cells expressing Nur77 was expected. In comparison, HY^{cd4} Gadd45β wildtype and knock-out male thymi have ~14% of DP thymocytes expressing Nur77 (**Fig. 4-21 B**). The similar frequencies of Nur77 expressing DP thymocytes suggested that Gadd45β does not function in a Nur77-driven manner to assist in apoptosis-mediated clonal deletion.

Finally, we examined the expression of the anti-apoptotic protein Bcl-2 in DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out mice. Within HY^{cd4} wildtype and Gadd45β knock-out female mice, DP thymocytes displayed an increase in Bcl-2 expression levels over B6 isotype levels (**Fig. 4-22 A**). This was expected, as we know that Bcl-2 levels are increased following positive selection (52). In the case of male mice, HY^{cd4} wildtype DP thymocyte expressed slightly higher levels of Bcl-2 compared to the HY^{cd4} Gadd45β knock-out DP thymocytes. The Bcl-2 expression levels of HY^{cd4} male mice were much higher than the non-transgenic, B6 DP thymocyte isotype control (**Fig. 4-22 A**), perhaps to offset an increase in pro-apoptotic intracellular activity or due to Bcl-2 expression being regulated by TCR signaling intensity. Overall, the similarity of Bcl-2 expression

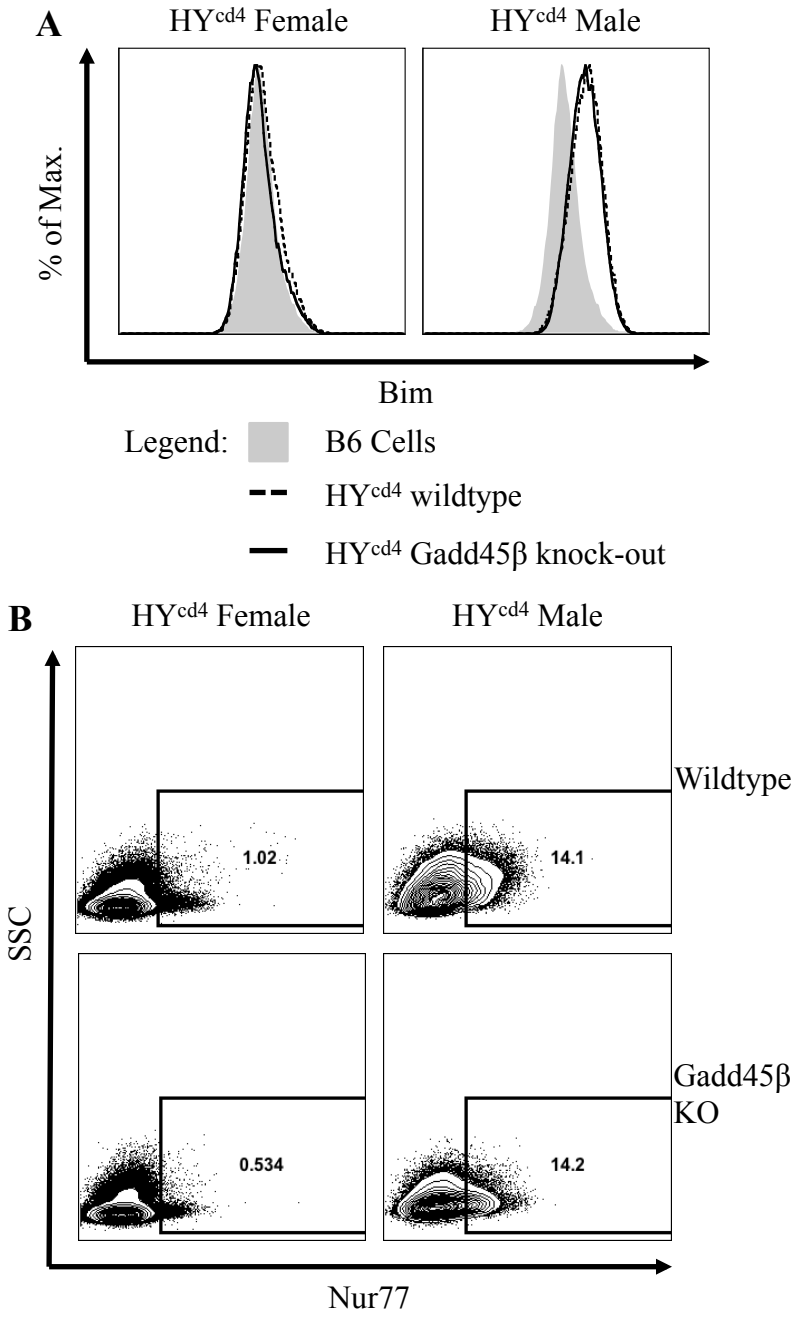


Fig. 4-21. Gadd45β deficiency does not alter the intracellular levels of the pro-apoptotic proteins Bim and Nur77 in T3.70+ DP thymocytes from HY^{cd4} female and male mice. **A.** Histogram overlays of intracellular Bim expression in DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out mice. The solid grey represents the non-transgenic B6 control, the dotted line represents the HY^{cd4} wildtype thymocytes, and the solid line represents the HY^{cd4} Gadd45β knock-out thymocytes. Representative of n=3. **B.** Representative flow cytometric plots of intracellular Nur77 expression in DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out mice. Representative of n=3.

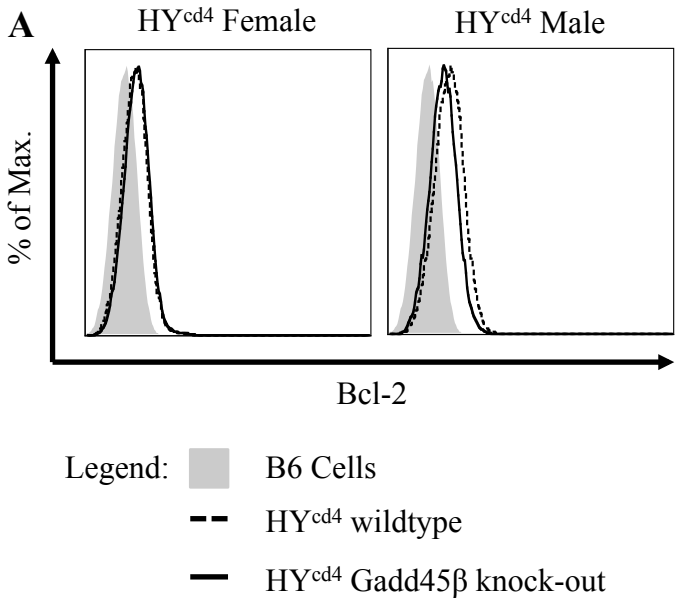


Fig. 4-22. Intracellular Bcl-2 levels within T3.70+ DP thymocytes from HY^{cd4} female and male mice are not altered in the absence of Gadd45β. **A.** Histogram overlays of intracellular Bcl-2 expression in DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out mice. The solid grey represents the non-transgenic B6 Bcl-2 isotype control, the dotted line represents the HY^{cd4} wildtype thymocytes, and the solid line represents the HY^{cd4} Gadd45β knock-out thymocytes. Representative of n=7.

in HY^{cd4} Gadd45β knock-out DP thymocytes to wildtype suggests that Gadd45β does not have an anti-apoptotic role in T cells by altering intracellular Bcl-2 levels. Based upon the expression of Bim, Nur77 and Bcl-2 collectively, it appears that Gadd45β does not contribute to negative selection by clonal deletion in the HY^{cd4} mouse model.

The similar surface expression of CD69 and PD-1 on DP thymocytes in the presence or absence of Gadd45β suggests that Gadd45β is dispensable for negative selection. Additionally, I have also shown that Gadd45β deficiency appeared to have no impact on the expression of various apoptotic mediators. However, in order to directly assess negative selection by clonal deletion I examined the frequency of DP thymocytes expressing the activated form of the executioner caspase, caspase 3. Within the DP thymocyte population of HY^{cd4} female mice, ~0.3% of cells expressed caspase 3 cleavage. This relatively small proportion of cells undergoing apoptosis reflected the fact that DP thymocytes in HY^{cd4} female mice undergo positive selection. In comparison, HY^{cd4} male mice exhibited a 4-fold increase in caspase 3 cleavage within DP thymocytes (**Fig. 4-23 A**). This drastic increase is characteristic of the negative selection events that DP thymocytes undergo upon encounter with their cognate antigen. In the absence of Gadd45β, DP thymocytes from both female and male mice exhibited similar frequencies of caspase 3 cleavage to their respective wildtype counterparts. Graphical analysis of multiple replicates of active caspase 3 expressing DP thymocytes showed significant increases in HY^{cd4} male mice compared to HY^{cd4} female mice. However, there was no significant difference in frequency of caspase 3 expressing DP thymocytes from HY^{cd4} wildtype and Gadd45β deficient female and male mice, respectively (**Fig. 4-23 B**). Therefore, Gadd45β deficiency did not appear to affect negative selection by clonal deletion in a caspase 3 dependent manner.

Gadd45β deficiency does not change the frequency or absolute number of T3.70+ T cells within the periphery of HY^{cd4} female and male mice

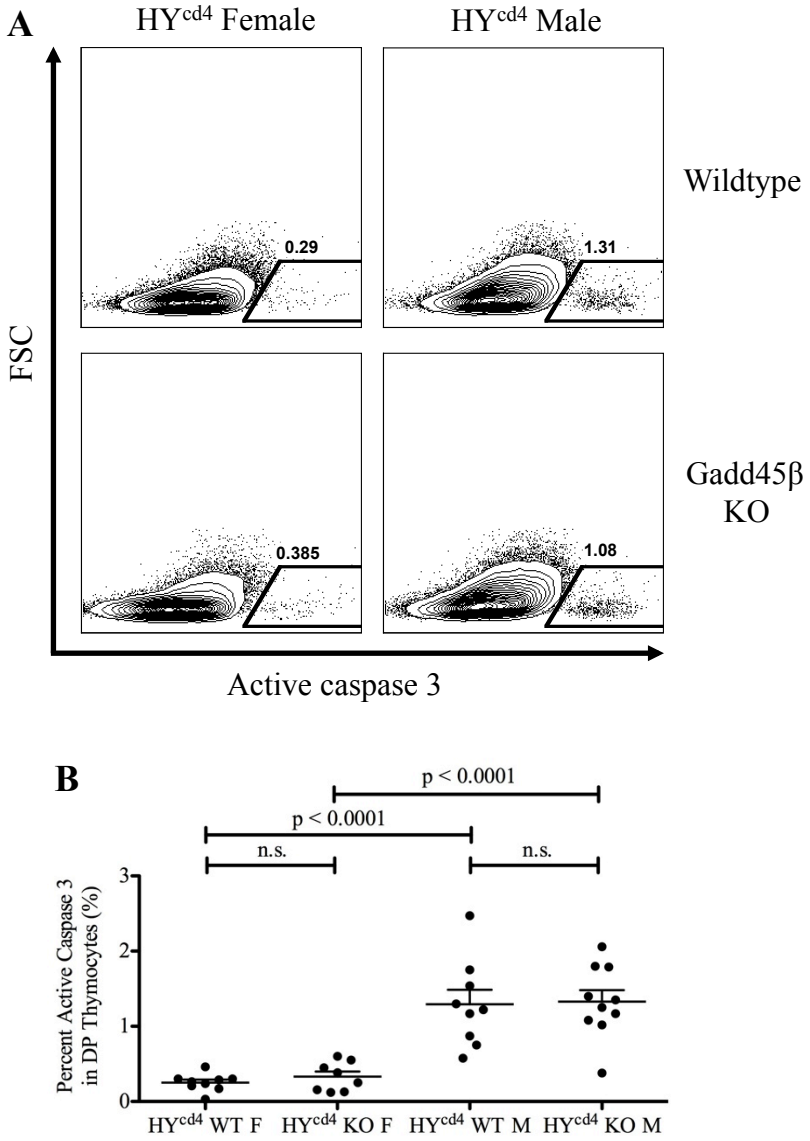


Fig. 4-23. T3.70+ DP thymocytes from HY^{cd4} female and male mice expressed similar levels of active caspase 3 despite the absence of Gadd45β, suggesting Gadd45β does not have a role in thymocyte apoptosis. **A.** Representative flow cytometric plots of active caspase 3 expressing DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out female and male mice. **B.** Graphical analysis of multiple replicates of caspase 3 expressing DP thymocytes from HY^{cd4} wildtype and Gadd45β knock-out female and male mice. HY^{cd4} WT F n = 9; HY^{cd4} Gadd45β KO F n = 8; HY^{cd4} WT M n = 8; HY^{cd4} Gadd45β KO M n = 10.

The peripheral T cell repertoire of HY^{cd4} mice is reflective of the selection events that occur during thymic development. Within HY^{cd4} female mice, a small proportion of the T cell repertoire was composed of $T3.70^{+}$ T cells that survived positive selection. HY^{cd4} Gadd45β knock-out female mice had a similar proportion of T3.70+ T cells compared to wildtype (**Fig. 4-24 A**). In comparison, HY^{cd4} male mice should lack a T3.70+ population within the periphery based on the broadened DP thymocyte population presumably undergoing negative selection and the absence of a CD8 SP thymocyte population **(Fig. 4-19 A)**. However, as demonstrated previously, approximately 12% of splenic T cells from HY^{cd4} male mice expressed the HY TCR (89). Within HY^{cd4} Gadd45β knock-out male spleens, the proportion of HY TCR+ T cells was similar at approximately 10% of total splenocytes (**Fig. 4-24 A**). Multiple replicates for absolute cell numbers of T3.70+ T cells showed a significant difference in the number of T3.70+ T cells between HY^{cd4} wildtype female and male mice, and Gadd45β knock-out female and male mice (**Fig. 4-24 B**). However, there was no statistically significant difference in T3.70+ splenocyte number between HY^{cd4} wildtype and Gadd45β knock-out female mice or male mice, respectively.

Identical analysis was performed within the lymph node compartment of HY^{cd4} wildtype and Gadd45β knock-out mice. HY^{cd4} female mice had a small proportion of T3.70+ T cells within the lymph node compared to male mice. This difference was also present between HY^{cd4} Gadd45β knock-out female and male mice. However, similar to what was observed within the spleen, the relative proportions of T3.70+ T cells appeared to be similar between HY^{cd4} wildtype and Gadd45β knock-out female mice and male mice, respectively (**Fig. 4-25 A**).

Graphical analysis of multiple replicates showed that there was a statistically significant increase in the absolute number of T3.70+ T cells in HY^{cd4} wildtype males when compared to females. However, surprisingly, there is no statistically significant difference in the number of T3.70+ T cells between HY^{cd4} Gadd45β knock-out males and females (**Fig. 4-25 B**). The data suggested that HY antigen

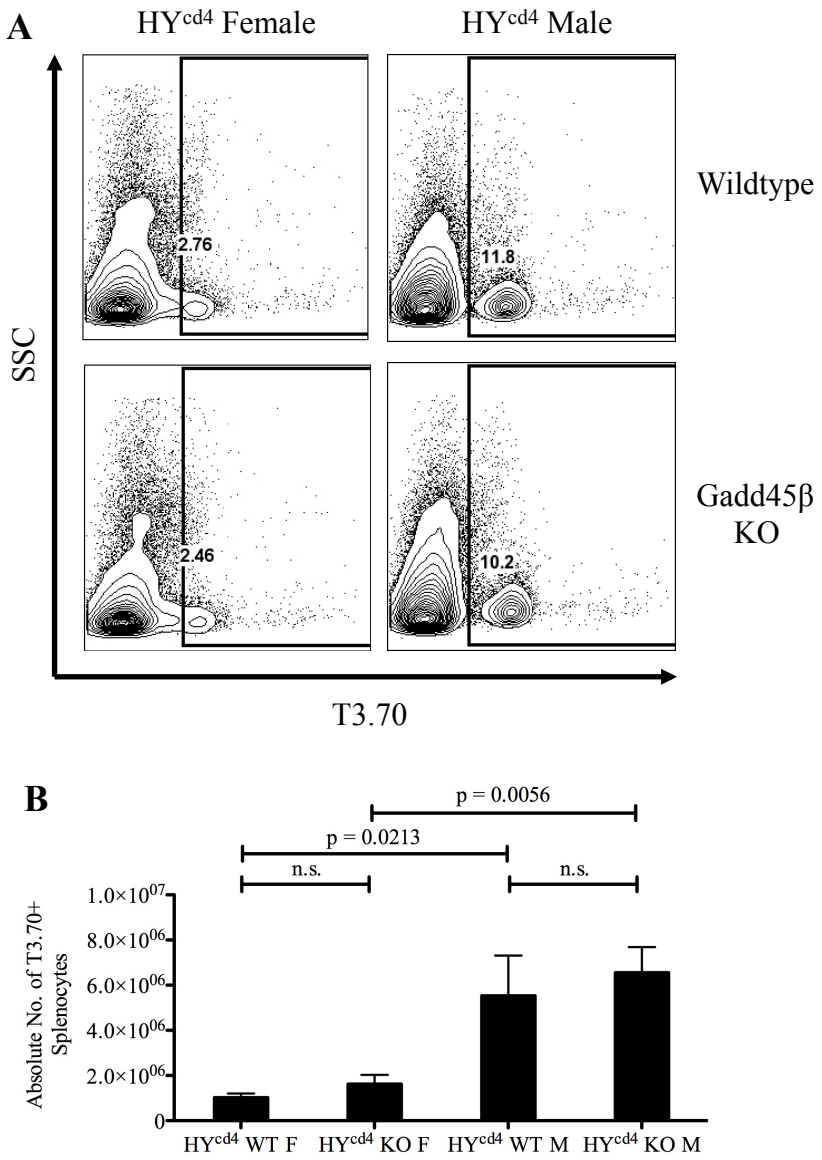


Fig. 4-24. Gadd45β deficiency did not alter the frequency and absolute number of T3.70+ T cells within the spleens of HYcd4 female and male mice. **A.** Representative flow cytometric plots of T3.70+ T cells from the spleen of HY^{cd4} wildtype and Gadd45β knock-out female and male mice. Graphical analysis of the absolute number of T3.70+ T cells in the spleen of HY^{cd4} wildtype and Gad45β knock-out female and male mice. n.s.: not significant. HY^{cd4} WT F n = 6; HY^{cd4} Gadd45β KO F n = 5; HY^{cd4} WT M n = 5; HY^{cd4} Gadd45β KO M n = 7.

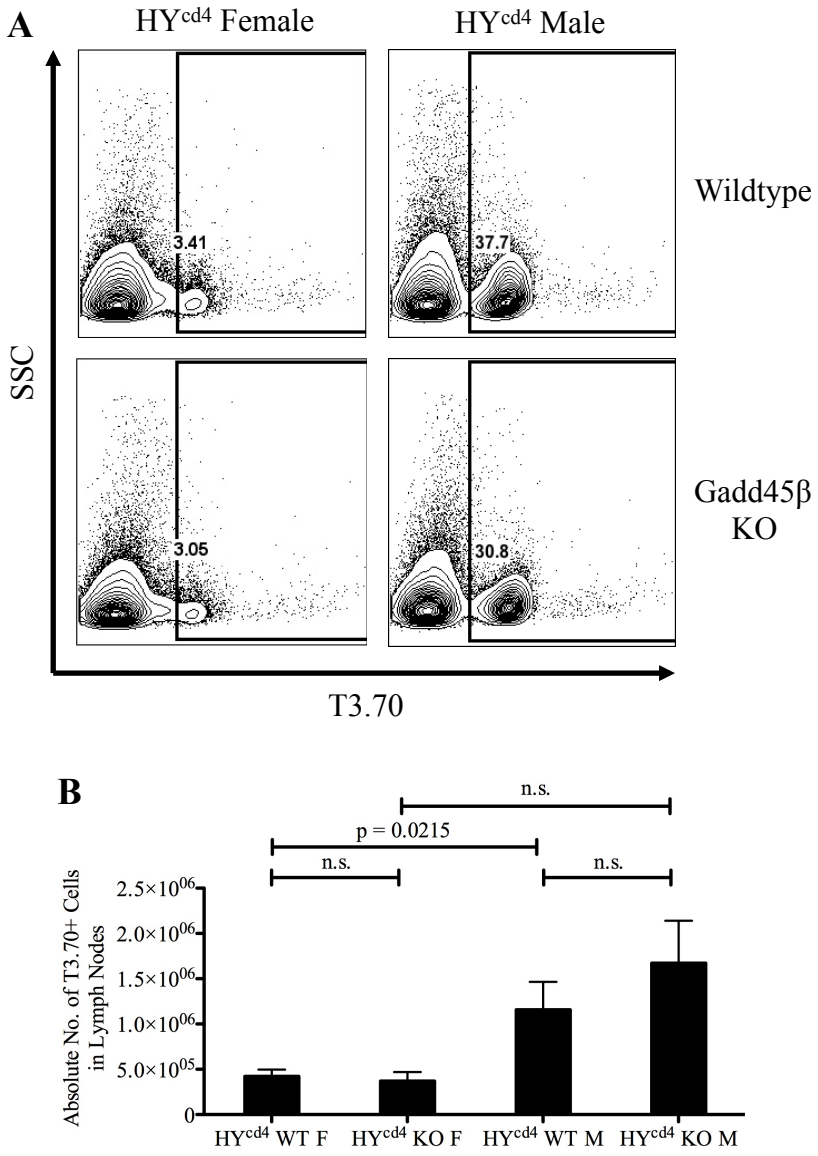


Fig. 4-25. Gadd45β deficiency did not alter the frequency and absolute number of T3.70+ T cells within the lymph nodes of HY^{cd4} female and male mice. **A.** Representative flow cytometric plots of T3.70+ T cells from the spleen of HY^{cd4} wildtype and Gadd45β knock-out female and male mice. **B.** Graphical analysis of the absolute number of T3.70+ T cells in the lymph node of HY^{cd4} wildtype and Gad45β knock-out female and male mice. n.s.: not significant. HY^{cd4} WT F n = 7; HY^{cd4} Gadd45β KO F n = 4; HY^{cd4} WT M n = 5; HY^{cd4} Gadd45β KO M n = 7.

specific T cells escape negative selection within both HY^{cd4} wildtype and Gadd45β knock-out male mice and that Gadd45β deficiency does not alter the proportion and frequency of antigen-specific T cells within the peripheral, monoclonal repertoire of HY^{cd4} mice.

The peripheral T3.70+ T cell population was largely composed of CD8+ T cells in both HY^{cd4} female and male mice, although male T cells had a slightly altered phenotype. CD8+ T cells accounted for approximately 80% of T3.70+ lymph node cells in HY^{cd4} Gadd45β wildtype and knock-out female mice (**Fig. 4-26 A**). In comparison, the proportion of CD8+ T cells ranged between 36-60% in HY^{cd4} Gadd45β wildtype and knock-out male mice. The CD8+ T cells had reduced levels of CD8 coreceptor, resulting in a shift in the population (**Fig. 4-26 A**). There was no difference in the frequency of T3.70+ CD8+ T cells in the presence or absence of Gadd45β. Calculation of the absolute numbers of CD8+ T cells in the lymph node of HY^{cd4} female mice showed no difference in comparison to HY^{cd4} Gadd45β knock-out female mice (**Fig. 4-26 B**). Similarly, there was no significant difference in the number of CD8+ T cells in the lymph nodes of HY^{cd4} males compared to Gadd45β knock-out males. Furthermore, although the graphical plot appeared to show a significant difference in CD8+ T cell number between HY^{cd4} females and males, in the presence or absence of Gadd45β, there was no statistical significance (**Fig. 4-26 B**). Therefore the data suggested that Gadd45β deficiency does not alter the proportion and absolute number of CD8+ T cells within the peripheral compartments of HY^{cd4} mice.

Finally, the memory T cell subsets of the CD8+ T cell population were assessed based on surface expression of CD62L and CD44. Quadrant gating of T3.70+ CD8+ T cells divided the subset into the following sub-populations: $CD62L^+$ $CD44^-$ (naïve); $CD62L^+$ $CD44^+$ (central memory); and $CD62L^-$ $CD44^+$ (effector memory). Phenotypic analysis of the CD8+ T cell population in HY^{cd4} female mice showed similar frequencies of a naïve CD8+ T cell population in the presence or absence of Gadd45β (**Fig. 4-27 A**). Within HY^{cd4} Gadd45β wildtype

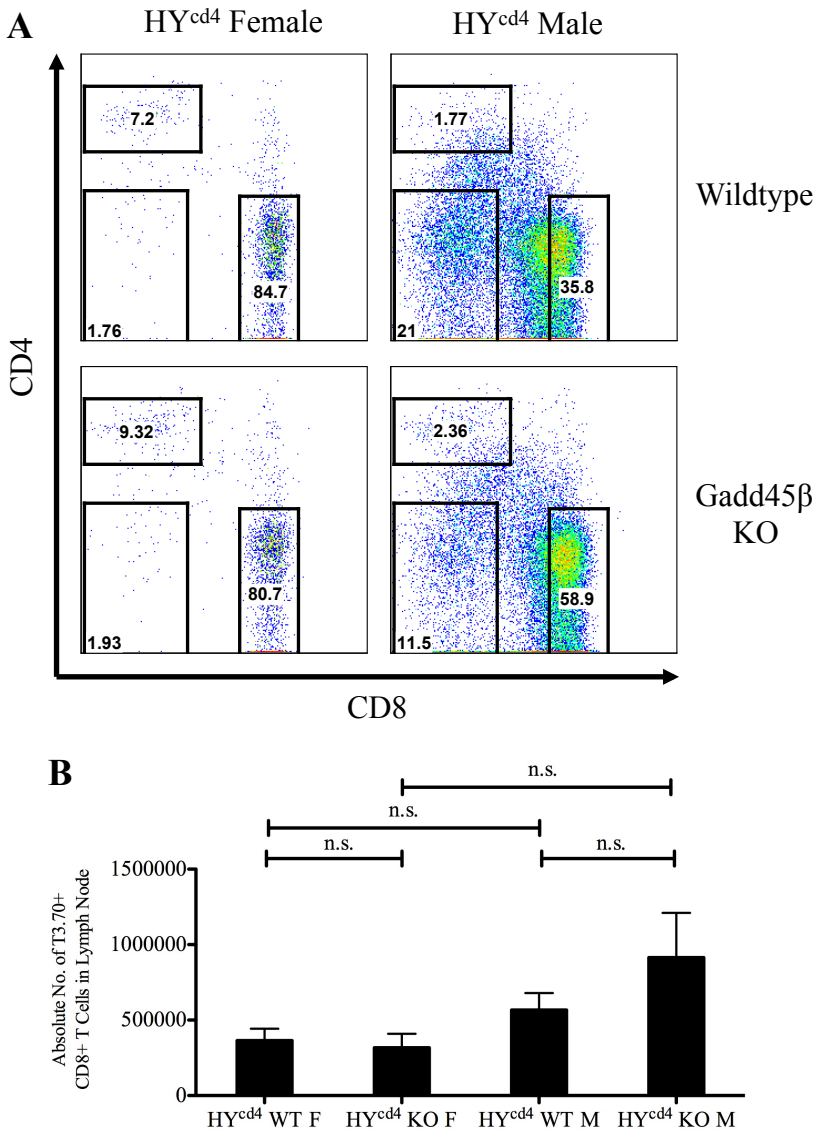


Fig. 4-26. The frequency of T3.70+ CD8+ T cells may be altered by Gadd45β deficiency but the calculation of the absolute number of CD8+ T cells did not show statistical significance. **A.** Representative flow cytometric plots of CD4 and CD8 coreceptor expression of T3.70+ T cells from the lymph nodes of HY^{cd4} wildtype and Gadd45β knock-out female and male mice. **B.** Graphical analysis of the absolute number of T3.70+ CD8+ T cells in the lymph nodes of HY^{cd4} wildtype and Gad45β knock-out female and male mice. n.s.: not significant. HY^{cd4} WT F n = 6; HY^{cd4} Gadd45β KO F n = 4; HY^{cd4} WT M n = 5; HY^{cd4} Gadd45β KO M n = 7.

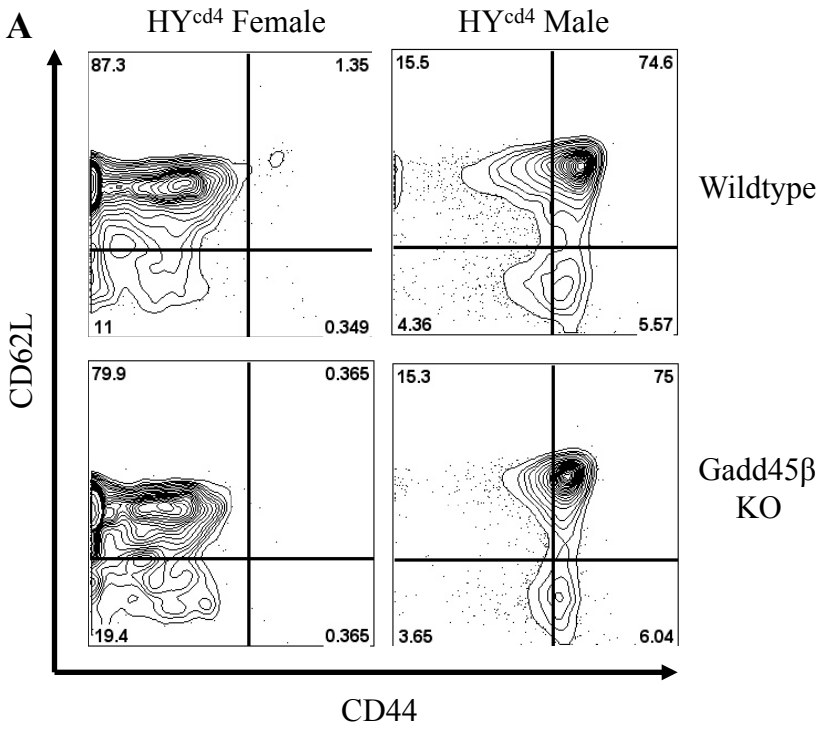


Fig. 4-27. The majority of T3.70+ CD8+ T cells from HY^{cd4} male mice have a central memory phenotype that is independent of Gadd45β. **A.** Representative flow cytometric plots of CD62L and CD44 expression of T3.70+ CD8+ T cells from the lymph nodes of HY^{cd4} wildtype and Gadd45β knock-out female and male mice. CD62L+ CD44- T cells are classified as naïve, CD62L+ CD44+ T cells are classified as central memory and CD62L- CD44+ T cells are classified as effector memory T cells. Representative of HY^{cd4} WT F n = 7; HY^{cd4} Gadd45β KO F n = 4; HY^{cd4} WT M n = 5; HY^{cd4} Gadd45β KO M n = 7.

male mice, CD8+ T cells had a central memory phenotype. Similarly, HY^{cd4} Gadd45β knock-out male CD8+ T cells expressed a similar phenotype and at a similar frequency to wildtype T cells (**Fig. 4-27 A**). Therefore, Gadd45β did not appear to affect the development of central memory T cells within the peripheral compartment of HY^{cd4} male mice.

Gadd45β deficiency does not appear to alter the T cell subsets within the thymus and spleen of B6 wildtype and HY^{cd4} female and male mice

Extensive flow cytometric analysis was performed to characterize the Gadd45β knock-out mouse on the B6 and HY^{cd4} transgenic backgrounds. T cell frequencies and absolute cell numbers within the thymic and peripheral T cell compartments were similar in wildtype and Gadd45β deficient mice. However, there was a statistically significant reduction in the number of NK-T cells in non-TCR transgenic Gadd45β knock-out thymi. Multiple markers of apoptosis have suggested that clonal deletion events were not altered by Gadd45β deficiency. Therefore, the analyses have provided evidence that Gadd45β expression is dispensable for normal thymic development of both the polyclonal T cell subsets of B6 wildtype mice and antigen-specific T3.70+ T cells of HY^{cd4} female and male mice.

Chapter 5: Discussion

Conclusions and critical discussion of studying negative selection in HY^{cd4} neonatal mice and Gadd45β deficient mice

Summary conclusions from the HY^{cd4} neonate and Gadd45β thesis projects

The overall goal of my thesis project was to increase our understanding of clonal deletion. Using the HY^{cd4} transgenic mouse model, I specifically analyzed the ontogeny of antigen-specific T3.70+ T cells and assessed the role of Gadd45β in T cell development. Our published data showed that despite clonal deletion being intact based on the absence of a CD8SP thymocyte population, HY^{cd4} male mice contain an antigen-specific T3.70+ peripheral T cell population. Since we know that negative selection is imperfect (157) we hypothesize that some thymocytes have escaped this process within HY^{cd4} male mice. However, these mice do not develop any overt autoimmunity. Therefore, we sought to understand the ontogeny of the antigen specific T3.70+ T cell population by analyzing HY^{cd4} female and male neonates at Days 3, 5 and 11 post-birth.

Our studies showed that DP T cells were present within the spleen at Day 3 post-birth, suggesting that negative selection is leaky. Furthermore, high levels of PD-1 on both T3.70+ DP and CD8+ T cells from the spleen suggest that its expression may have a role in peripheral tolerance within the neonatal mice. In addition, we found the characteristic CD8 down regulation that is apparent on peripheral T3.70+ CD8+ T cells from HY^{cd4} adult male mice occurs following entry into the periphery. PD-1 surface expression is absent on CD8+ T cells from HY^{cd4} adult male mice. Therefore, in the HY^{cd4} model it is possible that a PD-1 mediated peripheral tolerance mechanism is restricted to neonatal mice, until other mechanisms can be established in adult mice. Such mechanisms may include dominant suppression by tolerogenic dendritic cells or T regulatory cells, anergy or clonal deletion. Beyond the ontogeny studies, we wanted to further our

understanding of what molecular events are required to mediate negative selection and what proteins may be involved. A microarray analysis of DP thymocytes from HY^{cd4} adult male mice showed multiple genes whose expression was up regulated during negative selection. One of these proteins was Gadd45β. Therefore we analyzed thymocyte selection in non-TCR transgenic Gadd45β deficient mice and the HY^{cd4} Gadd45β deficient mice. Based on the evidence, Gadd45β deficiency did not alter the gross frequency or absolute number of T cell subsets within non-TCR transgenic or HY^{cd4} mice except within the thymic NK-T cell compartment of B6 mice. Therefore, the evidence suggested that the absence of Gadd45β did not alter any selection events we measured including negative selection. Collectively, the HY^{cd4} neonatal studies and Gadd45β analysis has allowed us to better understand the origin of the antigen-specific T3.70+ T cells and the contribution of Gadd45β to T cell selection. The evidence presented in this thesis has also raised many more interesting questions about the tolerance mechanisms imposed on the peripheral T3.70+ CD8+ T cells within HY^{cd4} male neonates and whether or not the affect of Gadd45β was masked by other proteins in the Gadd45 protein family.

Discussion of the DP T cells within the spleen of HY^{cd4} neonatal male mice: what mediates their escape, what is their fate and how are these cells regulated?

Are peripheral DP T cells unique to the HY^{cd4} model system?

We originally thought that the neonatal HY^{cd4} male mice would have a small T3.70+ CD8SP T cell population in the thymus and even fewer T cells in the spleen during the early days post-birth. However, our analysis of the neonatal mice showed the exact opposite and provided insight into the T cell development within the neonatal HY^{cd4} model system. We demonstrated that a DP T cell population was present within the T3.70+ T cell subset from the spleen of HY^{cd4} male neonates but was absent within B6 neonates. It is possible that the

appearance of antigen-specific T3.70+ T cells within the neonatal spleen is specific to the HY^{cd4} mouse model. Antigen-specific T3.70+ T cells have been also observed within the classical HY TCR transgenic background (129). Given the polyclonal-specificity of splenocytes from non-transgenic mice, it is difficult to make further conclusions about the presence of DP T cells being restricted to transgenic versus non-transgenic mouse models.

The HY^{cd4} model system of negative selection was derived from the classical HY TCR transgenic model but altered to regulate the HY TCRα chain expression and promote physiological expression of the TCR at the DP thymocyte stage. In comparison, the classical HY transgenic system prematurely expresses the TCRα chain during the DN thymocyte stage, causing inefficient and non-physiological selection events (126). Therefore, the HY^{cd4} model serves as a better system for analyzing thymocyte selection in a physiological manner.

The Shevach group showed that DP T cells were present within the lymph node, but not the spleen, in 3 day old HY male mice and reduced in frequency at approximately 10 days of age (129). This data coincided with our observations of gradually reduced frequencies of DP T cells within the spleen of HY^{cd4} male neonates between Day 3 to 11. Due to the premature expression of the TCR in classical HY mice, it was uncertain whether the appearance of DP T cells in the periphery was an artifact of early TCR expression. Our results with the HY^{cd4} model suggest that early TCR expression is not the cause of DP T cells in the spleen.

Furthermore, we considered whether or not the presence of the antigen-specific T cell population within the spleen was specific to the HY-derived transgenic model systems. The 2C TCR transgenic mouse model recognizes allogeneic MHC Class I L^d (ubiquitous self antigens) with high affinity, inducing negative selection (158, 159); yet these mice also contain antigen-specific T cells within the periphery (160). Therefore, as other TCR transgenic model systems display antigen-specific

T cells within the periphery, it is possible that thymic escape occurs in situations of negative selection against ubiquitous antigen. However the nature of self-antigen in directing the escape of T cells from negative selection events is unlikely since antigen-specific CD8+ T cells are also present within the periphery of the tissue-restricted antigen OT-1 TCR transgenic mouse model (161). Therefore, CD8+ T cells within the periphery of MHC Class I-restricted TCR transgenic mouse models appears to be a hallmark of these systems cementing the idea that central tolerance is not perfect.

What physiological factors mediate the escape of antigen-specific T3.70+ T cells from the thymus?

Immature thymic tissue in neonatal mice

Although the appearance of antigen-specific T cells within the periphery is not restricted to the HY^{cd4} model system and other TCR transgenic models also display this characteristic, there must be a reason for their escape from clonal deletion. I have showed that HY^{cd4} neonatal male mice have a frequency of self-reactive T3.70+ T cells within the spleen as early as 3 days post-birth and considered that the characteristics of the immature neonatal thymus were permitting T cell escape. If this were the case, the T3.70+ T cells likely escape during the neonatal stage of development and persist within the periphery until adulthood.

The presence of autoreactive T cells within the periphery of neonatal mice and the induction of autoimmunity has been shown in a variety of model systems. Breakdown of neonatal tolerance and induction of autoimmunity has been implicated following injection of bovine type-II collagen into ICR (CD-2) neonates, a typical outbred mouse strain, generating autoimmune chronic joint inflammation. This process was dependent on the presence of autoreactive T cells within the periphery (162). Furthermore, neonatal double transgenic INS-HA

TCR-HA, expressing the influenza viral protein hemagglutinin (HA) under control of the insulin promoter and MHC Class-II restricted T cells specific for the HA protein, have a naturally occurring autoreactive T cell population within the periphery. At Day 3 post-birth, approximately 25% of splenocytes were CD3+ T cells and lymphocytic infiltration into the pancreas had begun; by Day 7, the lymphocytic infiltration was extensive and the neonates had developed hyperglycemia (163). Both studies have implicated the peripheral autoreactive T cell population as the cause for developing autoimmunity but did not address the reason these cells escaped negative selection within the thymus. Evidence from the GFP-Foxp3 fusion protein expressing mouse model, $Foxp3^{gfp}$, has shown that the neonatal thymus is delayed in T cell ontogeny of the Foxp3+ T regulatory cell population. Immunohistochemistry analysis showed immature thymic medulla tissue at Days 1, 4 and 9 post-birth and was proposed to be the reason for delayed Foxp3+ regulatory T cell generation as well as less efficient negative selection at the neonatal stage (164). Therefore it is possible that the immature neonatal thymic tissue allows for the escape of potentially autoreactive T cells from negative selection. In order to definitely demonstrate that the immature thymus was not the factor in the escape of antigen-specific T3.70+ T cells, mixed bone marrow chimeras were generated with HY^{cd4} wildtype and PD-1 knock-out male bone marrow and male recipients. The presence of T3.70+ CD8lo T cells within the spleens of the adult male chimeras (**Fig. 3-27 A**) suggests that it is unlikely the immature nature of the thymus from neonatal mice is permitting the escape of T3.70+ T cells into the spleen. Rather, it is likely due to an intrinsic feature of the T cells themselves.

Lack of cognate male antigen expression within thymus

We considered that the antigen-specific T3.70+ T cells were able to escape thymic selection due to a lack of cognate antigen expression within the thymus. We think this is unlikely since the T3.70+ DP thymocyte population had a dulled phenotype, with reduced surface levels of CD4 and CD8 coreceptor expression,

suggestive of antigen encounter. Furthermore, the DP thymocytes showed CD69 induction directly *ex vivo*, which is a marker of TCR engagement with peptide-MHC complexes (pMHC). We showed that DP thymocytes had intracellular expression of active caspase 3 which is initiated following high affinity antigen encounter within the thymus. Finally, the Kisielow group showed that HY male antigen expression was detectable at embryonic day 16 within the thymic tissue (165). Therefore, leaky negative selection does not appear to be due to the lack of ubiquitous male antigen expression within the thymus.

Affinity of the HY TCR for antigen and agonist selection

I have demonstrated that the T3.70+ self-reactive T cells within the periphery of HY^{cd4} male mice are present due to imperfect negative selection events. It has been previously shown that the relative affinity of the TCR for its peptide-MHC complex may be sufficient for clonal deletion within the thymus but not for T cell activation within the periphery (166). However the absence of cognate HY male antigen within the thymus has been demonstrated to not be the source of antigen-specific T cell escape from selection events.

Alternatively, the antigen specific T3.70+ T cell population may be redirected from negative selection and undergo the alternative selection process known as agonist selection. It has been proposed that some high-affinity TCR expressing thymocytes do not undergo negative selection; instead they are positively selected by an agonist ligand (167). Various T cell populations are derived from agonist interactions with self ligand, and these cells include natural T regulatory cells, invariant natural killer T cells, natural CD8αα intraepithelial cells and natural T helper 17 cells (168). It is thought that these T cells require strong and sustained TCR:peptide-MHC signals to undergo agonist selection and are involved in immune cell regulation (167).

Experimental studies have provided significant evidence to suggest that autoreactive thymocytes can be diverted to an alternative, regulatory lineage and not induce autoimmunity. *In vitro* studies with homozygous 6F/6F mice, expressing a transgenic knock-in allele of a mutant TCR ζ chain and are therefore unable to signal downstream of the TCR, showed that thymocytes were incapable of undergoing negative selection but do not develop spontaneous autoimmunity. However, it appeared that these T cells developed into the regulatory T cell lineage as these mice had 1.5 to 2-fold more Foxp3+ CD4+ T cells within the thymus and spleen compared to controls. Therefore, it appeared that the high-affinity TCR clones that were unable to undergo negative selection developed a regulatory T cell fate and displayed substantial suppressive activity (169).

A regulatory function for DN T cells has also been implicated for cells that have escaped negative selection and entered the periphery. K14-mOVA mice, which express OVA in the skin, salivary glands and mammary gland epithelium, succumbed to graft versus host disease upon adoptive transfer of OVA-specific OT-1 T cells. However, K14-mOVA OT-1 double transgenic mice that contain a resident OT-1 T cell population did not develop graft versus host disease. When the skin draining lymph nodes and spleen were examined for regulatory T cell populations, there was an abundance of OT-1 DN T cells that exhibited regulatory T cell activity. The evidence suggested that these cells contributed to peripheral tolerance in the prevention of graft versus host disease (170). It is currently unclear if agonist selection is being employed within the HY^{cd4} male mouse model and the analysis within this thesis project did not closely examine this selection process. However, approximately 20% of self-reactive T3.70+ T cells within the spleen of HY^{cd4} male mice are DN T cells, compared to 5-8% in HY^{cd4} female mice (**Fig. 3-2 B**). HY^{cd4} male mice contain a higher proportion of DN T cells within the spleen, similar to what was observed within K14-mOVA OT-1 double transgenic mice and it is possible that these cells have a regulatory function within HY^{cd4} male mice. However, further experimental analysis is required to make this conclusion.

What is the fate of the peripheral T3.70+ DP and CD8+ T cells?

Possible development of DP T cells into a regulatory T cell lineage

Our evidence showed a substantial T3.70+ DP T cell population within the periphery that gradually reduced in frequency between Day 3 and 11 HY^{cd4} male neonates. However the HY^{cd4} neonatal studies did not show the developmental fate of the DP T cell population but it was considered that these cells might be agonistly selected and develop into an alternative lineage. It is known that recent thymic emigrants preferentially adopt the regulatory T cell lineage within a lymphopenic environment, similar to what is found within neonatal mice. Adoptive transfer of CD4+ CD8- Foxp3- cells from the thymus or lymph nodes of $Foxp3^{gfp}$ into $TCR\beta^{-/-}$ recipients showed that thymocytes readily differentiated into regulatory T cells upon entry into the periphery. This development was attributed to be an intrinsic property of thymocytes instead of being due to lymphopenia-induced proliferation. Injection of a 1:1 ratio of congenically labeled CD4+ CD8- Foxp3- T cells from the thymus and lymph nodes demonstrated a 5-10 fold increase in thymocyte derived regulatory T cells (171). Therefore it appears that the intrinsic properties of thymocytes alone can dictate their regulatory fate once they surpass the selection processes within the thymus. Therefore the DP T cell population in HY^{cd4} male neonates may further develop within the periphery to form a regulatory role. However, the experimental data presented here did not look at agonist selection of these cells. This would be an interesting future experiment within the HY^{cd4} neonate project.

Developmental fate of peripheral antigen-specific T3.70+ DP T cells into the CD8αα intestinal epithelial lymphocyte lineage

Based on the present evidence of a substantial proportion of T3.70+ DP T cells in the spleen of HY^{cd4} neonatal male mice, we considered that this population might be destined for the CD8αα intestinal epithelial lymphocyte (IEL) lineage. The

IEL cell subsets are located within the intestinal epithelium and lamina propria, are various T cell precursors and contribute to the overall intestinal immune function (172). It is known that T cells expressing the TCRαβ and the CD8αα homodimer are most abundant within the intestinal epithelium (173) therefore we assessed CD8α and CD8β coreceptor expression in total thymocytes of B6 and HY^{cd4} male neonates. Preliminary evidence showed no change in the proportion of CD8αα coreceptor expressing T3.70+ thymocytes in HY^{cd4} male neonates compared to the HY^{cd4} adult male. In contrast, B6 neonates had a large proportion of CD8αα homodimer coreceptor expressing thymocytes at Day 11 post-birth (**Fig. 5-1 A**). Approximately half of the CD8αα homodimer coreceptor thymocytes also express CD4 coreceptor, suggesting these may be CD4+ CD8αα+ thymus dependent precursors to the CD8αα IEL population (**Fig. 5-1 B**) (173). However, I cannot definitely conclude that these cells are CD8αα IEL precursors based on the current evidence.

Within the HY^{cd4} male neonates, although the T3.70+ thymocytes lacked CD8αα homodimer coreceptor expression, it does not preclude these cells developing to the CD8αα IEL lineage. It is known that autoreactive DP thymocytes that escaped clonal deletion down regulate both CD4 and CD8β coreceptor expression to become DN. It was shown that these cells then preferentially migrated to the intestine followed by re-expression of CD8α coreceptor (174). When I examined the DN thymocyte compartment of HY^{cd4} adult males, approximately 60% of DN thymocytes were CD3+ (**Fig. 5-2 A**). Furthermore, the proportion of CD8αα homodimer coreceptor expressing T3.70+ increased in Day 11 HY^{cd4} male neonates within the spleen, evidence that T cells that have escaped clonal deletion are adopting the CD8αα IEL fate within the periphery (**Fig. 5-2 B**). Although this evidence is preliminary, it may support the developmental progression of autoreactive neonatal thymocytes that escape clonal deletion into the alternative CD8αα IEL lineage in the HY^{cd4} male mouse model. More detailed experimental analysis of this population directly *ex vivo* and tissue homing experiments post-

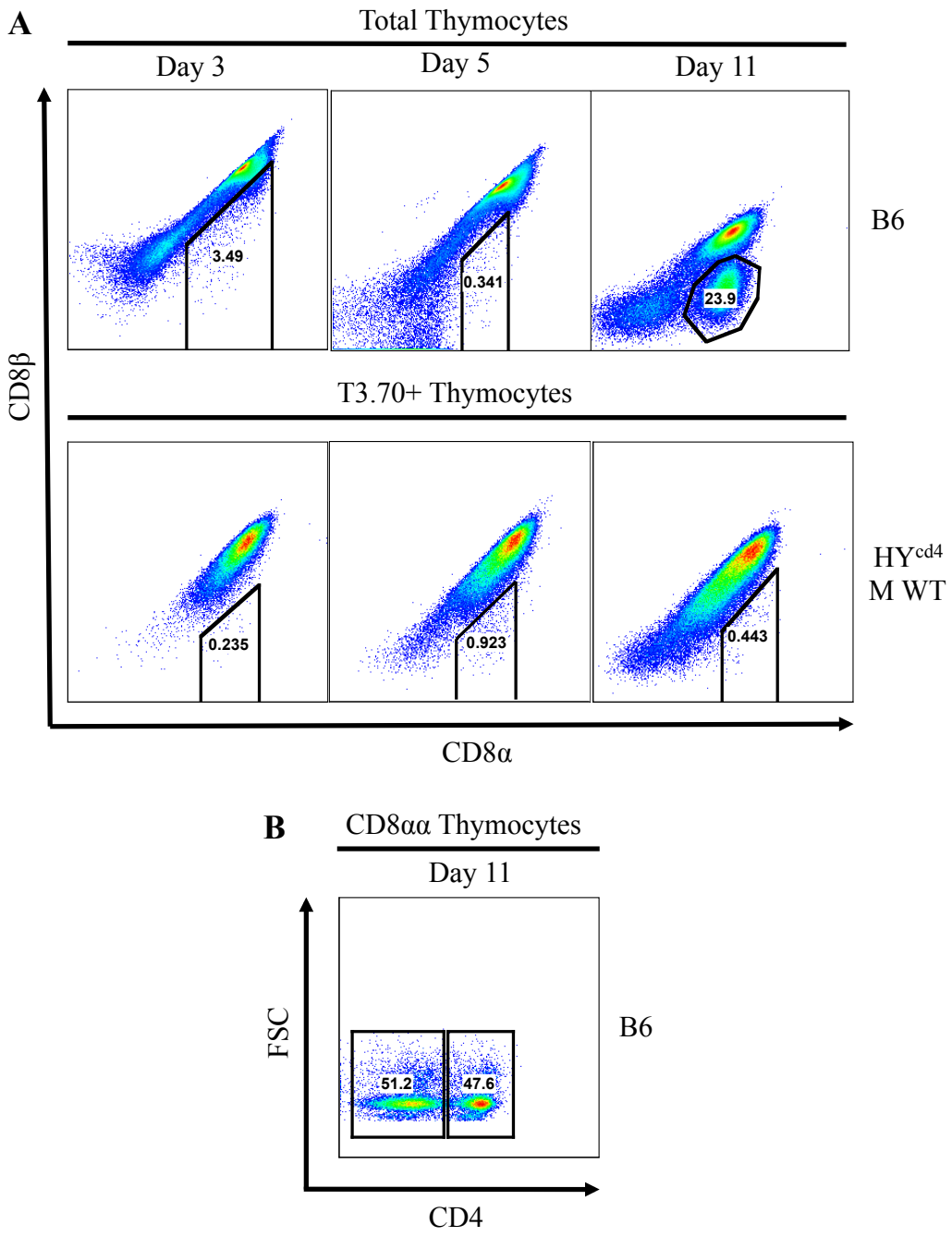


Fig. 5-1. Preliminary evidence from the thymus of Day 11 B6 neonatal mice showed a proportion of CD8α+ thymocytes that may contribute to a CD4+ thymus derived CD8αα intestinal epithelial lymphocyte population. **A.** Representative flow cytometric plots of CD8α and CD8β coreceptor expression to identify CD8αα homodimer coreceptor expressing thymocytes in B6 neonates and HY^{cd4} male neonates. **B.** Representative flow cytometric plot of CD4 coreceptor expression within the CD8αα homodimer coreceptor expressing thymocytes in the Day 11 B6 neonates.

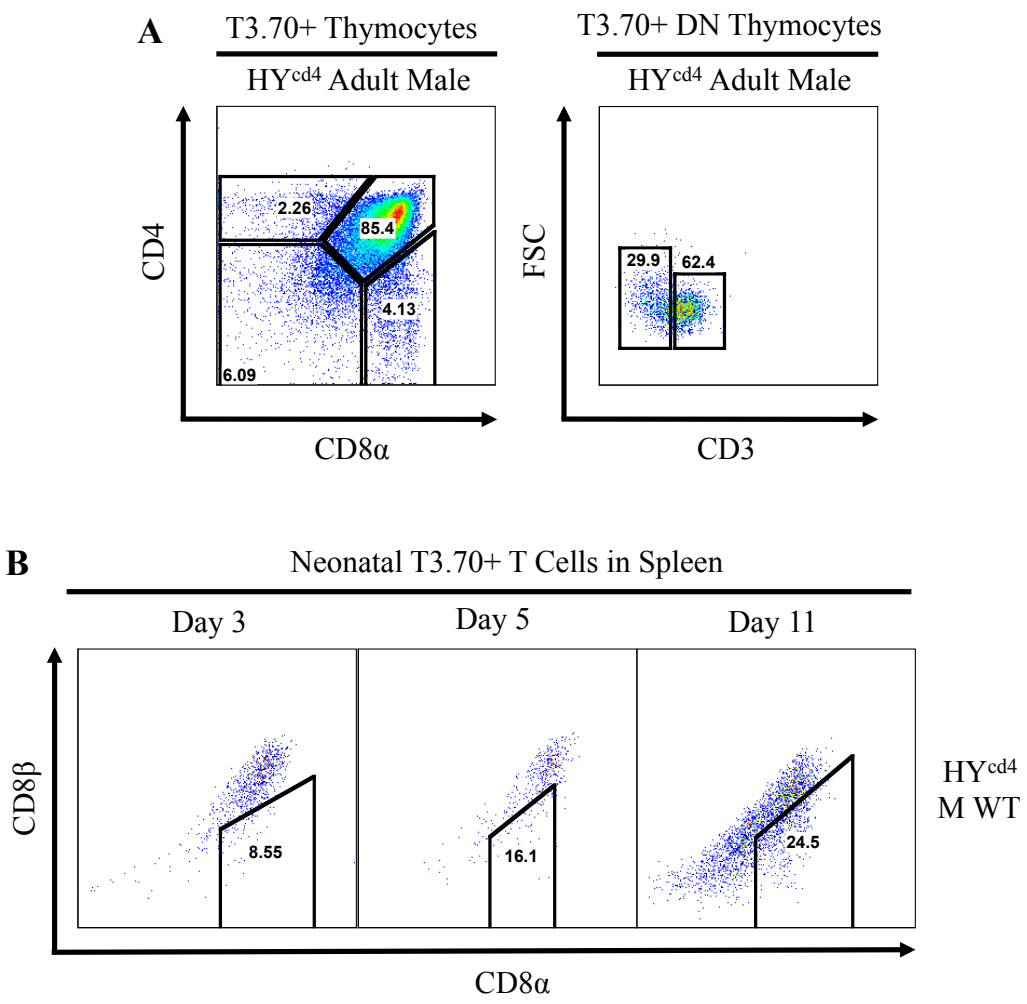


Fig. 5-2. Preliminary evidence within HYcd4 adult and neonatal male mice may coincide with the literature, suggesting that autoreactive DP thymocytes adopt a DN thymocyte fate following antigen encounter, then re-express CD8α coreceptor upon entry into the periphery and become a CD8αα intestinal epithelial lymphocyte. **A.** Representative flow cytometric plots of the T3.70+ thymocytes that have dulled CD4 and CD8 coreceptor expression, falling into the DN thymocyte gate and their surface expression of CD3 within a HY^{cd4} adult male mouse. **B.** Representative flow cytometric plots of CD8α and CD8β coreceptor expression to identify CD8αα homodimer coreceptor expressing splenocytes in HY^{cd4} male neonates.

adoptive transfer of T3.70+ DN and DP T cells from HY^{cd4} male neonates are required to definitively show the developmental fate of these cells.

Development of innate lymphocytes from antigen-specific T cell precursors

The present study of HY^{cd4} male neonates compared to HY^{cd4} male adults has shown a substantial proportion of antigen-specific CD8+ T cells within the spleen at both developmental stages that have escaped negative selection. The frequency of CD8+ T cells increases within the HY^{cd4} male neonates between Day 3 and Day 11 post-birth and remains fairly substantial into adulthood. However, these mice do not develop overt autoimmunity. Therefore it is possible that these cells might be destined to an alternative T cell fate. The evidence from Hung-Sia Teh's group that demonstrated peripheral CD8+ T cells were destined to an innate lymphocyte fate supports this hypothesis. They showed that HY TCR CD8lo T cells expressed NK cell markers DX5 and CD94 following antigen stimulation and IL-2 *in vitro* culture (175). Furthermore, cytotoxic activity of the HY TCR CD8lo T cells post-non cognate antigen stimulation with an *in vivo Listeria monocytogenes* bacterial challenge resembled that of NK cells (175, 176) supporting the hypothesis of CD8lo T cells' developmental fate into innate lymphocytes. Peripheral T cells from HY^{cd4} adult male mice also have reduced CD8 coreceptor expression and given the similarities between the classical HY and the HY^{cd4} model systems, HY^{cd4} CD8+ T cells may also develop into innate lymphocytes. However, it is unclear at this time whether or not CD8+ T cells are destined for this fate within the HY^{cd4} model system.

How are the peripheral T3.70+ T cells regulated to prevent autoimmunity?

We have found that despite a robust frequency of antigen-specific T3.70+ T cells within the periphery of HY^{cd4} male mice, they do not appear to cause overt autoimmunity. The cognate HY male antigen is ubiquitously expressed within the peripheral compartment; therefore it is not likely the lack of antigen encounter

that is preventing T cell activation. Therefore, other peripheral tolerance mechanisms may be active in regulating this self-reactive T cell population within the periphery.

Induction of anergy on T3.70+ T cells within the periphery of HY^{cd4} male mice

The HY male antigen is ubiquitously expressed within HY^{cd4} male mice, therefore there is opportunity for antigen encounter of peripheral T3.70+ T cells with their cognate antigen. The down regulation of CD8 coreceptor levels on T3.70+ CD8+ T cells provides evidence of TCR interactions *in vivo* (149). As mentioned above, our understanding of central memory T cell survival suggests they do not require peptide-MHC encounter within the periphery. Yet, if they were to frequently encounter antigen within the periphery, current evidence suggests that T cells that encounter chronic self-antigen undergo an initial clonal expansion, followed by long-term induction of anergy without peripheral clonal deletion (177).

T3.70+ CD8+ T cells become activated during *in vitro* stimulation assays and up regulate the activation marker CD69. Therefore it is unlikely that T3.70+ CD8+ T cells are anergic. The peripheral T3.70+ T cells respond to high levels of antigen in an *in vitro* peptide stimulation assay. Furthermore, while surface expression of the inhibitory receptor CTLA-4 is associated with anergy induction (178), preliminary evidence of HY^{cd4} CD8+ T cells showed no increase in CTLA-4 surface expression compared to a B6 control. The lack of T3.70+ T cell response may be due to insufficient sensitivity of the TCR to the endogenous levels of peptide-MHC *in vivo*.

Peripheral tolerance may be induced by surface expression of the inhibitory receptor PD-1

Other peripheral tolerance mechanisms may be imposed on T3.70+ T cells within HY^{cd4} male mice, and we speculate that surface expression of PD-1 may be

involved in tolerance. Our data showed that HY^{cd4} wildtype male neonates expressed high levels of PD-1 on the cell surface directly *ex vivo* compared to HY^{cd4} adult male and B6 adult female controls. The absence of PD-1 expression on T3.70+ CD8+ T cells within the periphery of HY^{cd4} adult male mice suggests that PD-1 expression is turned off at some point between the neonatal and adult stages of development. We know that PD-1 is not required for DP thymocyte development and fate, as PD-1 deficiency is not necessary for negative selection even though in wildtype HY^{cd4} male mice about 50% of DP thymocytes are PD-1 expressing (119). Evidence suggests that PD-1 may be regulating T cell trafficking in the periphery (179) but it is known that antigen-specific T3.70+ T cells lacking PD-1 surface expression are still able to enter the periphery within HY^{cd4} male mice.

Within a wildtype B6 mouse directly *ex vivo*, both effector memory T cell populations and recent thymic emigrants (RTEs) had drastically higher levels of PD-1 surface expression compared to controls (119). During the neonatal stage of development, a considerable proportion of the peripheral T cell population are likely to be recent thymic emigrants; therefore PD-1 may be required for regulating any self-reactive T cells that have recently exited the thymus. PD-1 mediated tolerance has been shown in an anti-CD3 therapy treated NOD mouse model, whereby mice treated with the monoclonal antibody anti-PD-L1 at 12 weeks rapidly developed diabetes whereas those lacking PD-1-PD-L1 pathway blockade remained healthy (180). Our studies with HY^{cd4} PD-1 knock-out adult mice have shown no gross differences in the peripheral T cell populations, suggesting that PD-1 may not have a dominant role in peripheral tolerance. We can speculate that the contribution of PD-1 expression within the HY^{cd4} mouse model may be subtle since CD8+ T cells from HY^{cd4} adult mice lack PD-1 surface expression. Further detailed experimental analysis of HY^{cd4} wildtype and PD-1 knock-out T cells is required to definitively suggest a role for PD-1 in peripheral tolerance of T3.70+ T cells.

Coreceptor tuning as a mechanism of peripheral tolerance

An interesting hallmark of the HY^{cd4} male mice is the down regulation of CD8 coreceptor on the cell surface of peripheral CD8+ T cells, while thymocytes express wildtype levels of CD8 coreceptor. The data from the present study showed CD8 coreceptor down regulation on HY^{cd4} neonatal male CD8+ T cells occurring gradually from wildtype levels at Day 3 post-birth to identical levels compared to HY^{cd4} adult mice at Day 11 post-birth. Based on this evidence, CD8 coreceptor down regulation is induced within the periphery in the few days post-birth. Therefore it is possible that the modulation of CD8 coreceptor levels can mediate peripheral tolerance.

This consideration is supported by the concept of coreceptor tuning whereby the modulation of CD8 coreceptor levels on the cell surface can alter the sensitivity of the TCR to antigen stimulation in an IL-7R signaling dependent manner. Coreceptor tuning would function as a peripheral tolerance mechanism by reducing CD8 coreceptor levels and consequently desensitizing a TCR for its cognate antigen within the periphery to prevent T cell activation (149). The analysis of coreceptor tuning within the HY^{cd4} model system, presented in the Appendix, found inconclusive evidence to suggest that T3.70+ CD8+ T cells were strictly regulated by coreceptor tuning. However, we were able to show that CD8 coreceptor levels on HY^{cd4} CD8+ T cells increased to wildtype levels following *in vitro* culture. Therefore, according to the model of coreceptor tuning, it is possible that these cells gained sensitivity to their cognate antigen. Further experimental analysis by adoptive transfer of the "untuned" cells into B6 male mice would provide the definitive evidence to show that coreceptor tuning alters sensitivity of T3.70+ T cells for their cognate antigen in the HY^{cd4} model system.

A model of T3.70+ T cell development and regulation in HY^{cd4} male neonatal mice

The HY^{cd4} neonatal mouse studies have improved our understanding of the generation of antigen-specific T3.70+ CD8+ T cells within HY^{cd4} male mice. This project has addressed a few curiosities about the development, role and regulation of neonatal DP T cells within the spleen but leaves many more questions to be answered.

Based on the evidence presented, I have a proposed model of T cell development of the antigen-specific T3.70+ T cell population and its possible roles within the periphery (**Fig. 5-3 A**). Within HY^{cd4} male neonates, self-reactive T3.70+ thymocytes escape clonal deletion either within the cortex or the medulla. These cells enter the periphery as either T3.70+ DP or CD8+ T cells expressing high levels of PD-1. Evidence within the scientific literature suggests that the unique T3.70+ DP T cells may develop into the CD8αα IEL lineage or the CD8+ innate lymphocyte lineage with NK cell-like cytotoxicity. Since antigen-specific T3.70+ T cells do not appear to cause overt autoimmunity within HY^{cd4} male mice, these cells may be regulated in three different ways. High levels of PD-1 surface expression can directly antagonize TCR signaling by cognate antigen within the periphery and thereby prevent T cell responses. Furthermore, chronic antigen encounter within the periphery may induce anergy of the T3.70+ CD8+ T cells. Finally, reduced CD8 coreceptor levels on the surface of T3.70+ CD8+ T cells provides some evidence that coreceptor tuning may be regulating this population.

In the interest of addressing other questions raised from this present data, an interesting experiment to perform would be an adoptive transfer system of the HY^{cd4} neonatal male DP T cells from the spleen into an adult B6 male mouse, in order to determine the fate of this interesting cell population. Furthermore, an ultimate goal of this project is to better understand the tolerance inducing signals on the antigen specific T3.70+ T cells present within both HY^{cd4} male neonatal and adult mice. In regards to this, it would be interesting to examine the surface expression of Fas and FasL within the spleen of both HY^{cd4} male neonatal and adult mice, since the interaction and engagement of Fas-FasL has been shown to

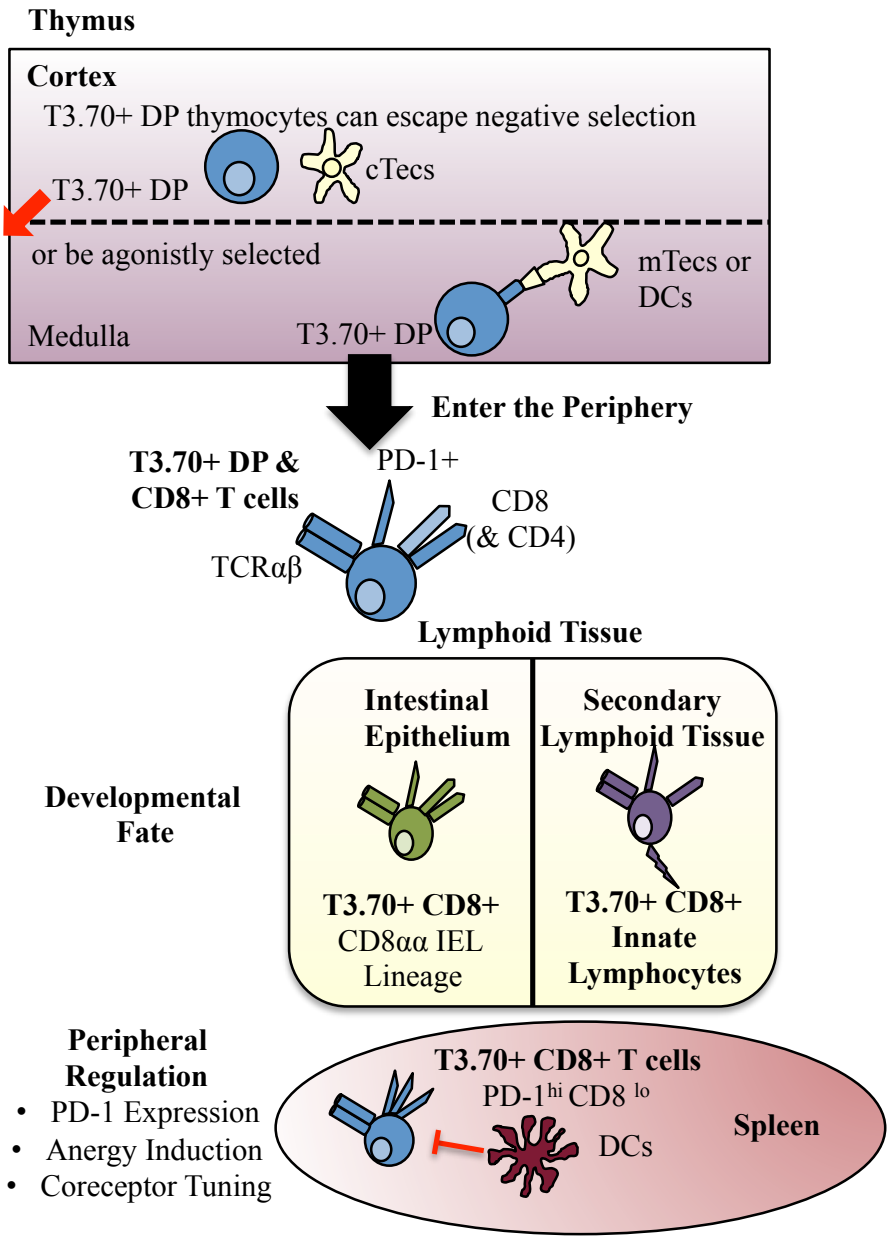


Fig. 5-3. A proposed model of T cell development and regulation of T3.70+ T cells within HY^{cd4} neonatal male mice. **A.** A detailed model of HY^{cd4} neonatal male T cell development based on the presented data and proposed developmental fate and regulatory mechanisms imposed on these antigen-specific T3.70+ T cells within the periphery. T3.70+ DP thymocytes either escape negative selection or are agonistly selected within the thymus. They enter the periphery as T3.70+ DP or CD8+ T cells, expressing high levels of PD-1. Within the periphery, it is proposed these T3.70+ T cells have a developmental fate into the CD8αα IEL lineage or innate lymphocyte lineage. These cells may be regulated by PD-1, anergy or coreceptor tuning.

be crucial for maternal T cell tolerance to fetal male HY antigen expression and induction of a hyporesponsive state of the maternal CD8+ T cells (181). Therefore, if the T3.70+ T cell population is expressing high levels of Fas or FasL, it may provide insight of an additional method of peripheral tolerance that is imposed *in vivo* within the HY^{cd4} male model.

Discussion of the role of Gadd45β in clonal deletion of self-reactive T cells within the HY^{cd4} mouse model

The role of Gadd45β in the stress response, the MAPK pathway and in clonal deletion of thymocytes

Gadd45β is a member of the Gadd45 protein family induced by stress response signals and has differential pro-survival or pro-apoptotic roles within varying cell types. Previous research with non-TCR transgenic mouse models suggested that Gadd45β expression in T cells was responsible for regulating p38 MAPK activation, production of inflammatory cytokines and autoimmunity (147, 148). Therefore, when we examined Gadd45β deficiency on the wildtype B6 and TCR transgenic HY^{cd4} backgrounds we sought to determine its contribution to clonal deletion of T cells. However, our evidence suggested that Gadd45β does not regulate cell death of developing thymocytes within the B6 wildtype and TCR transgenic HY^{cd4} models.

The contribution of Gadd45β to the clonal deletion pathway was initially considered following microarray analysis of DP thymocytes undergoing clonal deletion within HY^{cd4} male mice (128). Gadd45β was among many other genes up regulated during clonal deletion, therefore it was considered to have a crucial role in negative selection. Evidence from peripheral CD4+ T cells showed that Gadd45β deficiency directly caused reduced activation of the MAPK p38 (147). Furthermore, Gadd45β and Gadd45γ deficient mice spontaneously develop autoimmune lymphoproliferative disorders and systemic lupus erythematosus

(148). Given the role of the MAPKs p38 and JNK in clonal deletion (79, 80) and autoimmunity induction in Gadd45β deficient mice, we considered that Gadd45β was a possible target protein involved in the molecular events of negative selection.

However, our present data showed that Gadd45β deficiency within the B6 and HY^{cd4} backgrounds had no observable impact on the frequency or absolute number on the majority of T cell populations, except thymic NK-T cells. Furthermore, expression of high affinity antigen encounter markers CD69, PD-1 and the apoptotic marker active caspase 3 were similar in HY^{cd4} wildtype and Gadd45β knock-out male mice, suggesting Gadd45β had no impact on negative selection. In consideration of the similarities in clonal deletion despite Gadd45β deficiency, and its regulatory role on p38 activation, we considered that Gadd45β might be expressed too late to regulate p38 activity. This may be likely given the evidence of Gadd45β directly associating with the MAPK kinase family member directly upstream of p38, MTK1 (MEKK4) (182). Unfortunately, we were not able to assess Gadd45β deficiency on p38 activation within HY^{cd4} male mice by flow cytometry and therefore unable to demonstrate that Gadd45β regulates p38 in negatively selected thymocytes. Therefore it remains possible that Gadd45β plays differential roles in T cells from the thymus and the periphery.

Why was clonal deletion not altered in HY^{cd4} Gadd45β knock-out male mice?

The contribution of Bim, the dominant regulator of clonal deletion

Understanding the role of Gadd45β to negative selection may have been difficult to monitor if its contribution to clonal deletion was minimal compared to other proteins. The BH-3 only protein Bim is the dominant regulator of clonal deletion mediated by apoptosis, therefore the impact of Gadd45β deficiency may be masked by Bim activity (183). Therefore we speculate that Gadd45β may play a subtle role in clonal deletion or may function in an alternative, non-dominant,

Bim-independent pathway that was not apparent in our experimental analysis due to Bim expression.

Redundancy of Gadd45β by the other Gadd45 isoforms, Gadd45α and Gadd45γ

Evidence within the literature suggests that Gadd45β has an important role in the stress response and apoptosis induction within T cells. Unfortunately, our present data showed minimal differences in T cell development in the absence of Gadd45β, but it does not definitely exclude Gadd45β from having a role in clonal deletion. The Gadd45 protein family consists of three proteins, one of which is Gadd45β. The other two, Gadd45α and Gadd45γ, have high sequence homology with Gadd45β and are highly conserved evolutionarily (184). We found that HY^{cd4} Gadd45β knock-out T cells expressed the other Gadd45 family members, Gadd45α and Gadd45γ, by quantitative PCR. Therefore, it is possible that Gadd45β deficiency had no observable difference on clonal deletion within the HY^{cd4} model system due to redundancy by Gadd45α and Gadd45γ; and evidence within the literature may support this claim. It is known that Gadd45α knock-out T cells had spontaneously increased p38 activation, causing a hyperproliferative T cell response and development of a lupus-like autoimmune condition (185). Similarly, mice deficient for both Gadd45β and Gadd45γ spontaneously developed a lymphoproliferative disorder and systemic lupus erythematosus (148). Together, this evidence suggests that the activity of the Gadd45 isoforms α and γ alter T cell tolerance, and their roles may be similar to Gadd45β and possibly redundant. Therefore, the impact of Gadd45β deficiency on thymocyte selection within the HY^{cd4} mouse model may have been masked by expression and activity of the other isoforms, Gadd45α and Gadd45γ.

A model of the role of Gadd45β in clonal deletion of T3.70+ DP thymocytes in HY^{cd4} male mice

The Gadd45β protein has been shown to have differential functions within

varying cell types, and our present data has shown minimal impact of Gadd45β to clonal deletion of T cells. However, the prospect that redundancy may be masking the role of Gadd45β to negative selection is intriguing. I have developed a proposed model of the contribution of Gadd45β to clonal deletion of T cells and show how we believe redundancy may have a factor in Gadd45β mediated negative selection (**Fig. 5-4 A**). Within the thymus of HY^{cd4} adult male mice, T3.70+ DP thymocytes encounter high affinity antigen, sequestering various proteins to the TCR synapse that amplify TCR downstream signaling. The dominant pathway of apoptosis-mediated clonal deletion is regulated by Bim and may mask the impact of Gadd45β on clonal deletion. Gadd45β may contribute to an alternative, secondary pathway of apoptosis-mediated clonal deletion that is yet unknown. Furthermore, the impact of Gadd45 deficiency may be due to the other Gadd45 protein isoforms Gadd45α and Gadd45γ, which may have roles in clonal deletion.

In consideration of future experiments, assessment of T cell selection in Gadd45β knock-out animals supplemented with RNAi knock-down of the other Gadd45 isoforms would be of interest to fully appreciate the contribution of the Gadd45 proteins to negative selection.

The origin of T3.70+ T cells within the periphery of HY^{cd4} male mice and the contribution of Gadd45β to clonal deletion

Analysis of the HY^{cd4} neonatal mice and HY^{cd4} Gadd45β deficient mice within this thesis project has contributed to our understanding of clonal deletion and the function of T cells that escape that process. Although some of the data presented was inconclusive, both projects have raised more questions and intrigue about the thymic development and T cell escape from negative selection. Furthermore, the methods of peripheral tolerance regulating the antigen specific T3.70+ T cells within HY^{cd4} neonatal and adult male mice are still unclear. Whether or not these processes are dependent on PD-1 and Gadd45β remains to be determined but the

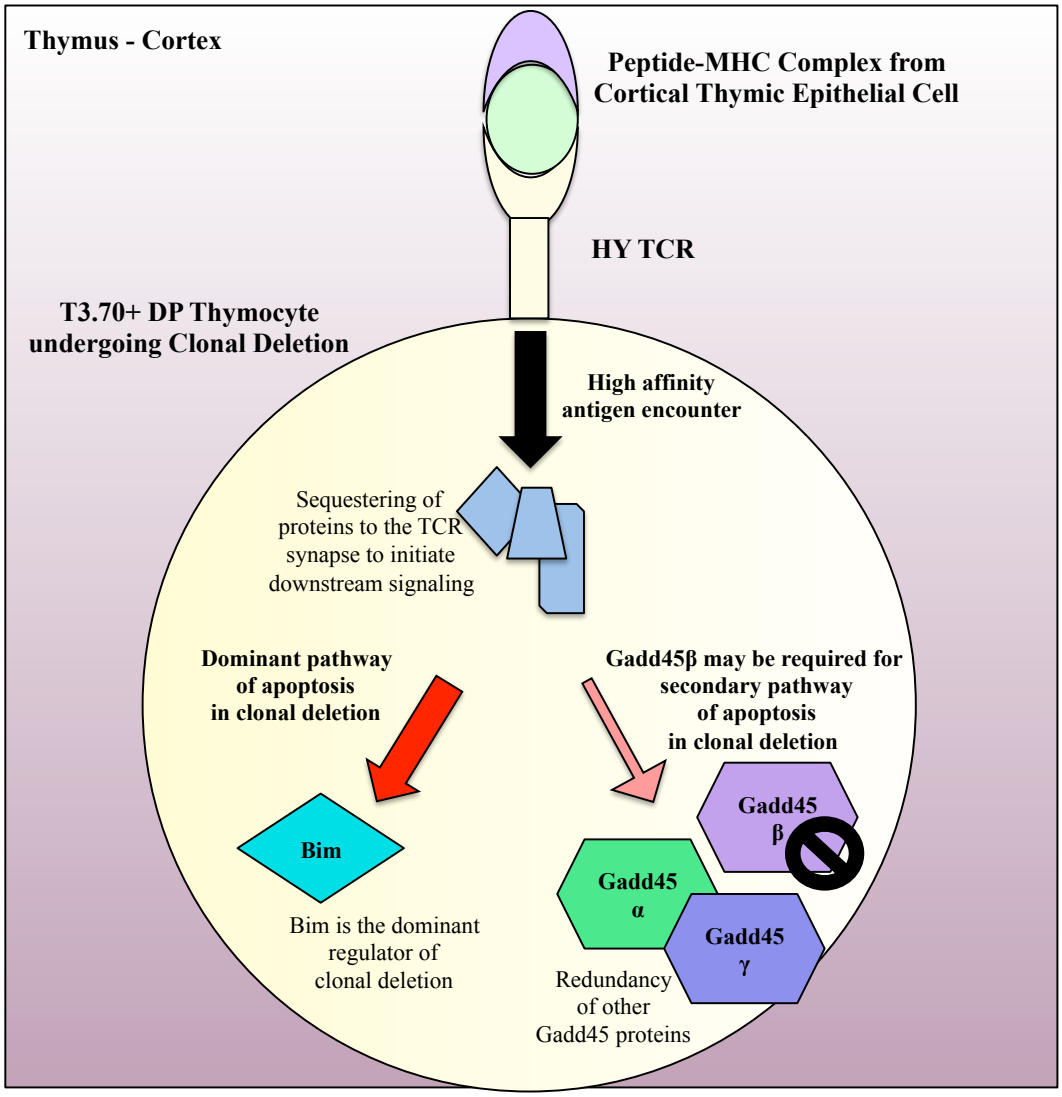


Fig. 5-4. A proposed model of clonal deletion of T3.70+ DP thymocytes from HY^{cd4} adult male mice and the contribution of the protein Gadd45β. **A.** A detailed model of HY^{cd4} adult male DP thymocytes undergoing clonal deletion following antigen encounter within the thymus and the possible contribution of Gadd45β to this process. The model demonstrates that Bim is the dominant regulator of clonal deletion and we speculate that Gadd45β may have a role in an alternative secondary pathway of clonal deletion. Furthermore, we propose that clonal deletion appeared to be intact in Gadd45β deficient mice due to redundancy of the other Gadd45 proteins, Gadd45α and Gadd45γ.

presented evidence does provide some consideration to the contribution of both PD-1 and Gadd45β to T cell development and regulation. At some point, future experimental analysis may shed more light on their roles in the molecular processes mediating central and peripheral tolerance.

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Chapter 7: Appendix

Coreceptor Tuning in the HY^{cd4} Mouse Model

Assessing Coreceptor Tuning as a Peripheral Tolerance Mechanism in HY^{cd4} Mice

In the HY^{cd4} mouse model of negative selection, HY^{cd4} male mice lack a CD8SP thymocyte compartment. However, flow cytometric analysis of the peripheral secondary lymphoid organs shows a substantial population of antigen-specific T cells composed of CD8+ T cells. Therefore, these cells must escape negative selection in some fashion. Phenotypic analysis of this population also shows down regulation of the CD8 coreceptor. As demonstrated in Chapter 3, HY^{cd4} male neonatal mice express B6 wildtype levels of CD8 coreceptor on CD8+ T cells at Day 3 post-birth, which gradually reduces to levels similar to HY^{cd4} adult male CD8+ T cells by Day 11. Since CD8+ T cells *in vivo* actively reduce CD8 coreceptor levels, it was considered that this might serve as a mechanism of peripheral tolerance for these antigen-specific T cells.

Coreceptor tuning is not the only tolerance mechanism in HY^{cd4} male mice

The Singer group has proposed that IL-7R mediated signaling regulates CD8 coreceptor expression at the transcriptional level thereby modulating T cell receptor sensitivity by altering cell surface expression of CD8 coreceptor. They have called this peripheral tolerance mechanism 'coreceptor tuning.' Upon antigen encounter within the periphery, downstream signaling through the TCR inhibits IL-7R signaling, and therefore reduces CD8 coreceptor levels (**Fig. 7-1 A**) (186). Therefore, T cells in female mice that are HY-responsive never encounter high affinity antigen, therefore the absence of TCR-signaling results in continual expression of CD8 coreceptor levels by an IL-7R mediated mechanism. In contrast, HY-responsive male T cells do encounter their cognate antigen within

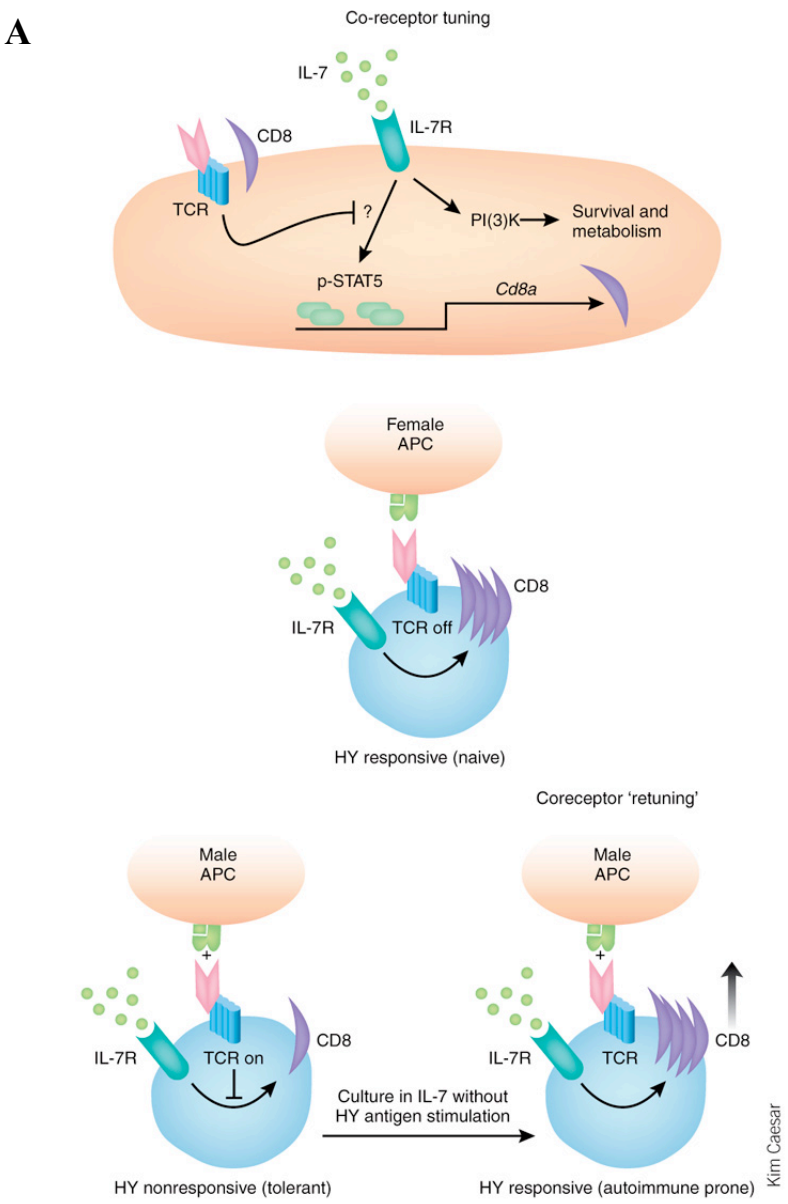

Fig. 7-1. Model of the peripheral tolerance mechanism, coreceptor tuning. **A.** A detailed model and outline of the phenomenon of coreceptor tuning, a proposed peripheral tolerance mechanism, involving the modulation of TCR sensitivity by reducing CD8 coreceptor levels. IL-7R downstream signaling directly targets the transcription of *Cd8α*. Within HY female mice that lack the cognate HY antigen, TCR signaling is off and IL-7R downstream signaling promotes the upregulation of CD8 coreceptor. Alternatively in HY male mice that do express the cognate HY male antigen, TCR downstream signaling is on. The downstream cascade inhibits IL-7R signaling, causing reduced CD8 coreceptor levels. However once cultured *in vitro*, these cells upregulate CD8 coreceptor once again. From Nature Immunology (2007) **8**: 1027-1028, Fig 1.

the periphery. Therefore, downstream TCR signaling inhibits the IL-7R signaling pathway causing reduced CD8 coreceptor levels. As the Singer group demonstrated, upon culture of these T cells *in vitro* with IL-7 in the absence of cognate antigen, CD8 coreceptor levels increase and are presumably autoimmune prone (**Fig. 7-1 A**).

In order to determine if coreceptor tuning was a process occurring within the HY^{cd4} male mouse model, we developed an experimental assay involving *in vitro* culture and stimulation with cognate antigen outlined below (**Fig. 7-2 A**). Pooled T cells were cultured *in vitro* in medium alone, followed by supplementation with IL-7 or not at 24 hours. Cells were cultured for 24, 48 or 72 hours total and then stimulated with cognate antigen *in vitro* for 18 hours. Upon removal of the HY^{cd4} male T cells from the *in vivo* environment and culture *in vitro* with IL-7, CD8 coreceptor levels began to shift upward at 48 and 72 hours compared to the directly *ex vivo* HY^{cd4} male control (**Fig. 7-3 A**). The increase in CD8 levels appeared to be a time-dependent process, as there was no difference observed in the presence or absence of IL-7 at 24 hours compared to the control. This delay can be accounted for within the coreceptor tuning model since IL-7R signaling transcriptionally regulates CD8 expression, therefore it would take time for CD8 coreceptor levels to increase. Based on the modulation of CD8 coreceptor levels *in vitro*, it appeared that once removed from high affinity antigen the HY^{cd4} male mice T cells were responsive to IL-7.

In addition to assessing CD8 coreceptor levels, surface expression of IL-7R (CD127) and IL-2Rβ (CD122) was examined to ensure that the CD8+ T cells were responsive to IL-7 and if surface expression levels of another common γ-chain cytokine receptor, IL-2R, changed. Following *in vitro* culture for 72 hours, cells cultured with IL-7 exhibited CD127 down regulation, as expected. In contrast, cells not treated with IL-7 expressed similar levels of CD127 to the *ex vivo* control. Furthermore, cells cultured in the absence of IL-7 exhibited reduced CD122 surface levels whereas those cultured with IL-7 expressed *ex vivo* levels of CD122 (**Fig. 7-3 B**). This reduction in CD122 levels could be due to

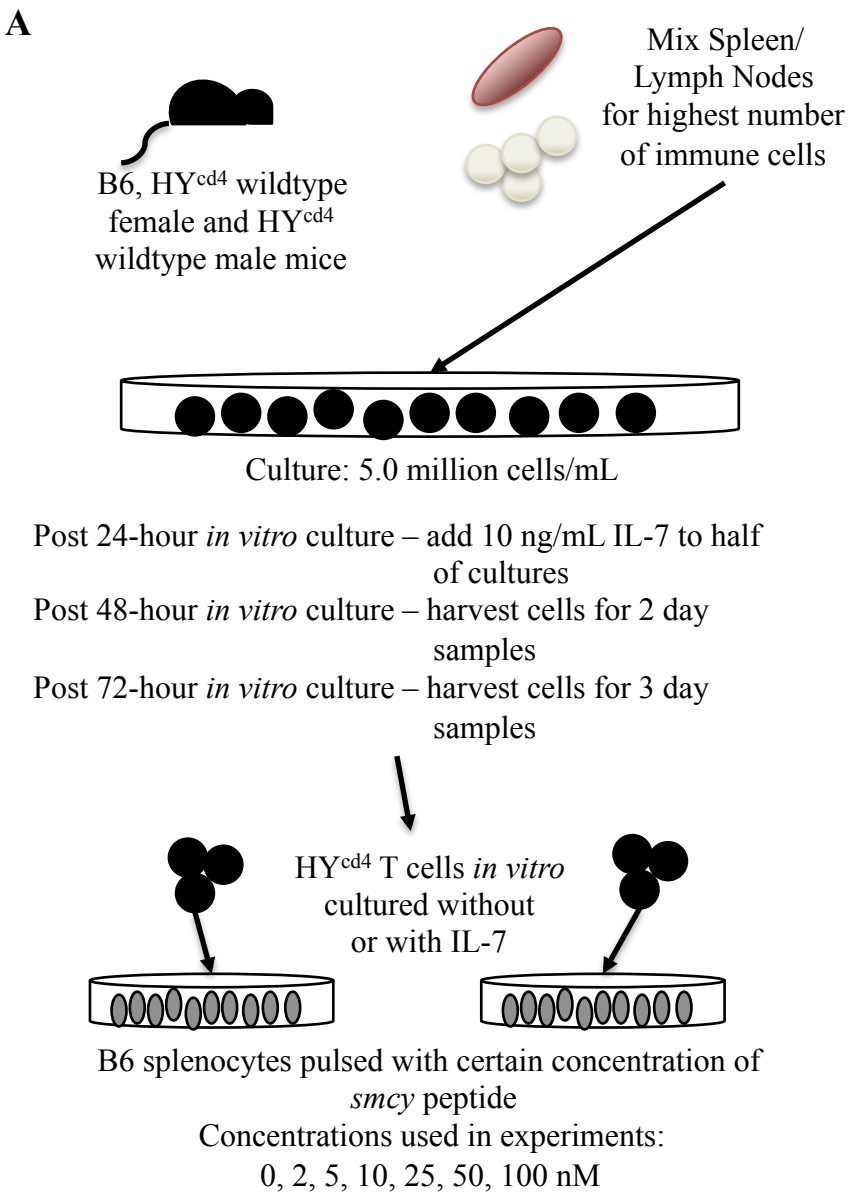

Fig. 7-2. A detailed outline of the experimental assay to evaluate the peripheral tolerance mechanism, coreceptor tuning, within the HY^{cd4} mouse model by *in vitro* culture with 10 ng/mL IL-7 in the absence of antigen stimulation. **A.** Detailed outline of the experimental assay examining coreceptor tuning within B6 and HY^{cd4} mice. The spleen and lymph node cell suspensions from B6, HY^{cd4} female and HY^{cd4} male mice were mixed and cultured at 5 x 106 cells/ml. After 24 hours of *in vitro* culture, 10 ng/mL of IL-7 was added to half of the cultures. Cells were harvested for the time points desired. Cells were then removed for flow cytometry or further *in vitro* stimulation with B6 splenocytes pulsed with the desired concentrations of *smcy* peptide.

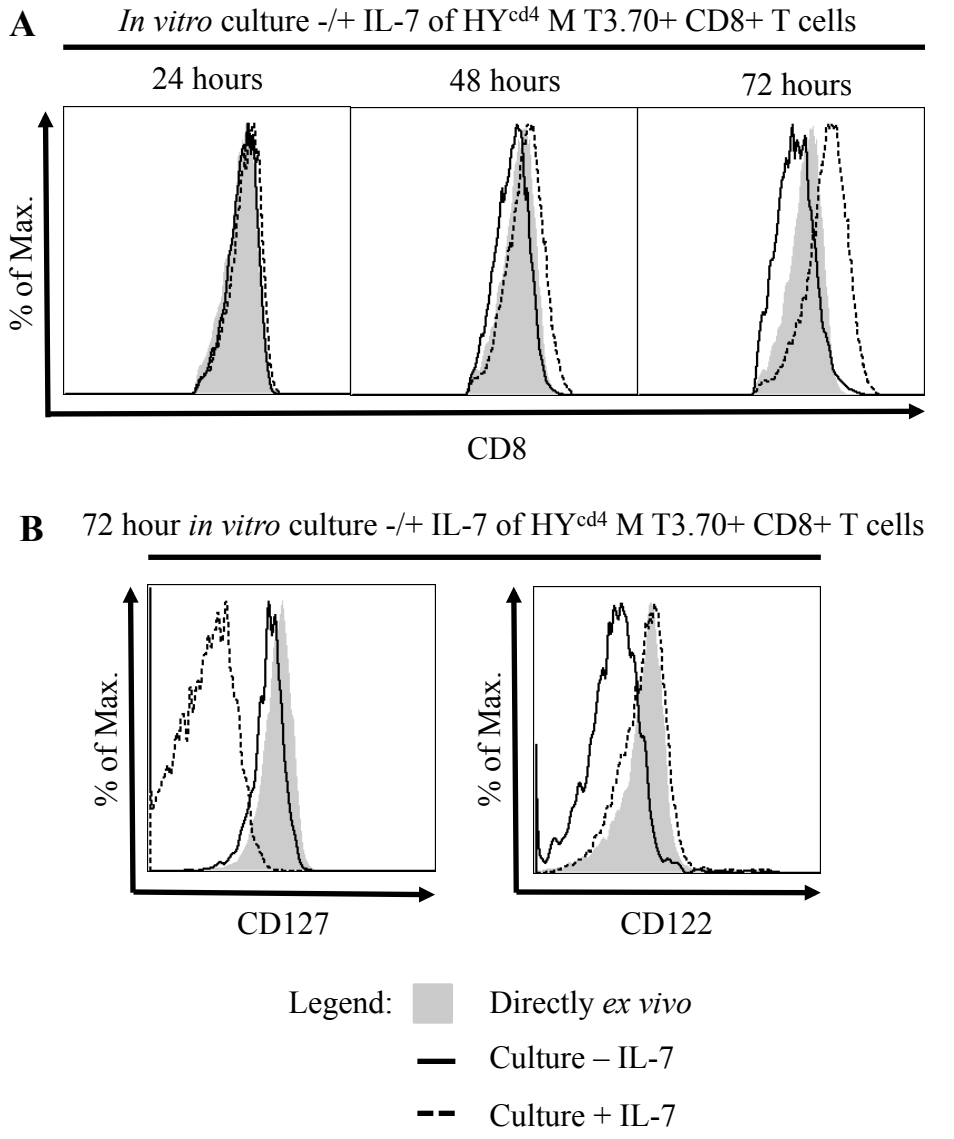

Fig. 7-3. *In vitro* culture with 10 ng/mL of IL-7 promoted the internalization of CD127 (IL-7Rα) and the up regulation of CD8 coreceptor on antigen-specific T3.70+ CD8+ T cells from HY^{cd4} male mice. **A.** Histogram overlays of CD8 coreceptor expression on T3.70+ CD8+ T cells from HY^{cd4} male mice cultured *in vitro* with or without 10 ng/mL IL-7 for 24, 48 or 72 hours. Representative of 24 hour culture: n = 2; 48 hour culture n = 4; 72 hour culture n = 6. **B.** Histogram overlays of CD127 (IL-7Rα) and CD122 (IL-2Rβ) on T3.70+ CD8+ T cells from HY^{cd4} male mice cultured *in vitro* for 72 hours with or without 10 ng/mL IL-7. Representative of n = 2.

consumption of IL-2 as those CD8+ T cells were cultured without IL-7, and both IL-2 and IL-7 cytokines are known to promote T cell survival (187).

Following *in vitro* culture with IL-7, HY^{cd4} male CD8+ T cells had increased levels of CD8 coreceptor expression. The model of coreceptor tuning suggests that T cells with increased CD8 expression gain TCR sensitivity; therefore we wanted to examine TCR sensitivity with *in vitro* stimulation B6 splenocytes pulsed with *smcy* peptide. TCR stimulation and downstream signaling was assessed by CD69 and PD-1 surface expression by flow cytometry. HY^{cd4} male T cells cultured in the absence or presence of IL-7 for 48 hours showed similar expression levels of CD69 when stimulated with 100 nM of *smcy* peptide. At 72 hours, however, T3.70+ CD8+ T cells previously cultured for 72 hours had slightly higher levels of CD69 compared to those not cultured with IL-7 (**Fig. 7-4 A**). This suggested that T3.70+ CD8+ T cells gained more sensitivity to TCR stimulation when cultured with IL-7 for 72 hours. Therefore it is possible that upon removal of the T3.70+ CD8+ T cells from the *in vivo* environment of HY^{cd4} male mice, TCR sensitivity has been increased to cognate antigen stimulation. Analysis of multiple replicates showed a gradual increase in the frequency of CD69 expressing CD8+ T cells following stimulation with varying concentrations of *smcy* peptide. However, the curve was fairly tight between T3.70+ T cells previously cultured in the presence or absence of IL-7 (**Fig. 7-4 B**). This suggested that the downstream signaling through IL-7R may not be responsible for the increase in TCR sensitivity and may be simply due to reducing amounts of peptide-MHC (pMHC) interactions.

The surface expression of the inhibitory receptor PD-1 was also assessed on T3.70+ T cells following *in vitro* stimulation with *smcy* peptide. The surface expression of PD-1 was drastically higher in cells stimulated with 100 nM *smcy* peptide compared to the directly *ex vivo* control, as expected. However, there was no difference in PD-1 levels on T3.70+ CD8+ T cells that had been previously cultured in the presence or absence of IL-7 for 48 hours. The same observation was made with CD8+ T cells that had been cultured for 72 hours (**Fig. 7-5 A**).

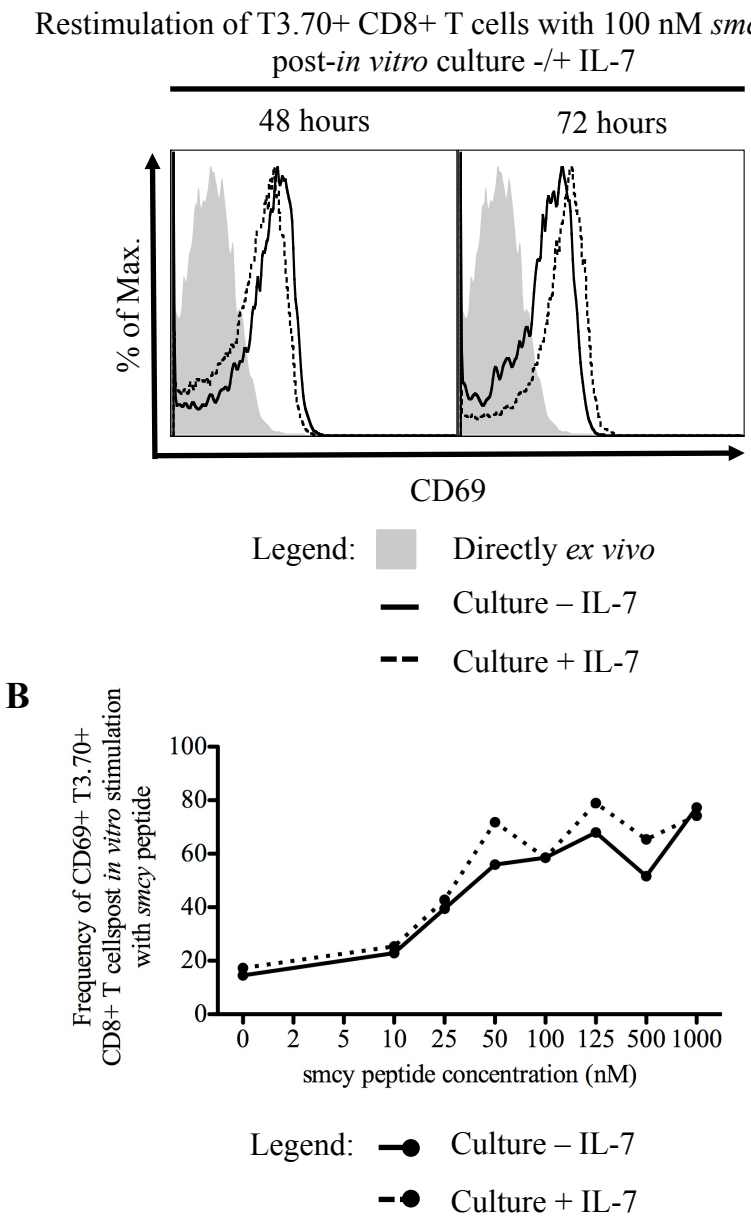

Fig. 7-4. Following *in vitro* restimulation with *smcy* peptide, cultured T3.70+ CD8+ T cells responded to similar extents based on CD69 surface expression despite the presence or absence of 10 ng/mL of IL-7. **A.** Representative flow cytometric plots of CD69 surface expression of T3.70+ CD8+ T cells, previously cultured for 48 or 72 hours with or without IL-7, stimulated with 100 nM of *smcy* peptide. Representative of 100 nM n = 3. **B.** Graphical analysis of the frequency of CD69+ T3.70+ CD8+ T cells, previously cultured with or without IL-7, following *in vitro* stimulation with varying concentrations of *smcy* peptide. Representative of 0 nM n = 7; 10 nM n = 5; 25 nM n = 3; 50 nM n = 4; 100 nM n = 3; 125 nM n = 2; 500 nM n = 2; 1000 nM n = 4.

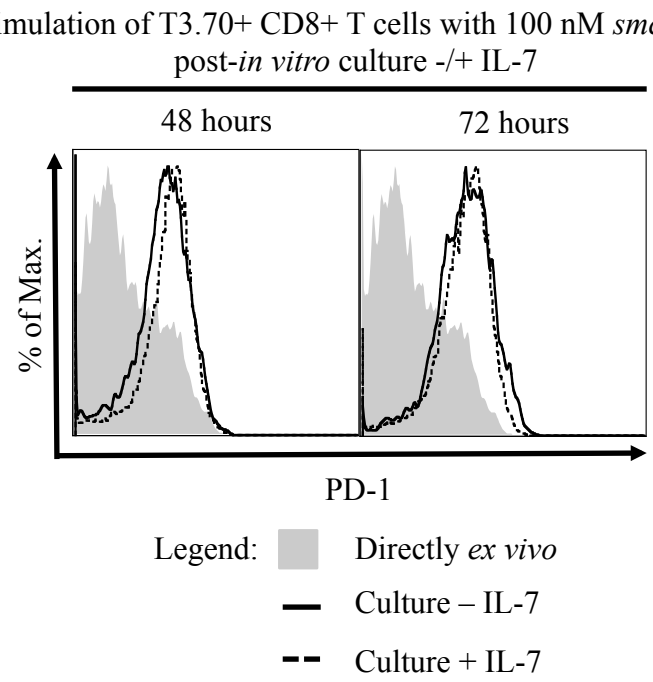

Fig. 7-5. Following *in vitro* restimulation with *smcy* peptide, cultured T3.70+ CD8+ T cells expressed similar levels of the inhibitory receptor PD-1 regardless of culture with or without 10 ng/mL of IL-7. **A.** Representative flow cytometric plots of PD-1 surface expression of T3.70+ CD8+ T cells, previously cultured for 48 or 72 hours with or without IL-7, stimulated with 100 nM of *smcy* peptide. Representative of 100 nM n = 3.

This data suggested that PD-1 surface expression levels were not influenced by increased TCR sensitivity. Furthermore, it appeared that the increase in TCR sensitivity was independent of an IL-7R driven mechanism, based on the lack of differences between CD8+ T cells cultured with or without IL-7.

Based on the *in vitro* assays examining coreceptor tuning in the HY^{cd4} mouse model, T3.70+ CD8+ T cells from HY^{cd4} male mice increased CD8 coreceptor levels upon removal from the *in vivo* environment and culture with IL-7 (**Fig. 7-3 A**). As suggested by the coreceptor tuning model, this appeared to be an IL-7R signaling dependent process. However when the antigen-specific T3.70+ CD8+ T cells were assayed for increased TCR sensitivity following IL-7 *in vitro* culture, there were minimal differences in both CD69 and PD-1 surface expression on cells cultured with or without IL-7. This evidences suggested that the TCR sensitivity threshold change was not due to the IL-7R mediated mechanism of coreceptor tuning. While the modulation of CD8 coreceptor by IL-7R signaling appeared to be intact within the HY^{cd4} mouse model, coreceptor tuning may not be the sole peripheral tolerance mechanism that regulates the antigen specific T3.70+ CD8+ T cells within HY^{cd4} male mice.