Contributions of Bim and Nur77 to multiple mechanisms of T cell tolerance following high affinity antigen encounter

by

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ABSTRACT

Prevention of autoimmune disease requires the elimination or inactivation of T cells that are highly reactive to self-peptides. During T cell development in the thymus, self-reactive thymocytes undergo negative selection upon receiving a high affinity T cell receptor (TCR) signal following encounter with self-antigens. Negative selection can occur in the cortex against ubiquitous antigens (UbA) and in the medulla against tissue-restricted antigens (TRA). While negative selection encompasses several mechanisms, most self-reactive thymocytes are removed from the repertoire by clonal deletion. Bim and Nur77 are two proteins upregulated during negative selection that are thought to be key mediators of clonal deletion due to their established pro-apoptotic functions. We therefore sought to characterize the contributions of Bim and Nur77 to UbA and TRA-mediated clonal deletion *in vivo* using the well established HY^{cd4} and OT-I Rip-mOva TCR transgenic mouse models, respectively. Despite being a major mediator of thymocyte apoptosis, we found that Bim is ultimately not required for UbA-mediated clonal deletion in the HY^{cd4} model. Nur77 was also dispensable for UbA-mediated deletion and could not compensate for Bim deficiency in inducing caspase-3 activation. In fact, our data suggests that the apoptotic function of Nur77 may be inhibited in the presence of TCR signalling. However, deficiency in both Bim and Nur77 tended to result in a greater number of antigen-specific thymocytes, which appeared to be due to thymocyte-intrinsic and extrinsic factors. In contrast to UbA-mediated clonal deletion, Nur77 deficiency was sufficient to modestly impair TRA-mediated deletion in the OT-I Rip-mOva model, and Bim deficiency completely abrogated deletion. Furthermore, our data suggests a novel role for Nur77 in anergy induction in this model. These results reveal differences in the molecular mechanisms of negative selection

against UbA vs. TRA. In addition, we examined negative selection in a diverse T cell repertoire by examining Bim and Nur77 deficiency in unmanipulated mice. In polyclonal mice lacking Bim and Nur77, we found an accumulation of thymocytes that had experienced high affinity TCR signalling, suggesting that clonal deletion was impaired. This was accompanied by increases in regulatory T cells and anergic phenotype T cells, two alternative fates that can occur upon high affinity antigen encounter. Nevertheless, polyclonal $Bim^{-/-}Nur77^{-/-}$ mice tended to develop a phenotype suggestive of excessive inflammation, whereas this was never observed in Bim or Nur77 single knockout mice. Similarly, only OT-I $Bim^{-/-}Nur77^{-/-}$ Rip-mOva chimeras developed autoimmune diabetes. These results indicate that deficiency in two key molecules involved in negative selection greatly increases the risk of autoimmune disease. Collectively, these studies shed light on the multiple mechanisms that work in concert to maintain self-tolerance.

PREFACE

This thesis is an original work by Qian Hu. It contains content co-authored by collaborators and republished with permission:

- Hu, Q., A. Sader, J. C. Parkman, and T. A. Baldwin. 2009. Bim-mediated apoptosis is not necessary for thymic negative selection to ubiquitous self-antigens. *J Immunol* 183: 7761-7767. ***Copyright 2009. The American Association of Immunologists, Inc.***
- Hu, Q. and T. A. Baldwin. 2015. Differential roles for Bim and Nur77 in thymocyte clonal deletion induced by ubiquitous self-antigen. *J Immunol* (in press). ***Copyright 2015. The American Association of Immunologists, Inc.***

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "UNDERSTANDING THE CELLULAR AND MOLECULAR MECHANISMS UNDERLYING NEGATIVE SELECTION IN THE THYMUS", Protocol No.220, 2014.

DEDICATION

This thesis is dedicated to my father, Jie Hu, who has always encouraged curiosity and a love of learning, and has imparted to me the importance of education.

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LIST OF RECURRING ABBREVIATIONS

Aire – autoimmune regulator

APC – antigen presenting cell

AV – annexin V

B6 – C57BL/6 wildtype mice

BH3 – Bcl-2 homology domain 3

Bim – Bcl-2-interacting mediator of death

BKO – $Bim^{-/-}$

CICD – caspase-independent cell death

CFSE – carboxyfluorescein diacetate succinimidyl ester

CLP – common lymphoid progenitor

cTEC – cortical thymic epithelial cell

DC – dendritic cell

DKO – double knockout ($Bim^{-/-}Nur77^{-/-}$)

DN – double negative ($CD4^{-}CD8^{-}$)

DP – double positive ($CD4^{+}CD8^{+}$)

Egr – early growth response (family includes Egr2 and Egr3)

ERK – extracellular signal-regulated kinase

ETP – early thymic progenitor

F – female

Foxp3 – forkhead box p3

FR4 – folate receptor 4

Het/Het – refers to $Bim^{+/-}Nur77^{+/-}$

HSC – hematopoietic stem cell

HY – male antigen

IEL – intraepithelial lymphocyte

IFNγ – interferon γ

IL – interleukin

ITAM – immunoreceptor tyrosine-based activation motif

JNK – Jun N-terminal kinase

LAT – linker for the activation of T cells

LMPP – lymphoid multipotent progenitor

LSK – $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{hi}$

M – male

MAPK – mitogen-activated protein kinase

MFI – mean fluorescence intensity

MHC – major histocompatibility complex

mTEC – medullary thymic epithelial cell

NK – natural killer

NKO – $Nur77^{-/-}$

NR4A – nuclear receptor subfamily 4 group A (family includes Nur77, Nor-1, Nurr1)

Nur77-FL – full-length Nur77 transgenic mice

Nur77-GFP – mice expressing green fluorescent protein placed under control of Nur77 promoter

Ova – chicken ovalbumin

PD-1 – programmed death-1

pMHC – peptide-MHC

RAG – recombination-activating gene

Rip-mOva – mice with transgenic rat insulin promoter driving membrane-bound Ova expression

SSC – side scatter

Smcy – gene from which the male HY antigen is derived; also used to refer to the peptide recognized by the HY TCR

SP – single positive ($CD4^-CD8^+$ CD8SP or $CD4^+CD8^-$ CD4SP)

Tfh – T follicular helper cell

TMRE – tetramethylrhodamine ethyl ester

Treg – T regulatory cell ($Foxp3^+$ $CD4^+$)

TNFα – tumour necrosis factor α

TCR – T cell receptor

TRA – tissue-restricted antigen

UbA – ubiquitous antigen

WT – wildtype

Z-VAD-FMK – pan-caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone

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CHAPTER 1: INTRODUCTION

T cells are an integral part of the immune system

The mammalian immune system is a complex network of many cell types and proteins that collectively coordinate a protective response against foreign entities. In addition to epithelial and mucosal barriers to the outside environment, the immune system proper has traditionally been divided into two categories: innate and adaptive immunity. Adaptive immunity, primarily mediated by T and B cells, first arose in jawed vertebrates and has several features distinct from the more ancient innate immune system (1). While innate immune responses are rapid due to the non-specific recognition of conserved pathogen associated molecular patterns, the adaptive immune response is delayed due to requisite expansion of T and B cells that recognize highly specific antigen epitopes. After a primary immune response, a small fraction of activated T and B lymphocytes survive as memory cells, which respond in a more rapid and robust manner to subsequent encounters with the same antigen. Coordination of the innate and adaptive immune responses is key to providing efficient and complete protection. Natural killer (NK) cells, which derive from lymphoid progenitors yet have innate immune functions, have been considered an evolutionary bridge between innate and adaptive immunity. We now know that natural killer cells are one of several types of innate lymphoid cells (2).

Our highly evolved immune system offers formidable protection against pathogens and cancers, with T cells playing varied and critical roles in host defense. The T cell compartment is composed of many different subsets, including but not restricted to $CD8^+$ cytotoxic T cells, $CD4^+$ T helper cells – divided further into Th1, Th2, Th17, and T follicular helper (Tfh) lineages, and

$CD4^+$ T regulatory cells (Treg). The majority of T cells express a T cell receptor (TCR) composed of an α and β glycoprotein chain, while a minority express a γδ pair instead. Conventional $\alpha\beta TCR^+$ T cells recognize peptide antigens presented on major histocompatibility complex molecules (MHC). The CD8 and CD4 co-receptors facilitate the interaction between the TCR and the peptide-MHC (pMHC) complex, with $CD8^+$ T cells being MHC class I-restricted and $CD4^+$ T cells being MHC class II-restricted.

Diversity in the TCR repertoire

Because each TCR recognizes a pMHC complex, the diversity of the TCR repertoire is necessarily vast in order to account for the myriad of antigens encountered in a lifetime. TCR diversity is achieved through recombination of genetic segments, nucleotide additions and deletions, and pairing of different α and β chains to form the complete receptor. However, of a theoretical 10^{15} different combinations, a survey of mouse splenocytes indicates that the actual mature T cell repertoire contains as few as 2×10^6 different clones (3). Though the random nature of TCR generation is critical for pathogen protection, it inevitably generates TCRs that cannot interact with self-MHC as well as TCRs specific for self-antigens. In order to establish a healthy immune system, the T cell repertoire must be shaped to select for useful clones while eliminating those that have the potential to mediate autoimmunity. This is achieved through positive and negative selection, respectively, during T cell development in the thymus. Thymic selection and later competition in the periphery are among the factors that reduce the size of the TCR repertoire.

Early stages of T cell development

All cells of the blood ultimately derive from hematopoietic stem cells (HSCs) found in fetal liver and adult bone marrow. HSCs are lineage negative (Lin^-), stem cell antigen-1 positive ($Sca\text{-}1^+$), and have high expression of tyrosine kinase receptor c-Kit– collectively abbreviated LSK (**Fig. 1-1**). Additionally, they lack expression of the fms-related tyrosine kinase 3 receptor (Flt3). Development from HSCs into terminal lineages involves a series of differentiation steps with successive loss of potential to develop into other lineages. One model posits that the first lineage commitment step results in the separation of erythrocyte-megakaryocyte progenitors and myeloid-lymphoid progenitors. The latter are termed lymphoid multipotent progenitors (LMPPs) and are characterized by acquisition of high Flt3 expression. Following this model, LMPPs give rise to granulocyte-macrophage progenitors and common lymphoid progenitors (CLPs). CLPs can be detected in blood and have been proposed to be the initial bone marrow-derived progenitor (4). However, due to the extremely low numbers of thymus-seeding progenitors, the identity of the progenitors are uncertain, and there may be multiple progenitors capable of differentiating into T cells in the thymus. After the initial thymus-seeding cells enter the thymus, they become known as early thymic progenitors (ETPs). A recent study reported similarities between ETPs and LMPPs as well as CLPs (5), raising the possibility that LMPPs and/or CLPs can seed the thymus.

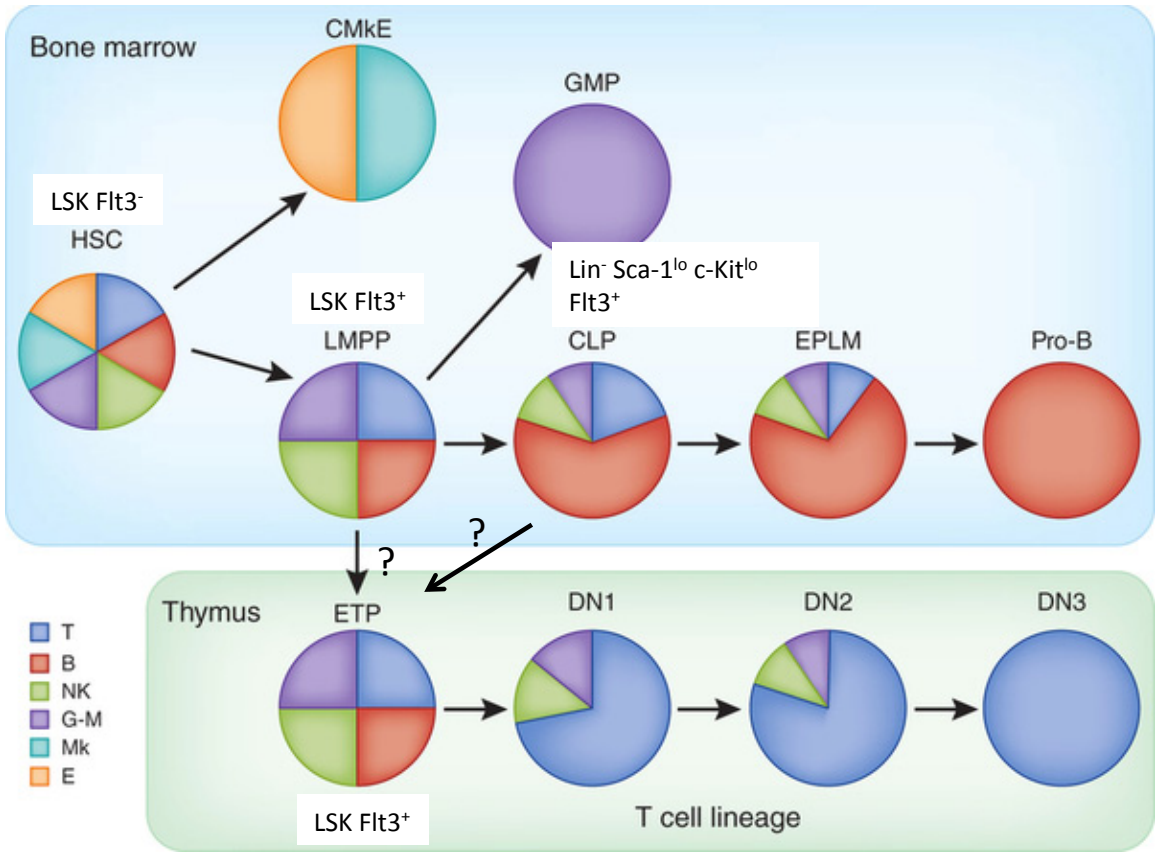


Figure 1-1. T lymphopoiesis from bone marrow-derived progenitors. Hematopoietic stem cells (HSCs) give rise to all blood cells via a series of differentiation steps with successive loss of potential to develop into other lineages. This model posits that the first divergence is between the erythrocyte-megakaryocyte lineages and the myeloid-lymphoid lineages. The latter progenitor – lymphoid multipotent progenitor (LMPP) – gives rise to granulocyte-macrophage progenitors and common lymphoid progenitors (CLPs). Early thymic progenitors (ETPs) share similarities with LMPPs and CLPs, suggesting that LMPPs and/or CLPs could be thymus-seeding progenitors. LSK, Lin$^-$Sca-1$^+$c-Kit^{hi}; Flt3, fms-related tyrosine kinase 3; CMkE, common megakaryocyte and erythroid progenitor; GMP, granulocyte-macrophage progenitor; EPLM, early progenitor with lymphoid and myeloid potential; Pro-B, committed pro-B cell; DN1–DN3, stages of CD4$^-$CD8$^-$ double negative thymocytes; NK, natural killer; G-M, granulocyte-macrophage; Mk, megakaryocyte; E, erythroid. Figure modified from Ceredig (2012) Nat Immunol 13: 309-310. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology 13: 309-310, copyright 2012.

The thymus is broadly divided into two compartments: the cortex and the medulla. Bone marrow-derived progenitors enter the thymus at the cortico-medullary junction. Signaling through the chemokine receptors CCR7 and CCR9 has been shown to be important for homing of progenitors to the thymus (6). Additionally, thymic entry is regulated by interaction between platelet (P)-selectin glycoprotein ligand 1 (PSGL1) on circulating progenitors and P-selectin on thymic endothelium (7). The earliest thymocytes lack expression of CD4 and CD8 coreceptors and are thus known as double negative (DN) thymocytes. As thymocytes progress through the DN1 to DN4 stages of development, distinguished by expression of CD44 and CD25, they move towards the outer subcapsular zone of the cortex (**Fig. 1-2**). DN thymocytes receive developmental cues through interactions with cortical thymic epithelial cells (cTECs). Signaling through Notch receptors on thymocytes delivered by Delta-like ligands on cTECs is critical for T cell lineage commitment (8, 9). In addition to Notch, interleukin-7 (IL-7) produced by cTECs is important for development of DN2 and DN3 thymocytes (10, 11). In DN2 and DN3 thymocytes, recombination-activating gene (RAG) 1 and 2 enzymes mediate rearrangement of the TCRβ, TCRγ, and TCRδ loci (12, 13). Thus, divergence of $\gamma\delta^+$ T cell and $\alpha\beta^+$ T cell fates occurs at the DN3 stage. On DN thymocytes, the TCRβ chain is paired with a pre-TCRα chain (14). The pre-TCR and mature TCR associate with a CD3 signaling complex consisting of a CD3γ/CD3ε heterodimer, CD3δ/CD3ε heterodimer, and CD3ζ homodimer (**Fig. 1-3**). Pre-TCR signaling is required for allelic exclusion at the TCRβ locus, rescue from apoptosis, proliferation, and further differentiation; hence, this critical checkpoint for functional TCRβ rearrangement is called β-selection (15).

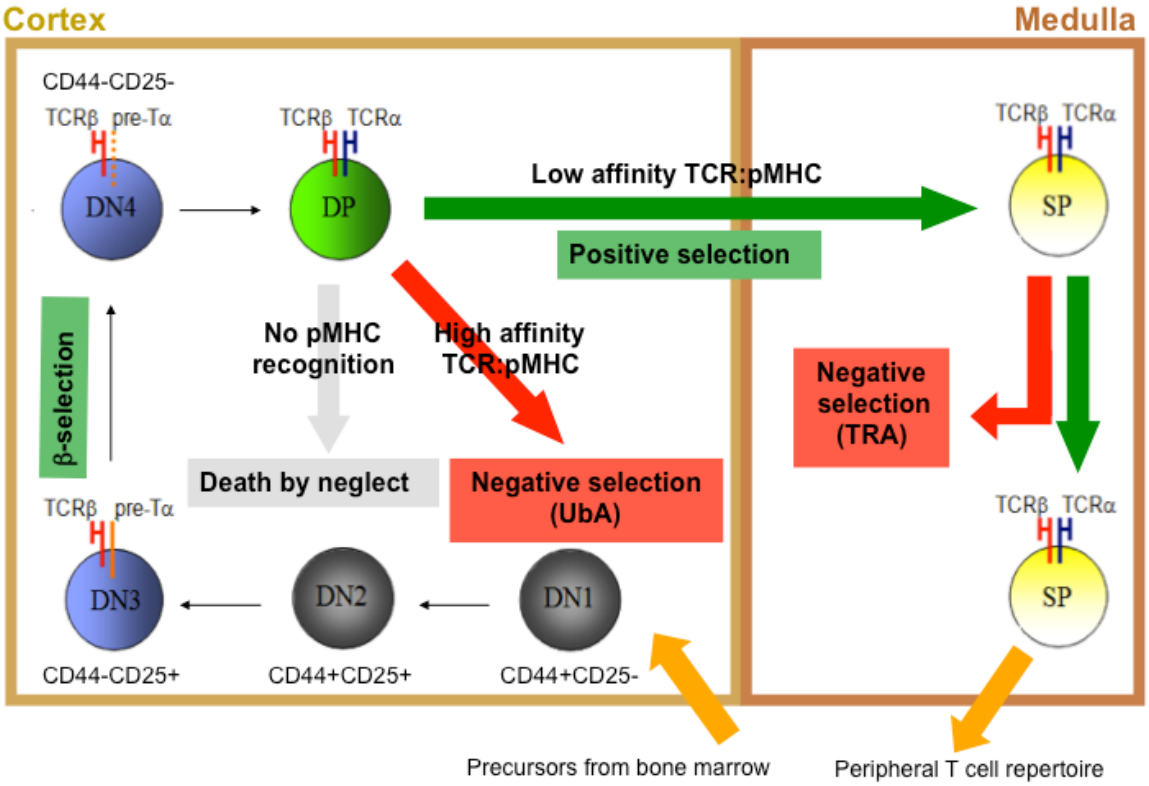


Figure 1-2. Development of αβT cells in the thymus. T cell progenitors enter the thymus at the cortico-medullary junction. The earliest $CD4^{-}CD8^{-}$ double negative (DN) thymocytes progress through four stages of development defined by differential expression of CD44 and CD25. Only thymocytes expressing a functional TCRβ chain survive the β-selection checkpoint at the DN3 to DN4 transition and are permitted to continue development into $CD4^{+}CD8^{+}$ double positive (DP) thymocytes. Depending on the affinity of its TCR for self-peptide-MHC (pMHC), DP thymocytes undergo death by neglect, negative selection, or positive selection. Positively selected thymocytes differentiate into $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ single positive (SP) cells and migrate to the medulla. While negative selection can occur in the cortex against ubiquitous antigens (UbA), the medulla is an exclusive site for negative selection against tissue-restricted antigens (TRA). The thymocytes that enter the peripheral T cell repertoire are mostly self-tolerant. Figure modified from Hu and Baldwin (2013) Programmed Cell Death in T Cell Development, ISBN: 978-953-51-1133-7, InTech, DOI: 10.5772/48607.

Following β-selection, thymocytes successively enter the DN4, immature CD8 single positive, and double positive (DP) stages. During this time, thymocytes reverse direction and traffic away from the subcapsular zone. Low-level rearrangement of the TCRα locus occurs in DN4 thymocytes but full-scale rearrangement does not occur until the DP stage (16). DP thymocytes only have a lifespan of 3-4 days (17) in which they must generate an αβTCR that can bind self-pMHC expressed by cTECs. Thymocytes that fail this self-MHC restriction checkpoint undergo death by neglect, maximizing the utility of the T cell repertoire. It was previously shown that up to 90% of DP thymocytes undergo death by neglect (18). Recent studies suggest that the frequency of DP thymocytes that experience TCR-induced death is higher than previously appreciated (19, 20), meaning that the frequency of thymocytes that undergo death by neglect is likely lower.

TCR signaling pathways in positive and negative selection

The fate of DP thymocytes is dependent on the affinity of its TCR for self-pMHC. Of the TCRs that successfully engage pMHC, low affinity interactions result in positive selection and high affinity interactions in negative selection. The polyclonal TCR repertoire in unmanipulated mice makes it difficult to study antigen-specific selection due to the enormous diversity of clones. By introducing a transgenic TCR specific for a known antigen, it becomes possible to investigate a low or high affinity response in a large population of antigen-specific thymocytes. The initial experiments demonstrating that TCR affinity for antigen determines positive vs. negative selection fates utilized the transgenic OT-I TCR specific for chicken ovalbumin peptide 257-264 (SIINFEKL, henceforth referred to as Ova) presented on MHC class I H-2K^{b}. By adding

peptides to OT-I fetal thymic organ cultures incapable of endogenous antigen presentation, it was found that variants of the antigenic Ova peptide were capable of inducing positive selection (21). Direct measurement of binding affinity between the OT-I TCR and various peptide/K^b complexes demonstrated a range of affinities that mediate positive selection, and that the threshold between positive and negative selection is narrow (22, 23). A recent study identified the protein Schnurri-2 as a regulator of positive vs. negative selection outcomes but the mechanism of its action remains undefined (24). In addition, a protein called Themis attenuates TCR signaling through recruitment and activation of phosphatases, enabling positive selection of thymocytes (25-27).

In order to understand how TCR affinity for different ligands translates into positive and negative selection outcomes, it is necessary to put into context the main molecules involved in TCR-proximal signaling (**Fig. 1-3**). Upon binding to pMHC, tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) in cytosolic domains of the CD3 chains are phosphorylated by lymphocyte-specific protein tyrosine kinase (Lck). One model proposes that interaction with CD4 and CD8 coreceptors brings Lck into proximity with the TCR signaling complex, where sufficient clustering triggers trans-autophosphorylation and activation of Lck (28). Phosphorylated ITAMs serve as binding sites for zeta chain-associated protein kinase 70 (Zap70), which is subsequently activated via phosphorylation by Lck. Zap70 phosphorylates the scaffold protein linker for the activation of T cells (LAT), allowing recruitment of phospholipase Cγ (PLCγ) and the adaptor protein growth factor receptor-bound protein 2 (Grb2) among others (29). Subsequently, several mitogen-activated protein kinase (MAPK) pathways are activated, ultimately leading to transcriptional changes.

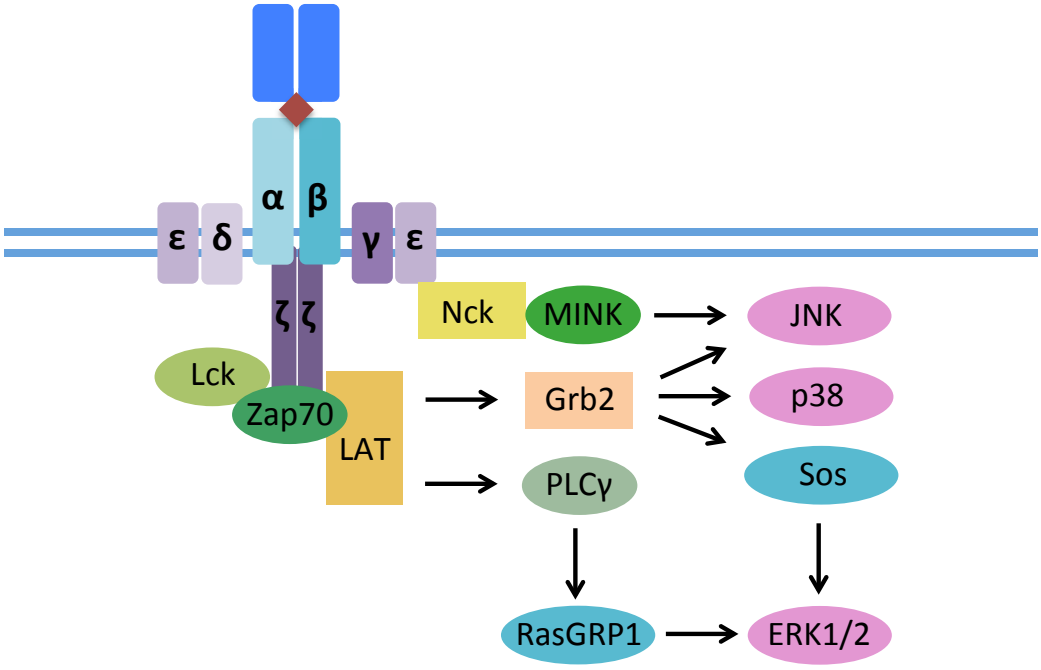


Figure 1-3. Molecules involved in proximal TCR activation. The TCR transduces signals through a complex comprised of CD3 chains (γ, δ, ε, ζ). Activation of kinases Lck and Zap70 leads to phosphorylation of the adaptor protein LAT, allowing recruitment of downstream molecules and activation of MAPK pathways. Differential phosphorylation of LAT is thought to contribute to differential MAPK activation, and consequently positive and negative selection outcomes. In addition, the protein Themis associates with Grb2 and recruits SHP1 and SHP2 phosphatases (not depicted), resulting in downregulation of kinase activity. Thus, Themis permits the low affinity TCR signal required for positive selection. During negative selection, JNK recruitment to the TCR complex is also thought to occur via the adaptor protein Nck and kinase MINK. The pathways leading to high induction of Bim and Nur77 during negative selection require more clarification (not depicted). Lck, lymphocyte-specific protein tyrosine kinase; Zap70, zeta chain-associated protein kinase 70; LAT, linker of activated T cells; PLCγ, phospholipase Cγ; Grb2, growth factor receptor-bound protein 2; Sos – Son of Sevenless; RasGRP1, Ras guanyl-releasing protein 1; MAPK, mitogen-activated protein kinase; Nck, non-catalytic region of tyrosine kinase adaptor protein; MINK, misshapen/NIK-related kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular-signal-regulated kinase. Figure modified from Hu and Baldwin (2013) Programmed Cell Death in T Cell Development, ISBN: 978-953-51-1133-7, InTech, DOI: 10.5772/48607.

Though our understanding of how low and high affinity TCR stimulation is translated into positive and negative selection is incomplete, the prevailing model is based on differential activation of the MAPKs extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), and p38. Inhibition of JNK activity by a dominant-negative mutant has been shown to inhibit peptide-induced deletion *in vivo* (30). Likewise, addition of a p38 inhibitor to a TCR transgenic fetal thymic organ culture impairs peptide-induced deletion (31). While JNK and p38 are important for negative selection, they are not required for positive selection (30-32). By contrast, inhibition of ERK1/2 or their upstream activator blocks positive selection but does not affect negative selection (31, 33-35).

Grb2 deficiency impairs JNK and p38 activation but does not affect ERK1/2 activation (36). This is because Ras, an upstream regulator of ERK, can be activated by two families of guanine nucleotide exchange factors in T cells: Sos, which is recruited by Grb2, and RasGRP, which is recruited by diacylglycerol generated by the PLCγ pathway (37). RasGRP1-deficient mice exhibit blocked positive selection (36, 38), consistent with the critical role of ERK1/2 in positive selection. While reaffirming that Grb2 is dispensable for ERK1/2 activation, a study showed that $Grb2^{-/-}$ mice are impaired in both negative and positive selection (39). One of the reasons that Grb2 is important for positive selection may be due to a role for Grb2 in amplifying TCR signaling by promoting Lck activation (39). In addition, Themis associates with Grb2 and may consequently be regulated by proteins in the signaling complex (25-27, 40).

Though ERK1/2 is dispensable for negative selection, high affinity TCR signaling induces robust but transient ERK activation. In contrast, positive selection is characterized by sustained ERK

signaling of lower intensity (41). This is thought to be due to differential localization of RasGRP1 induced by positive vs. negative selection ligands (23). During negative selection, RasGRP1 is localized at the plasma membrane, where it may be more subject to negative regulation. In contrast, RasGRP1 is localized at the Golgi during positive selection, allowing for sustained activation of the Ras/ERK pathway. In addition to activating Ras by catalyzing the formation of Ras-GTP, RasGRP primes Sos activation since Sos is activated by its own product Ras-GTP. The RasGRP and Sos positive feedback loop enables T cells to maximally respond to low-level stimulation (42).

Another protein that may play a role in differentially transducing positive vs. negative selection signals is misshapen/NIKs-related kinase (MINK). Stimulation of TCR transgenic thymocytes with high affinity antigen induces an association between CD3ε, the adaptor protein non-catalytic region of tyrosine kinase (Nck), and MINK (43). MINK was shown to be important for JNK activation but did not affect ERK1/2 activation. Consequently, MINK deficiency impaired negative selection but not positive selection.

Positive selection and lineage commitment

Positively selected thymocytes receive cues for survival, migration to the medulla, and differentiation into CD4 or CD8 single positive (SP) thymocytes (**Fig. 1-2**). The anti-apoptotic proteins Bcl-2 and Mcl-1 have been shown to be important for survival during positive selection (44-48). TCR signaling also induces CCR7 upregulation on DP thymocytes, allowing them to migrate towards the medulla in response to CCL19 and CCL21 produced by medullary thymic epithelial cells (mTECs) (49). CD4 and CD8 coreceptors are thought to influence lineage

commitment by regulating the strength of TCR signaling. While Lck binds both coreceptors, CD4 binds to Lck with higher affinity than CD8 (50). Reducing Lck activity redirects MHC class II-restricted thymocytes to the $CD8^+$ lineage and increasing Lck activity redirects MHC class I-restricted thymocytes to the $CD4^+$ lineage (51). While the intermediate steps in TCR signaling that lead to these different fates remain unclear, the transcription factors Gata3 and ThPOK are critical for development of $CD4^+$ T cells and Runx3 for $CD8^+$ T cells (52). The prevailing model proposes that stronger TCR signaling in MHC class II-restricted thymocytes induces ThPOK expression, which relieves Runx3-mediated suppression of uncharacterized CD4-lineage genes. In contrast to ThPOK-deficient mice, Gata3-deficient mice largely do not exhibit redirection to the CD8 lineage but are blocked in development of MHC class II-restricted thymocytes (52). This suggests that the role of Gata3 in CD4 lineage commitment precedes that of ThPOK.

Though the cortex contains other antigen-presenting cells (APCs) such as dendritic cells (DCs), cTECs are considered the chief mediators of positive selection (53, 54). One of the unique features of cTECs is their exclusive expression of a proteasome catalytic subunit called β5t (55). $\beta5t^{-/-}$ mice exhibit defective positive selection of $CD8^+$ T cells, suggesting that presentation of certain types of ligands is important for positive selection. Furthermore, it was shown that positive selection remains impaired in $\beta5t^{-/-}$ $CCR7^{-/-}$ mice, indicating that migration out of the cortex or negative selection in the medulla was not the reason for the reduction in CD8SP thymocytes (56). Expression of the surface glycoprotein CD5 has been shown to correlate with TCR signaling strength (57). Since $\beta5t^{-/-}$ mice exhibit CD8SP thymocytes with higher expression of CD5, it has been suggested that β5t-containing proteasomes generate peptides that bias the T cell repertoire away from high affinity for self-antigens (58).

Loading of self-peptides on MHC class II occurs in late endosomes through the action of cathepsin proteases, which degrade the MHC class II-associated li chain and allow exchange of the li-derived CLIP peptide with other peptides. cTECs highly express cathepsin L, whereas other thymic APCs preferentially express cathepsin S (59, 60). Deficiency in cathepsin L results in a severe reduction in the development of CD4SP thymocytes, indicating that high expression of cathepsin L is another way in which cTECs are specialized for positive selection. In addition to cathepsin L, high expression of thymic-specific serine protease and a high rate of autophagosome formation in cTECs compared to other thymic APCs are implicated in positive selection of CD4SP thymocytes (61).

Negative selection involves multiple mechanisms and settings

Negative selection is the process by which thymocytes bearing highly self-reactive TCRs are prevented from developing into mature, functional T cells. Negative selection can be achieved by clonal deletion, functional impairment (anergy), TCR editing, or clonal diversion of autoreactive thymocytes into regulatory lineages. Perhaps due to the finite resources and space available to support the T cell repertoire, clonal deletion is considered the predominant mechanism of negative selection (62).

Whereas positive selection occurs in the cortex, negative selection occurs in both the cortex and medulla. Clonal deletion of DP thymocytes in the cortex requires interaction with DCs, as cTECs are unable to efficiently induce deletion despite interacting with thymocytes (63, 64). Deletion in the medulla is different from in the cortex in several ways. Because migration to the medulla

requires positive selection to occur first, thymocytes undergoing negative selection in the medulla are of the more mature SP stage. Presentation of tissue-restricted antigens (TRA) is also unique to the medulla, whereas those self-antigens presented in the cortex are referred to as ubiquitous antigens (UbA). A critical regulator of TRA expression is the transcription factor Autoimmune Regulator (Aire), exclusively expressed in medullary thymic epithelial cells (mTECs) (65-68). Mutation of the Aire gene is responsible for the multi-organ autoimmune disease autoimmune polyendocrinopathy-candidiases-ectodermal dystrophy (APECED) in humans (69, 70), highlighting the critical role of clonal deletion to self-tolerance. TRA-mediated deletion is mediated by mTECs directly, as well as by DCs which cross-present the antigens (71, 72). In addition to deletion, mTECs are also able of inducing anergy and Treg cells (73-75). Lastly, the medulla contains higher numbers and different subsets of DCs than the cortex (76). Collectively, these differences argue that studies on the molecular mechanisms of negative selection should be considered for the context under which it occurred.

TCR transgenic models

In our study of negative selection, we utilized two MHC class I-restricted TCR transgenic models: HY^{cd4} for UbA-mediated selection and OT-I Rip-mOva for TRA-mediated selection. While older TCR transgenic mouse models provide a powerful means to study thymocyte development *in vivo*, TCR expression has been shown to occur prematurely at the DN stage (77). This results in premature selection under non-physiological circumstances (78, 79). The HY^{cd4} model was developed to correct for this problem by using CD4-cre to conditionally express the transgenic HY TCRα chain at the DP stage (**Fig. 1-4**) (77). Based on the classical HY model (80), the HY TCR is specific for an epitope from the male-specific smcy gene (KCSRNRQYL)

presented by H-2D^{b}. Antigen-specific T cells are detected with the T3.70 monoclonal antibody against the Vα17 HY TCRα chain. While expression of endogenous TCRα chains is known to occur, all TCRs include the transgenic Vβ8 chain. Clonal deletion of HY-specific DP thymocytes occurs in the cortex of HY^{cd4} male (M) mice, while positive selection occurs in HY^{cd4} female (F) mice generating T3.70^{+} CD8SP thymocytes (64, 81). Since DN and DP thymocytes differ in gene expression, proliferative ability, and signaling thresholds (82-84), correction of premature TCR expression in the HY^{cd4} model allows for investigation of physiological UbA-mediated deletion.

The OT-I TCR is composed of Vα2 and Vβ5 chains, and is specific for Ova peptide presented on MHC class I H-2K^{b} (21). Transgenic expression of a membrane-bound form of the neo-self-antigen Ova in the medulla of mice has been achieved by placing it under the control of the rat insulin promoter (Rip-mOva mice) (85). Thus, while OT-I mice have premature expression of the transgenic TCR, negative selection against Ova does not occur until a post-positive selection stage in the medulla. Transfer of OT-I bone marrow into Rip-mOva recipients creates chimeras in which negative selection occurs in a TRA-mediated manner, while transfer into wildtype (WT) recipients results in positive selection of Vα2^{+} CD8SP thymocytes. By using OT-I donors deficient in genes of interest, the bone marrow chimera strategy offers the ability to discriminate between whether the genes affect hematopoietic or stromal cells in the thymus.

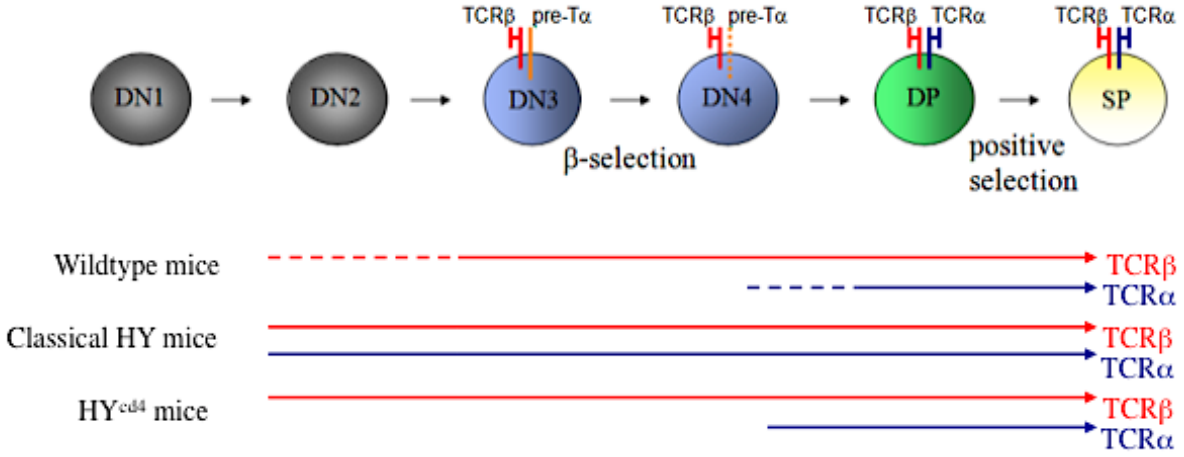


Figure 1-4. The timing of transgenic TCR expression is physiological in HY^{cd4} mice. The HY^{cd4} model utilizes a cd4-cre system to conditionally express the HY TCRα chain at the DP stage. This allows positive and negative selection to occur between the DP and SP transition as in wildtype mice, an important distinction from premature TCR expression and selection that occurs in classical HY mice. Figure author: Troy A. Baldwin.

The role of the intrinsic apoptosis pathway in clonal deletion

Clonal deletion is widely held to occur through apoptosis of autoreactive thymocytes. Many studies have examined the role of death receptor-induced 'extrinsic' apoptosis pathways in clonal deletion. In various models of negative selection, mice defective for Fas or Fas ligand (FasL) (86-88), or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (89-91), have been shown to have normal deletion of autoreactive thymocytes. Many of these studies utilized models of negative selection in which exogenous antigen is injected *in vivo*. This can cause non-specific thymocyte deletion by the secreted products of activated peripheral T cells (92). Because activation-induced cell death of peripheral T cells is impaired in the absence of death receptors, results derived from these model systems may be complicated by involvement of extrathymic factors (87, 88). Importantly, transgenic expression of dominant-negative Fas-associated protein with death domain (FADD), which interacts with both Fas and TRAIL, or the viral caspase-8 inhibitor cytokine response modifier A (CrmA) does not impair clonal deletion (93-95). Collectively, these data suggest that the extrinsic apoptosis pathway is not required for clonal deletion.

The intrinsic apoptosis pathway is initiated by release of cytochrome c from mitochondria to the cytosol, which through association with apoptotic protease activating factor 1 (Apaf1), results in cleavage and activation of caspase-9. Caspase-9 in turn activates executioner caspases by cleavage – chief among them caspase-3. Activation of caspase-3 is observed in thymocytes stimulated *in vitro* and from TCR transgenic models of negative selection (96, 97). In contrast, the active forms of executioner caspases-6 and -7 are not detected after stimulation of TCR transgenic thymocytes with cognate peptide but can be induced by non-specific stimulation with

staurosporine (98). Furthermore, comparison of caspase-$3^{-/-}$, caspase-$7^{-/-}$, and double knockout thymocytes indicates that caspase-3 is mainly responsible for DNA fragmentation in response to anti-CD3ε and anti-CD28 stimulation (99). Therefore, caspase-3 appears to be the predominant caspase that mediates clonal deletion. Though it normally plays a major role, caspase-3 activation is not strictly required for clonal deletion *in vivo* (97, 100). Furthermore, studies using pan-caspase inhibitors *in vitro* and *in vivo* have shown that TCR-induced thymocyte death can occur in the absence of caspase activity, albeit to reduced levels in some systems (96, 98, 101, 102). These data argue that clonal deletion can also be achieved through caspase-independent pathways.

The role of mitochondria in caspase-independent cell death (CICD)

It is now clear that several types of programmed cell death other than caspase-mediated apoptosis exist in cells. In some cases, such as necroptosis and autophagy, specific molecules in the pathway have been identified (103). Otherwise, CICD is established by observations of cell death under conditions where caspase activity is blocked by genetic ablation or inhibitors. Thus, while CICD has been demonstrated in thymocytes and mature T cells under experimental conditions (104), little is known about CICD during physiological processes like negative selection.

Though multiple as-of-yet to be discovered pathways may be encompassed by studies of CICD, a commonly reported feature is loss of mitochondrial membrane potential. As in caspase-mediated apoptosis, members of the Bcl-2 family are critical gatekeepers of the mitochondrial outer membrane. Members of this family are related in one or more regions called Bcl-2

homology (BH) domains (**Fig. 1-5**). The current body of work suggests that the Bax and Bak proteins oligomerize to form channels in the mitochondrial outer membrane (105). Anti-apoptotic Bcl-2 family members oppose this process through interaction with Bax and Bak. Another class of pro-apoptotic Bcl-2 proteins containing only the BH3 domain counteracts inhibition by anti-apoptotic Bcl-2proteins through direct binding which requires the BH3 domain (105). While some BH3-only proteins only function by sequestration of anti-apoptotic Bcl-2 members, others such asBcl-2-interacting mediator of cell death (Bim) have also been shown to directly bind and activate the oligomerization potential of Bax and Bak (106-109).

Through the channels formed by Bax and Bak, or other channels formed as a result of their activation, molecules are released from the mitochondrial intermembrane space. Two proteins released from mitochondria that have been shown to mediate CICD are apoptosis-inducing factor (AIF) and endonuclease G (endoG). AIF cleaves DNA into large fragments of 50 kilobase pairs (110), while endoG appears to mediate internucleosomal DNA cleavage (111). Alternatively or in addition to release of cytotoxic proteins, it has been argued that CICD results from loss of mitochondrial respiratory function following membrane permeabilization (112). The mitochondrial permeability transition pore is a channel composed of a complex of proteins that has been implicated in caspase-independent mitochondrial membrane permeabilization. Various members of the Bcl-2 family have been shown to inhibit or activate opening of this channel, emphasizing the importance of the Bcl-2 family to multiple types of cell death.

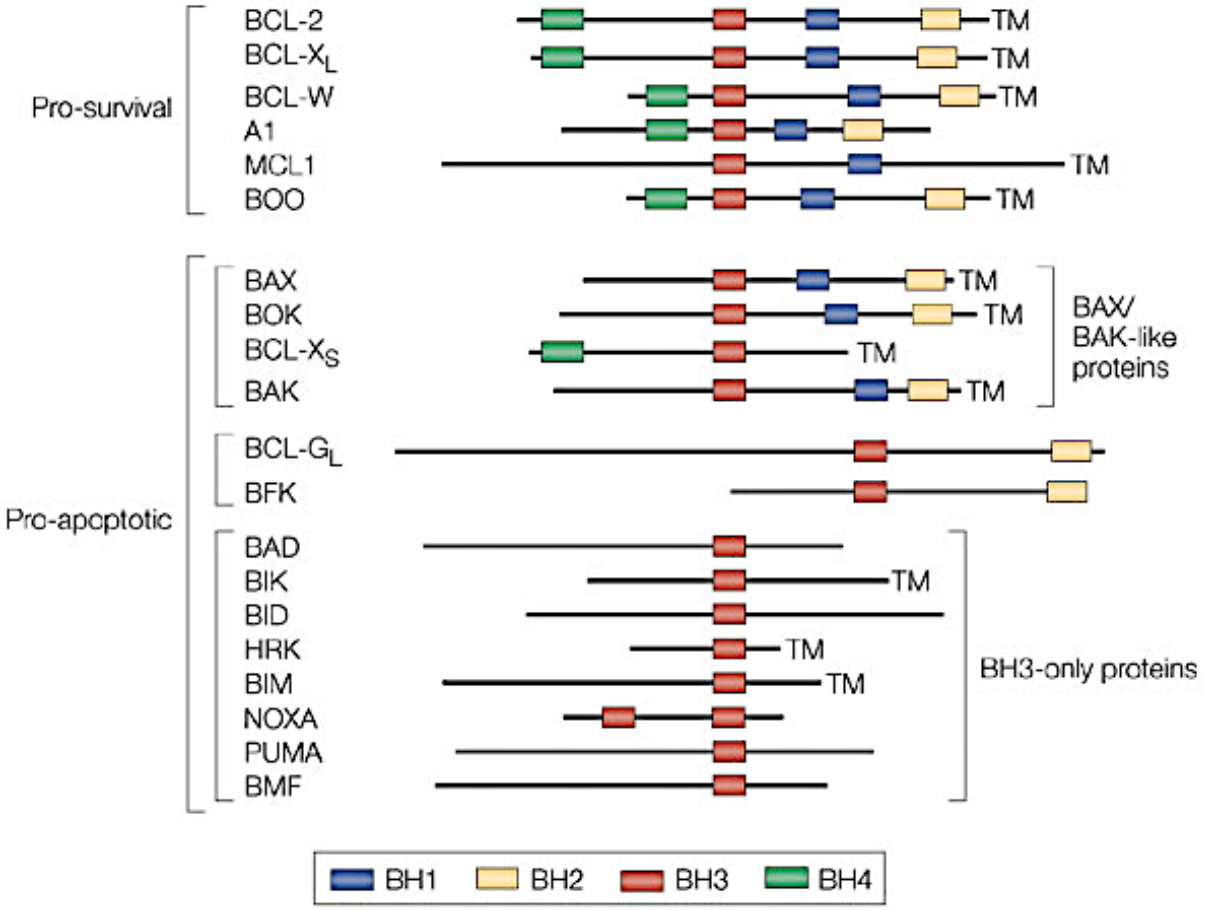


Figure 1-5. Members of the Bcl-2 family. The Bcl-2 family includes pro-survival and pro-apoptotic members, all containing one or more related regions called Bcl-2 homology (BH) domains. Oligomerization of Bax and Bak results in mitochondrial outer membrane permeabilization. The pro-survival Bcl-2 members inhibit cell death by binding Bax and Bak to prevent their oligomerization. The pro-apoptotic BH3-only members promote cell death by sequestration of pro-survival members, and in some cases, by direct binding and activation of Bax and Bak. Not all of the Bcl-2 proteins depicted here have been shown to be important in regulating cell death. Bcl-, B cell lymphoma-; Mcl-1, myeloid-cell leukemia sequence 1; Bax, Bcl-2-associated X protein; Bak, Bcl-2-antagonist/killer; Bim, Bcl-2-interacting mediator of cell death; Puma, p53-upregulated modulator of apoptosis; TM, transmembrane domain. Figure taken from Strasser (2005) Nat Rev Immunol 5: 189-200. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology 5: 189-200, copyright 2005.

The events following mitochondrial membrane permeabilization are unclear and may vary between different pathways falling under the CICD umbrella. Since internucleosomal DNA fragmentation, chromatin condensation, mitochondrial dysfunction, plasma membrane permeabilization, and exposure of phosphatidylserine have all been demonstrated under caspase-independent conditions (113, 114), the activation of caspases is the strictest distinguishing factor between classical apoptosis from other forms of programmed cell death.

Molecules induced during negative selection

Studies have attempted to characterize the molecular mechanisms of negative selection by identifying genes that are differentially regulated during positive vs. negative selection. Many such proteins are used as markers of TCR signaling, and some are known to function in regulating TCR signaling. The proteins used in our analyses include the cell surface markers CD5, CD69, and programmed death-1 (PD-1). In brief, CD5 is a negative regulator of TCR signaling. Since CD5 expression correlates with TCR signal strength (57), the level of CD5 expression can be used as a gauge of low vs. high affinity TCR signaling.

CD69 is a C-type lectin that induced early after TCR stimulation (115), including on DP thymocytes during positive and negative selection (116). Its upregulation is widely used to demonstrate that TCR signaling is intact. Despite being a common marker of T cell activation, little is known about its function. Some argue that it has no role in thymocyte selection, but may play a role in thymocyte egress (117, 118). However, another study has argued that CD69 expression promotes positive and negative selection (119).

PD-1 is a co-inhibitory receptor induced on DP thymocytes upon TCR stimulation. As with CD69, its expression is higher during negative selection than positive selection (64). Since maximal induction of PD-1 on DP thymocytes occurs after 48 h after TCR stimulation (64), its expression may provide insight on the kinetics of deletion. PD-1 is not required for clonal deletion, and the absence of this co-inhibitory receptor may enhance deletion in some models (120, 121). Despite being dispensable for thymic deletion, a large body of work shows that PD-1 deficiency results in autoimmunity due to the involvement of this protein in peripheral T cell tolerance (122).

In addition to the surface molecules described above, we utilize the transcription factor Helios, a member of the Ikaros family of zinc finger proteins, to verify that thymocytes are undergoing negative selection. Helios was originally characterized as a Treg marker; however, its role in Treg biology remains unclear since Helios^{+} and Helios^{-} Treg appear to have equal suppressive capability (123, 124). Recently, Helios has been used as a marker that specifically distinguishes thymocytes experiencing high affinity TCR signaling from those experiencing low affinity TCR signaling (19). Using Helios as a marker is advantageous since other markers are induced to lesser degrees by low affinity TCR signaling.

While the aforementioned proteins are used to characterize thymocytes undergoing negative selection, they are not known to mediate negative selection. The work presented in this thesis focuses on two proteins identified as upregulated during negative selection in various models including HY^{cd4} mice: Bim and Nur77 (81, 125-129). We have chosen to investigate the role of

these two proteins in negative selection due to their role in cell death in the literature. Thus, they are hypothesized to be key mediators of clonal deletion.

The role of Bim in clonal deletion

Bim, also known by the less common name Bod, is a pro-apoptotic BH3-only member of the Bcl-2 family. While numerous splice variants of the Bim locus have been reported, three major isoforms are readily detected at the protein level: Bim short (Bim_S), Bim long (Bim_L), and Bim extra long Bim_{EL}) (130). Molecular work in a number of cell types has demonstrated that additional regions in Bim_L and Bim_{EL} allow them to be post-translationally regulated by MAPK pathways (**Fig. 1-6A**) (131). However, in DP thymocytes stimulated with anti-CD3, neither the subcellular localization nor the amount of Bim protein was altered (132). This suggests that Bim regulation by TCR signaling during thymocyte selection may primarily be at the transcriptional level. Bim expression is positively regulated by the JNK pathway (133, 134), consistent with the importance of JNK to clonal deletion. The transcription factor forkhead box O3a (FoxO3a), a mediator of JNK signaling, has been implicated in Bim induction in T cells during cytokine withdrawal (135). Conversely, repression of Bim by pro-survival IL-2 signaling is thought to be due to inactivation of FoxO3 (136). In the HY^{cd4} model, Bim induction is 3.9-fold higher in HY^{cd4} M mice undergoing negative selection than in HY^{cd4} F mice undergoing positive selection (81).

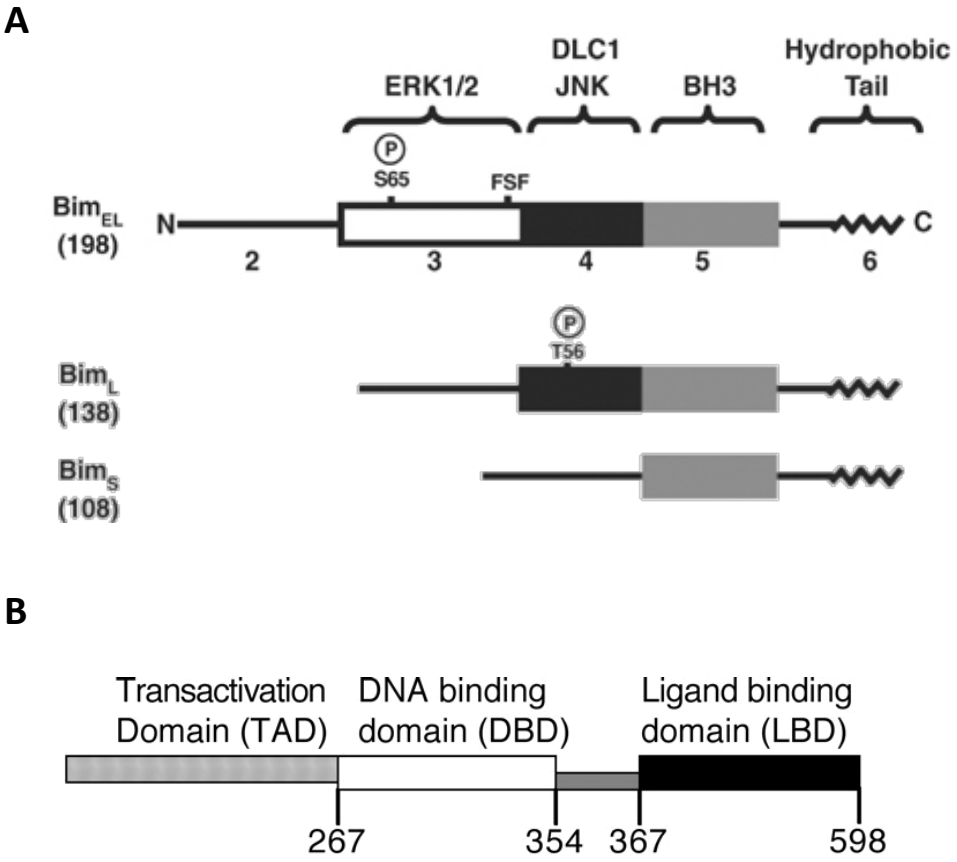


Figure 1-6. Functional domains of Bim and Nur77. (A) The three major isoforms of Bim are Bim short (Bim_S), Bim long (Bim_L), and Bim extra long (Bim_{EL}). All contain a BH3 domain, which is critical for their pro-apoptotic function. Bim_L and Bim_{EL} contain regions that subject them to regulation by MAPKs. DLC1, dynein light chain 1; FSF, ERK1/2 docking domain. Figure modified from Ley et al. (2005) Cell Death and Diff 12: 1008-1014 and reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation 12: 1008-1014, copyright 2005. **(B)** The functional domains of Nur77 are depicted. Nor-1 and Nurr1 are highly related in homology and function. Figure © Zeng et al., 2006, originally published in J Exp Med 203(3): 719-729.

Bim deficiency results in 2-4 fold increases in the number of myeloid and lymphoid cells (137). Over half of $Bim^{-/-}$ mice are reported to exhibit systemic autoimmunity after 12 months of age (137). Pathology in $Bim^{-/-}$ mice has many characteristics of humoral mediated-autoimmunity such as increased numbers of plasma cells, autoantibodies, and immune complex deposits in the kidneys. Since Bim plays a role in B cell negative selection and T cell contraction after an immune response (138, 139), it is unclear how much of the autoimmunity can be attributed to impaired thymocyte negative selection. It has been suggested that Bim deficiency in DCs promotes enhanced T cell responses and autoantibody production (140).

In several model systems of varying physiological representation, Bim deficiency was shown to impair deletion of DP thymocytes (141). However, Bim is largely dispensable for deletion against superantigens (142), indicating that it is not essential for all types of negative selection. Prior to the work presented in the third chapter of this thesis, the role of Bim in a physiological *in vivo* model of pMHC-induced clonal deletion had not been examined. We know now that the requirement for Bim in clonal deletion against UbA vs. TRA is different (97, 143, 144), emphasizing the importance of the context under which selection occurs. In addition to its role in central tolerance, Bim may affect the function of mature T cells in an apoptosis-independent manner. One study has shown a defect in global cytokine production by $Bim^{-/-}$ T cells, and Bim deficiency resulted in less severe disease in a model of experimental autoimmune encephalomyelitis (145).

The NR4A family

The nuclear receptor subfamily 4 group A (NR4A) family of nuclear receptors is comprised of three members: Nur77 (NR4A1), Nurr1 (NR4A2), and Nor-1 (NR4A3). Of the three, Nur77 has been the most extensively studied. Nur77 is also known as NGFI-B due to studies on its role in nerve growth factor induced signaling, as well as TR3 due to its role in thyroid hormone signaling (146). The members of this family are closely related in structure and function (**Fig. 1-6B**). All members bind an octanucleotide motif (5'-A/TAAAGGTCA) as monomers or homodimers, as well as an everted repeat of this element as homodimers. In addition, Nur77 and Nurr1 are known to form heterodimers with certain RXR receptors during retinoid signaling (147). In T cells polarized under Treg inducing conditions, all NR4A family members can be detected at the forkhead box p3 (Foxp3) promoter where they exert positive regulation of Foxp3 expression (148). Given the high homology of the DNA binding domain between NR4A family members, deletion of the transactivation domain of Nur77 creates a dominant-negative mutant that interferes with the transcriptional activity of all NR4A family members (149).

The NR4A proteins are considered orphan receptors since they bind DNA in a ligand-independent manner. For example, this has been demonstrated in studies where a Nur77 truncation mutant lacking most of the ligand-binding domain retained transcriptional activity similar to WT Nur77 (150). No ligands for NR4A receptors have been identified. However, various stimuli regulate their transcription and function. Of note, inflammatory stimuli including lipopolysaccharide, interferon γ (IFNγ) and tumour necrosis factor α (TNFα) induce Nur77 expression in macrophages (151). Nur77 is associated with protection in atherosclerosis in part due to its promotion of an anti-inflammatory phenotype in macrophages (152-154).

The other major role ascribed to Nur77 of interest to our studies is apoptosis. The role of Nur77 in apoptosis was first identified during TCR-induced death of thymocytes and DO11.10 T cell hybridomas (155, 156). Nur77 plays a role in the death of cancer cells in response to numerous apoptosis-inducing agents (146). Its role appears to have clinical significance since downregulation of Nur77 is associated with metastasis of various solid tumours in patients (157).

The mechanism of Nur77-mediated death

The mechanism by which Nur77 induces cell death is of great controversy and may differ depending on the cell type. In support of a transcription-dependent role of Nur77 in apoptosis, the transcriptional activity of Nur77 has been shown to correlate with its ability to induce thymocyte death (158). Until recently, target genes of Nur77 known to possess apoptotic function were those involved in the extrinsic apoptosis pathway, such as FasL and TRAIL (159). The only apoptotic protein involved in the intrinsic pathway that has shown to be regulated by Nur77 is Bim (160). However, Bim induction following TCR stimulation is only partly dependent on Nur77.

A transcription-independent role for Nur77 in apoptosis was first characterized in cancer cells. In response to treatment with various apoptosis-inducing stimuli, Nur77 was found to translocate from the nucleus to mitochondria, resulting in cytochrome c release (161). This has been corroborated in several other types of cancers (146). Subsequent work showed that in cancer cells, Nur77 converts Bcl-2 into a pro-apoptotic form by exposing its BH3 domain (162). This has also been reported in TCR-stimulated thymocytes (163); however, this finding is more

questionable since transgenic Bcl-2 overexpression was used. Previous studies have shown Nur77 to remain in the nucleus of stimulated thymocytes, while nuclear export of Nur77 in mature T cells is thought to protect them from apoptosis (164). In stimulated DO11.10 cells, nuclear export of Nur77, but not interaction between Bcl-2 and Nur77, was observed (165). The pathways responsible for regulation of Nur77 nuclear export are unclear. In DO11.10 cells, phosphorylation of serine 354 by ribosomal S6 kinase (RSK), downstream of the ERK1/2, is necessary for nuclear export (165). This is contested by another study that found protein kinase C but not ERK1/2 to be required for mitochondrial translocation (166). The fact that ERK1/2 (31, 33-35) and protein kinase C (167, 168) are not required for clonal deletion adds doubt to the significance of the transcription-independent mechanism of Nur77 to clonal deletion.

There is also little consensus on the events downstream of gene transactivation or mitochondrial targeting that result in cell death. Studies in T cell hybridomas and other cancer cells have shown cytochrome c release, suggesting activation of the intrinsic apoptosis (161, 162, 165). However, z-VAD treatment only partially rescued Nur77-induced cell death in T cell hybridomas, suggesting that Nur77 may also mediate a CICD mechanism (165). A role for Nur77 in CICD has also been shown in other cell types (169-171). Furthermore, transgenic Nur77 expression is not sufficient to induce cytochrome c release (159). Yet, mice that express transgenic Nur77 have severely reduced thymic cellularity and an increased frequency of thymocytes showing DNA fragmentation (149, 172).

The role of Nur77 in clonal deletion

Induction of Nur77 following TCR stimulation has long been established (155, 156), but the precise mechanism leading to its expression remains unclear. The ERK5 pathway has been reported to induce Nur77 expression in thymocytes and DO11.10 cells (32, 173). This has been called into question by others who show that ERK5 is not required for Nur77 induction in response to TCR stimulation (174). ERK5 is also not required for T cell development in a polyclonal repertoire, but its role in clonal deletion remains to be examined in a TCR transgenic model. In cancer cells, p38 signaling promotes Nur77 induction via the transcription factor MEF2 (175). Though this has not yet been shown in thymocytes, the p38 pathway is known to be important for negative selection.

Nur77 and Nor-1, but not Nurr1, are induced upon stimulation of thymocytes (149). Nur77 and Nor-1 have also been found to co-localize in thymic tissue (147). In the HY^{cd4} model, Nur77 induction in thymocytes undergoing negative selection is 11.3-fold higher than during positive selection (81). The sensitivity of Nur77 expression in response to TCR signaling has led to the generation of a Nur77-GFP reporter mouse (176).

Initial studies indicated that $Nur77^{-/-}$ mice did not exhibit alterations in T cell development (177). In contrast, expression of dominant-negative Nur77 partially impairs clonal deletion in some TCR transgenic models, leading to the suggestion that Nor-1 serves redundant functions in thymocytes (172, 178). Recently, Nur77 single deficiency has been shown to impair TRA-mediated clonal deletion (160), emphasizing the importance of context. This also demonstrated that Nur77 and Nor-1 are not always fully redundant.

Tolerance mechanisms other than thymic deletion

While the majority of self-reactive T cell clones are eliminated in thymus, those that escape clonal deletion have the potential to cause autoimmunity in the periphery (179, 180). Thus, several other mechanisms, both intrinsic and extrinsic to the autoreactive clones, are critical to the maintenance of self-tolerance. These tolerance mechanisms can be initiated in the thymus or periphery. Intrinsic mechanisms include immunological ignorance, peripheral deletion, anergy, and diversion into regulatory cells. Ignorance is the phenomenon by which autoreactive T cells remain naïve to cognate antigens expressed by tissues due to anatomical separation, immaturity of T cells and/or APCs, or low avidity (181). In contrast, the other intrinsic tolerance mechanisms occur as a result of high affinity antigen encounter. While self-specific T cells can adopt regulatory functions, thereby being intrinsically removed as an autoimmune threat, the suppressive action they exert on effector autoreactive cells comprises the extrinsic arm of tolerance. Regulatory T cell subsets are thought to include $Foxp3^+$ $CD4^+$ Treg, regulatory DN T cells, and $CD8\alpha\alpha^+$ intraepithelial lymphocytes (IELs). In addition to the role of DCs in deletion of autoreactive thymocytes, DCs can adopt a tolerogenic phenotype if they are exposed to antigen in the absence of full maturation stimuli. Tolerogenic DCs are important in downregulation of immunity, in part through inducing regulatory T cells (182).

Clonal diversion: $Foxp3^+$ Treg

A clear demonstration of the importance of tolerance mechanisms other than clonal deletion comes from cases of Treg deficiency. Though subsets of $Foxp3^-$ T cells with regulatory capacity exist, expression of Foxp3 defines, and is required for the development and function of, cells of

the "Treg" lineage. Humans with a mutation in the Foxp3 gene develop a severe multi-organ autoimmune disease (183). Similarly, mice that lack Foxp3 expression develop lethal autoimmunity. Prior to discovery of Foxp3 as the lineage-specifying factor, Treg were identified as $CD4^+CD25^+$ T cells. Treg that are generated during thymic selection are referred to as natural Treg, while those that are converted from $CD4^+$ T cells in the periphery are called induced Treg. Natural and induced Treg have been shown to contribute non-redundantly in ameliorating inflammation (184). While natural Treg are thought to raise the activation threshold for all immune responses, induced Treg appear to be specialized in mediating tolerance to oral and environmental antigens (185, 186).

This thesis is concerned primarily with the selection of natural Treg in the thymus. TCR repertoire analysis of $Foxp3^+$ Treg and conventional $Foxp3^-$ $CD4^+$ T cells indicates that the majority of TCR usage does not overlap between the two populations, supporting differential selection of Treg compared to conventional T cells (187-189). The repertoire overlap could in part be explained by the presence of induced Treg, which by definition come from the conventional $CD4^+$ pool. The first demonstration that TCR specificity determined Treg selection showed that Treg cells only developed in the thymus of TCR transgenic mice when the cognate antigen was also expressed (190). Subsequent experiments have shown that Treg selection in TCR transgenic mice is actually inefficient due to a limited niche for Treg (191). Using intrathymic injection and low frequency mixed bone marrow chimeras, it was demonstrated that Treg selection increases as intraclonal competition decreases. Because the niche for natural Treg is limited, it has been proposed that Treg are selected against TRA. While natural Treg specific

for TRA have been identified, UbA-mediated Treg selection in a polyclonal repertoire has also been demonstrated (192, 193).

Unlike anergy and clonal diversion to DN thymocytes described below, costimulation through CD28 is important for Treg selection. Since the TCR repertoires of Treg are similar in the presence or absence of CD28, it appears that the CD28 costimulation enhances selection efficiency or survival rather than increasing TCR signaling strength to a threshold sufficient for Treg selection (194, 195). Stronger TCR signaling in Treg cells than in positively selected conventional thymocytes has recently been demonstrated using Nur77-GFP reporter mice (176). Studies have also shown that altering the amount of cognate antigen or MHC class II expression on mTECs can control whether Treg selection or clonal deletion occurs (71, 196). The term 'agonist selection' has been used to describe the selection of Treg and other lineages at a window of TCR avidity between that of positive selection and clonal deletion (**Fig. 1-7**). While clonal deletion and agonist selection may involve different degrees of TCR signal strength, there is likely overlap between the two fates. Thus, it is conceivable that modulation of TCR signaling could alter the selection threshold, resulting in 'clonal diversion' to alternative fates instead of clonal deletion. In addition, failure to undergo clonal deletion may result in agonist selection. This may underlie the observations of an increase in the frequency of Treg in the absence of Bim or Nur77 (20, 160).

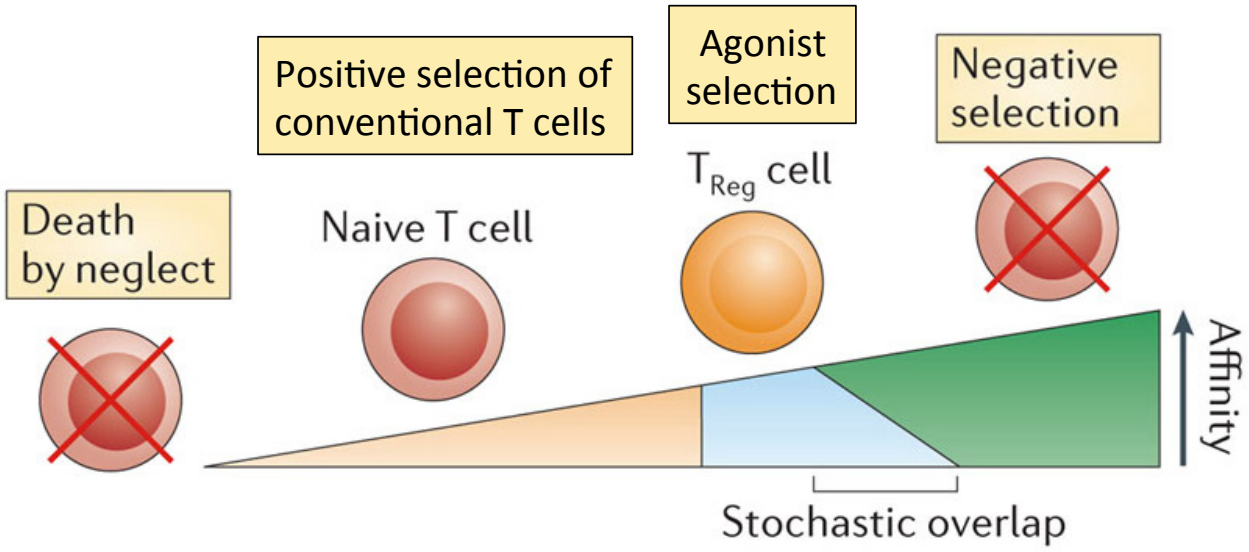


Figure 1-7. The affinity model of thymocyte selection. The affinity of the TCR for self-pMHC determines the fate of the thymocyte. Weak interactions are required for positive selection of conventional T cells; elsewise, death by neglect results due to inability to receive survival and differentiation signals through the TCR. Strong interactions cause negative selection by clonal deletion. High affinity agonists can also cause clonal diversion into Treg and other lineages; this process is termed agonist selection. The current affinity model holds that Treg selection occurs optimally within a window between positive selection and negative selection. The demarcation between clonal deletion and clonal diversion is plastic and subject to stochastic influences. Figure modified from Klein *et al.* (2014) Nat Rev Immunol 14*:* 377–391. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology 14*:* 377–391, copyright 2014.

Clonal diversion: CD8αα$^+$ IELs

In addition to CD8$^+$ T cells that express the conventional CD8αβ heterodimer, subsets of αβTCR$^+$ and γδTCR$^+$ CD8$^+$ T cells exclusively express the CD8αα homodimer. CD8αα$^+$ IELs are enriched in mucosal surfaces, particularly the small intestinal epithelia, where they are proposed to mediate tolerance to gut antigens (197). While γδTCR$^+$ CD8αα$^+$ IELs can largely be generated extrathymically, the number of αβTCR$^+$ CD8αα$^+$ IELs is considerably reduced in athymic mice compared to euthymic mice (198, 199). αβTCR$^+$ CD8αα$^+$ cells are nearly undetectable in the thymus, suggesting that they are derived from a thymic precursor. Using conditional expression of a GFP reporter driven by Rorγt, a transcription factor induced in DP thymocytes during positive selection, it was found that most αβTCR$^+$ CD8αα$^+$ IELs expressed GFP whereas γδTCR$^+$ CD8αα$^+$ IELs did not (200). Since this thesis is concerned with thymic selection, CD8αα$^+$ IELs will refer to those expressing the αβTCR from hereon.

The TCR repertoire of CD8αα$^+$ IELs have long been known to contain forbidden Vβ chains specific for endogenous superantigens (201), suggesting selection based on self-reactivity. In support of this, HY M mice contain at least 100-fold expansion of CD8αα$^+$ IELs compared to HY F mice (202, 203). Furthermore, it was shown that a rare population of CD8αα$^+$ DP thymocytes isolated from HY F differentiated into CD8αα$^+$ IELs when exposed to high but not low doses of HY antigen *in vitro*, and generated CD8αα$^+$ IELs when injected into recipients *in vivo* (204). In contrast, conventional DP thymocytes did not survive. The CD8αα$^+$ DP thymocytes do not express detectable surface αβTCRs, suggesting early commitment to the IEL lineage.

Separate studies had previously identified αβTCR$^+$ DN thymocytes expressing the IL-2/IL-15Rβ chain (CD122) as precursors for CD8αα$^+$ IELs (205). In polyclonal mice, their TCR repertoires contained overrepresentation of forbidden Vβ chains, and their generation in TCR transgenic mice depended on the presence of cognate antigen. Other studies have shown that αβTCR$^+$ DN thymocytes have high expression of CD5, indicative of high affinity TCR selection, though CD5 is downregulated as they differentiate into CD8αα$^+$ IELs (204). HY^{cd4} M mice, which contain much lower numbers of TCR$^+$ DN, do not generate as many CD8αα$^+$ IELs as classical HY M mice (77). It has been proposed that CD8αα$^+$ DP and αβTCR$^+$ DN represent sequential precursors in the same differentiation pathway, but this has not been established (206). A recent study showed that in the absence of CD28 costimulation, strong TCR signaling diverts DP thymocytes from clonal deletion into CD122$^+$ αβTCR$^+$ DN thymocytes (207). When cultured with IL-15 *in vitro*, the αβTCR$^+$ DN thymocytes upregulate CD8αα. These data suggest that generation of CD8αα$^+$ IELs can result from high affinity selection of conventional DP thymocytes. This model is of particular interest to this thesis because Bim$^{-/-}$ mice are known to contain an elevated frequency of αβTCR$^+$ DN thymocytes (208), which may reflect increased clonal diversion due to impaired clonal deletion.

As a final note, not all post-selection αβTCR$^+$ DN thymocytes may be destined for the CD8αα$^+$ IEL lineage. Emerging evidence suggests that there may be multiple DN T cell subsets with regulatory capability. In several studies, regulatory DN T cells can be generated in the periphery from conventional CD4$^+$ or CD8$^+$ T cells, suggesting a different lineage from regulatory CD8αα$^+$ IELs (209). These other DN T cell subsets may also have a thymic origin, but since they have not yet been described as agonist-selected, we will not elaborate on them here.

Anergy

In general terms, anergic T cells can be defined as those that have become intrinsically functionally unresponsive after antigen encounter. Among the most commonly reported characteristics of functional unresponsiveness are decreased IL-2 production and proliferation (210). Importantly, anergy is a relative state, and it is unknown to what degree an impairment in anergy would break tolerance in a physiological setting. Historically, anergy has been induced after TCR stimulation in the absence of CD28 costimulation (211). This type of *in vitro* anergy is considered distinct from the T cell unresponsiveness that occurs *in vivo,* with one of the differences being whether the T cells involved were previously activated or naïve. The first demonstration of anergy induction in TCR transgenic model involved transfer of naïve HY F mature $CD8^+$ T cells into athymic HY M recipients. Following proliferation and contraction, a small population of T cells survives and do not proliferate when restimulated with male antigen (212). Proliferation in these cases are likely in part lymphopenia-induced, since transfer of OT-II T cells into lymphoreplete recipients given soluble Ova results in limited proliferation but still cell death followed by survival of an anergic population (213). Other characteristics thought to distinguish *in vivo* anergy from *in vitro* anergy include the requirement for persistent antigen to maintain unresponsiveness and defective production of cytokines other than IL-2 (211).

In addition to anergy induction in the periphery, thymocytes can also be anergized during negative selection. Early studies using bone marrow chimeras showed that anergy induction to superantigens in the thymus is mediated by epithelial cells, whereas clonal deletion occurred when antigen was presented by hematopoietic cells (214). Further studies established that mTECs in particular induced anergy against alloantigen (215). Using HY TCR transgenic mice

to study pMHC-induced tolerance, it was shown that thymocytes that escape thymic deletion establish in the periphery but are unresponsive to male antigen (216, 217). The HY-specific T cells show downregulation of TCR and CD8, which we also see in *ex vivo* mature T cells from HY^{cd4} M mice (97). Similar events have been reported in MHC class II-restricted TCR transgenic models (211).

The term anergy likely encompasses multiple biochemically distinct forms of unresponsiveness. Much more is known about anergy induction in peripheral T cells than anergy induced during thymic negative selection. The early growth response (Egr) 2 and 3 transcription factors are important in initiating the anergy program *in vivo* and *in vitro* (218, 219). Among the targets of Egr2 or Egr3 is the E3 ubiquitin ligase Cbl-b. Cbl-$b^{-/-}$ mice develop spontaneous autoimmunity, and loss of Cbl-b exacerbates collagen-induced arthritis (220, 221). Cbl-b deficiency renders T cells resistant to anergy induction *in vitro* and *in vivo* through restoring the reduced calcium mobilization that normally occurs in anergized cells. The co-inhibitory molecules PD-1 and CTLA-4 are also important for peripheral anergy induction (222, 223). While upregulation of PD-1 occurs during thymic negative selection (64), it seems to reflect high affinity TCR signaling since it is induced on most antigen-specific thymocytes. Studies with PD-1 deficiency report intact clonal deletion, precluding analysis of its role in thymic-induced anergy (120, 121).

One of the forms of anergy in $CD4^+$ T cells is characterized by high expression of folate receptor 4 (FR4) and CD73. Adoptive transfer of TCR transgenic $CD4^+$ T cells into lymphoreplete recipients expressing cognate antigen resulted in the majority of cells becoming $FR4^{hi}CD73^{hi}$. Recovery of the TCR transgenic cells and re-introduction into antigen-expressing hosts resulted

largely in failure to proliferate (224). Foxp3^{+} Treg also express high levels of FR4 and CD73 (225, 226), but the anergic CD4^{+} T cells in this model were Foxp3^{-}. However, the presence of Foxp3^{+} Treg was required for induction of anergic FR4^{hi}CD73^{hi} CD4^{+} T cells. CD73 is an ecto-5′-nucleotidase that generates adenosine, which signals via the adenosine A_{2A} receptor on T cells to suppress effector functions (227, 228). FR4 mediates the transport of folic acid into T cells, and blockade of the receptor results in depletion of Treg *in vivo* (226). Thus, it has been suggested that Treg are highly dependent on this vitamin for maintenance. FR4 may serve a similar role in maintaining non-regulatory anergic T cells.

Rationale and Objectives

1. Given the ability of Bim and Nur77 to induce apoptosis, studies have examined the role of these two molecules in clonal deletion in a number of TCR transgenic models of UbA-mediated negative selection (141, 172, 178, 229). However, these studies are limited by premature expression of the transgenic TCR. In order to characterize clonal deletion of DP thymocytes in a physiological setting, we will utilize "on time" HY^{cd4} TCR transgenic mice deficient in Bim, Nur77 or both genes.
2. In addition to UbA-mediated clonal deletion, the medulla is an exclusive site for TRA-mediated deletion (66). Because the stage of thymocyte maturation and the surrounding environment in which selection occurs are different between the two contexts, the molecular mechanisms of negative selection may also differ. To study TRA-mediated selection, we will examine the effects of Bim and/or Nur77 deficiency using OT-I TCR transgenic bone marrow chimeras.

3. In addition to clonal deletion, other mechanisms make critical contributions to maintaining self-tolerance. Many specifically involve MHC class II-restricted thymocytes, such as $FR4^{hi}CD73^{hi}$ anergic $CD4^+$ T cells (224) and $Foxp3^+$ Treg (230, 231). In addition to these alternative fates for CD4SP thymocytes, post-selection TCR^+ DN thymocytes are implicated as precursors of $CD8\alpha\alpha^+$ IELs (207), and may also develop into other regulatory DN subsets (209). These DN thymocytes may also be MHC class II-restricted, precluding their analysis in our TCR transgenic models. Therefore, we will examine the role of Bim and Nur77 in clonal deletion and other forms of tolerance in unmanipulated mice with a polyclonal TCR repertoire.

Overall Theses

1. The molecular mechanisms of clonal deletion, specifically with respect to the role of Bim and Nur77, differ between UbA- versus TRA-mediated negative selection.
2. When clonal deletion is impaired, other mechanisms are utilized to maintain self-tolerance. However, impairment in multiple mechanisms of self-tolerance significantly increases the risk of developing autoimmunity.

CHAPTER 2: MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD) or Jackson Laboratories (Bar Harbor, ME). HY^{cd4} mice (77) and $Bim^{-/-}$ mice (141) have been previously described. Nur77-transgenic (Nur77-FL) mice (172) were a gift from Dr. Astar Winoto (University of California, Berkeley). $Nur77^{-/-}$ mice (177) and Rip-mOva mice (85) were purchased from Jackson Laboratories. OT-I mice (21) were a gift from Dr. Maureen McGargill (St. Jude Children's Research Hospital). Mice were interbred to generate the strains used in our studies. As byproducts of breeding, mice that were negative for transgenes or deletions were sometimes used as WT controls. Mice were bred and maintained in our colony at the University of Alberta in accordance with protocols approved by the University of Alberta Animal Care and Use Committee, and generally used between 4-12 weeks of age for experiments.

Bone marrow chimeras

To study the effect of transgenic Nur77 expression on T cell lymphopoiesis: $CD45.1^{+}$ WT and $CD45.2^{+}$ Nur77-FLbone marrow were mixed 50:50 and transferred into lethally irradiated $CD45.1^{+}$ WT recipients.

To study positive and negative selection using HY^{cd4} TCR transgenic mice: HY^{cd4}, HY^{cd4} $Nur77^{-/-}$, or HY^{cd4} $Bim^{-/-}$ F bone marrow was mixed with non-TCR transgenic (WT) F or M bone marrow at a ratio of 40:60 and transferred into lethally irradiated WT F or M recipients (sex-

matched based on WT donor bone marrow). For HY^{cd4} WT and DKO mixed chimeras, we transferred HY^{cd4} F ($CD45.1^{+}CD45.2^{+}$), HY^{cd4} $Bim^{-/-}$ $Nur77^{-/-}$ F ($CD45.1^{-}CD45.2^{+}$), and WT F or M ($CD45.1^{+}CD45.2^{-}$) bone marrow in a 20:20:60 ratio into WT ($CD45.1^{+}CD45.2^{-}$) F or M recipients.

To study positive and negative selection using OT-I TCR transgenic mice: OT-I $Nur77^{+/-}Bim^{+/-}$, OT-I $Nur77^{-/-}Bim^{+/-}$, OT-I $Bim^{-/-}Nur77^{+/-}$, or OT-I $Nur77^{-/-}Bim^{-/-}$ bone marrow was transferred into lethally irradiated WT or Rip-mOva recipients.

T cells were depleted from bone marrow donors by intraperitoneal administration of 100 μg of anti-Thy1.2 (clone 30H12) 1 and 2 days prior to bone marrow harvest, and additionally on day +1 in HY^{cd4} WT and $Bim^{-/-}Nur77^{-/-}$ mixed recipients and on days +1 and +2 in OT-I recipients. Irradiation of recipients was achieved by two exposures to 450 rad four hours apart. Chimeras were given water containing neomycin trisulfate (40 mg/L) and polymixin B sulfate (15 mg/L), both purchased from Sigma-Aldrich, or water containing novo-trimel (via University of Alberta Animal Services) for the first 4 weeks post-transplantation. Chimeras were harvested between 8-14 weeks after reconstitution for analysis.

Blood glucose monitoring

OT-I chimeras were monitored weekly for blood glucose levels using a One-Touch UltraMini system with OneTouch Ultra Test Strips starting from 2 weeks and up to 14 weeks after bone marrow reconstitution. Blood samples were obtained by tail vein bleeding. If blood glucose

exceeded 15 mmol/L, another reading would be taken 24 h later. Mice were considered diabetic upon two consecutive readings of over 15 mmol/L.

Tissue collection

Mice were anesthetized with isoflurane and subsequently euthanized via CO_2 asphyxiation. Thymus, spleen, and lymph nodes were harvested and gently ground into wire mesh screens filled with Hank's Balanced Salt Solution (HBSS) to achieve single cell suspensions. If samples were needed for cell culture, cells would be collected in sterile RP10 (RPMI 1640 with 2.05 mM L-glutamine from HyClone, 10% fetal calf serum (FCS), 5 mM HEPES, 50 mM 2-mercaptoethanol, 50 mg/mL penicillin/streptomycin, 50 mg/mL gentamycin sulfate) instead. Cellularity was determined by Trypan Blue exclusion in a hemocytometer (Fisher Scientific) under a light microscope (Zeiss).

***In vitro* apoptosis assay**

Wells were coated with functional grade anti-CD3ε (10 μg/mL), anti-CD28 (50 μg/mL), and anti-CD2 (10 μg/mL), all purchased from eBioscience, or with PBS overnight at 4°C. Wells were washed three times with PBS and blocked with 2% BSA/PBS for 30 minutes at 37°C prior to seeding with primary thymocytes freshly isolated from mice. Thymocytes were cultured for 6 h or 24 h at 37°C and 5% CO_2 in RP10 cell culture media, in the presence of 50 μM Z-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK) (Promega) or an equivalent volume of dimethyl sulfoxide (DMSO) vehicle control (Sigma). After culture, cells were labeled with 10 nM tetramethylrhodamine ethyl ester (TMRE) (Invitrogen) in RP10 for 15 minutes at 37°C prior to antibody staining. Annexin V (AV) (eBioscience) and cleaved caspase-3 (Cell Signaling Technology) staining was performed as per manufacturer's instructions.

Stimulation of HYβ splenocytes

Splenocytes were harvested from various HYβ strains, mixed with WT F splenocytes at a 4:1 ratio, and stimulated with various concentrations of smcy peptide (KCSRNRQYL; ProImmune). The cultures were harvested following 24 h of stimulation and CD69 induction was assessed by flow cytometry.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and stimulation of OT-I splenocytes

Stimulator splenocytes were harvested from B6 mice, washed, and treated with ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood cells. The desired number of stimulator splenocytes were resuspended at 2 x 10^7 cells/mL in pure FCS and pulsed with 100 nM Ova peptide (SIINFEKL; gift from Dr. Hanne Ostergaard) or left unloaded for 1 h at 37°C. After Ova peptide loading, stimulators were washed and resuspended in RP10 for culture. Effector splenocytes from OT-I chimeras were harvested, treated with ACK lysis buffer, washed with PBS, and labeled with 1.25 μM CFSE in PBS at 1 x 10^7 cells/mL for 10 minutes at 37°C. After CFSE labeling, effectors were quenched with RP10 and resuspended in RP10 for culture. Effectors were mixed with stimulators at a ratio of 4:1 and cultured at 37°C. Cells were harvested at 24 h, 48 h, and 72 h post-stimulation to be analyzed for CD69 induction and CFSE dilution by flow cytometry.

Cytokine assay

OT-I effectors and B6 stimulators were cultured as described above. On day 5 post-stimulation, those wells that were originally stimulated with Ova were resuspended in 1:1000 Brefeldin A (eBioscience) in RP10, with or without 100 nM of Ova. Cells were cultured for 4 h at 37°C and then utilized for intracellular cytokine staining by flow cytometry.

Antibodies and flow cytometry

For each staining sample, 2 x 10^6 cells were aliquoted per well in 96-well plates. Prior to antibody staining, cells were incubated with anti-Fc receptor (supernatant from 24G.2 hybridoma cells) at a 1:200 dilution in FACS Buffer (PBS, 1% FCS, 0.02% sodium azide, 1 mM EDTA, pH 7.2) for 10 minutes at 4°C. Cells were stained in FACS buffer with antibodies or reagents according to manufacturer's instructions or empirically derived concentrations. Experiments involving TMRE utilized FACS Buffer without sodium azide. Intracellular staining for non-transcription factor proteins was performed using the BD Fixation/Permeabilization kit. Staining for BH3 Bcl-2 and total Bcl-2 additionally involved incubation with 5% goat serum as previously described (163). Intracellular staining for transcription factors was performed using the eBioscience Foxp3 Staining Buffer Set. D^b/smcy pentamers were purchased from ProImmune and used as previously described (77). Anti-cleaved caspase 3 (Asp175) and anti-Bim (clone C34C5) were purchased from Cell Signaling Technology. Anti-Bcl-2 (BH3 domain specific) was purchased from Abgent. Secondary antibodies were purchased from Invitrogen and Jackson Laboratories. CXCR5-biotin was purchased from BioLegend. Vβ8-biotin and anti-Bcl2-PE (total Bcl-2) was purchased from BD Pharmingen. All other antibodies were purchased from

eBioscience. Cell events were collected on a BD FACSCanto II or BD LSRFortessa and analyzed with FlowJo software (Treestar Inc.).

In gating for specific populations, refer to each figure legend for the markers used. Unless otherwise stated, lineage markers are not excluded. For staining of DN thymocytes and T cells, anti-CD8α was used.

Statistical analysis

Mean, standard deviation and p-values were determined using Prism software (GraphPad Software Inc.). P-values were calculated using a two-tailed unpaired *t* test with 95% confidence interval.

CHAPTER 3: RESULTS - The role of Bim and Nur77 in MHC class I-restricted UbA-mediated negative selection

This chapter contains content from the following sources, republished with permission:

- Hu, Q., A. Sader, J. C. Parkman, and T. A. Baldwin. 2009. Bim-mediated apoptosis isnot necessary for thymic negative selection to ubiquitous self-antigens. *J Immunol*183: 7761-7767. ***Copyright 2009. The American Association of Immunologists, Inc.***
- Hu, Q. and T. A. Baldwin. 2015. Differential roles for Bim and Nur77 in negative selection against ubiquitous self-antigen. *J Immunol* (in press). ***Copyright 2015. The American Association of Immunologists, Inc.***

Previous studies have examined the roles of Bim and Nur77 in negative selection using a variety of model systems. Given that Bim and Nur77 are linked to apoptosis, studies have evaluated deletion of DP thymocytes when characterizing their contribution to negative selection (141, 172, 178, 229). By examining thymocyte numbers, studies have demonstrated a role for Bim in clonal deletion (141, 229). However, negative selection is intact if thymocytes bearing self-reactive TCRs are ultimately prevented from entering the peripheral pool as mature and functional T cells (62). By this definition, the absence of Bim does not significantly impair negative selection, as evidenced by limited increases in the number of superantigen-reactive CD4SP and CD8SP thymocytes (142). An important drawback common to these studies is that the model systems used do not reflect pMHC-mediated negative selection at the DP stage. Likewise, the role of Nur77 in negative selection was also evaluated using non-physiological TCR transgenic models (172, 178). Therefore, we revisited the role of Bim and Nur77 in

negative selection using the physiological HY^{cd4} mouse model designed to correct the problem of premature TCR expression and selection (77).

Bim is not required for UbA-mediated clonal deletion in the HY^{cd4} model

Differing conclusions on the role of Bim in clonal deletion indifferent model systems (141, 142) prompted us to examine the matter using the physiological HY^{cd4} model of UbA-mediated negative selection. While both HY^{cd4} F and HY^{cd4} $Bim^{-/-}$ F mice generated antigen-specific ($T3.70^{+}$) DP and CD8SP populations, HY^{cd4} M and HY^{cd4} $Bim^{-/-}$ M mice contained mostly DP thymocytes and largely lacked CD8SP thymocytes (**Fig. 3-1A**). In either HY^{cd4} M or HY^{cd4} $Bim^{-/-}$ M mice, the absolute number of $T3.70^{+}$ CD8SP was greatly reduced compared to female counterparts (**Fig. 3-1B**). These data indicate that Bim is not required for UbA-mediated clonal deletion. We also observed a reduction in the number of $T3.70^{+}$ DP thymocytes in HY^{cd4} M compared to HY^{cd4} F control mice (**Fig. 3-1C**). In contrast, the number of $T3.70^{+}$ DP thymocytes was similar between female and male Bim-deficient mice, suggesting that deletion is delayed in the absence of Bim. Though HY^{cd4} $Bim^{-/-}$ M mice contained a modest increase in the number of $T3.70^{+}$ CD8SP thymocytes compared to HY^{cd4} M mice, HY^{cd4} $Bim^{-/-}$ M mice also contained a higher number of $T3.70^{+}$ DP thymocytes as well. By controlling for the effect of Bim deficiency on precursor populations, clonal deletion efficiency was ultimately similar between HY^{cd4} M and HY^{cd4} $Bim^{-/-}$ M mice, as evidenced by a similar reduction in the number of $T3.70^{+}$ CD8SP relative to female controls. Furthermore, the proportion of $T3.70^{+}$ CD8SP bearing a mature $CD24^{lo}$ phenotype was ultimately similar in Bim-sufficient or deficient male mice (**Fig. 3-1D**). Whereas Bim deficiency had no significant effect on clonal deletion, positive selection efficiency was increased in the absence of Bim, as evidenced by similar numbers of $T3.70^{+}$ DP precursors

yet significantly higher numbers of T3.70^{+} CD8SP in HY^{cd4} Bim$^{-/-}$ F compared to HY^{cd4} F mice (**Fig. 3-1B and 3-1C**). Additionally, a higher proportion of T3.70^{+} CD8SP in HY^{cd4} Bim$^{-/-}$ F mice were CD24^{lo} compared to those found in HY^{cd4} F mice (**Fig. 3-1D**). We speculate that Bim deficiency may extend the lifespan of DP thymocytes, thereby increasing their chance of encountering positively-selecting ligands, but does not prevent negative selection from occurring.

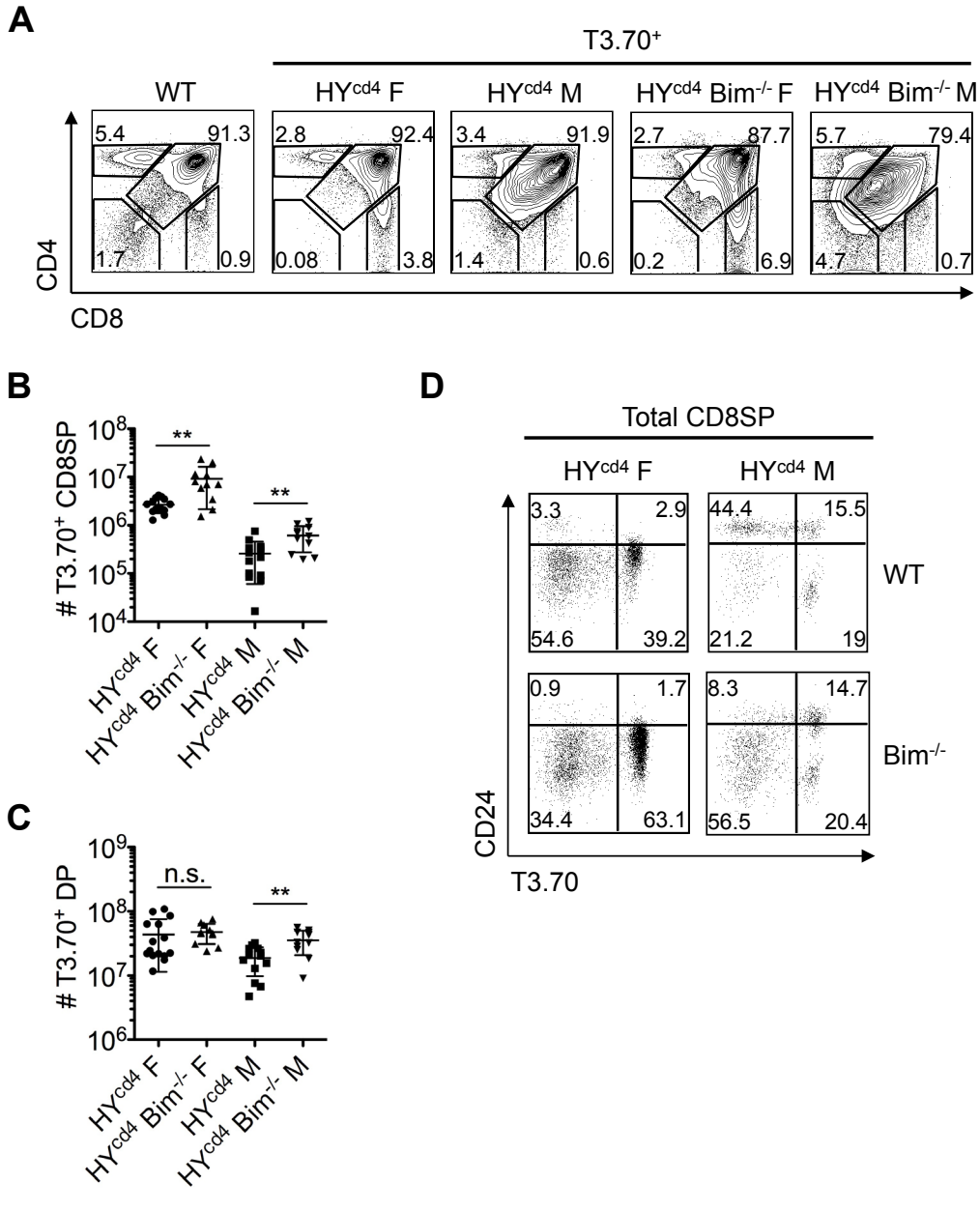


Figure 3-1. Bim is not required for UbA-mediated clonal deletion in the HY^{cd4} model. (A) Representative CD8 by CD4 profiles of total (WT mice) or T3.70^{+} (HY^{cd4} mice) thymocytes from each mouse strain. **(B)** The absolute number of T3.70^{+} CD8SP and **(C)** T3.70^{+} DP from indicated strains. Data are representative of a minimum of 11 mice per strain from 11 independent experiments. (**D)** Expression of CD24 and T3.70 among total CD8SP thymocytes, representative of at least 5 mice per strain from 5 independent experiments. Data is depicted as mean ± SD; ** $p < 0.01$; n.s. not significant.

Bim is not required for UbA-mediated clonal deletion in an oligoclonal repertoire

We sought to confirm that UbA-mediated clonal deletion remains intact in the absence of Bim by characterizing negative selection of male-reactive thymocytes in an oligoclonal repertoire. To this end, we utilized Vβ8 TCR transgenic mice that express the TCRβ chain derived from the HY TCR (henceforth referred to as HYβ). In female HYβ mice, pairing of the transgenic TCRβ with endogenous TCRα chains generates T cells with various affinities for male antigen (smcy peptide in particular), among other specificities (180). Using pentamers of H-2D^{b}/smcy complexes, we also found an elevated frequency of male-reactive CD8^{+} T cells in HYβ F compared to B6, and a reduction in this population in HYβ M mice that is indicative of clonal deletion (**Fig. 3-2A and 3-2B**). Consistent with observations in the HY^{cd4} model, Bim deficiency did not impair elimination of male-reactive T cells in the HYβ model. Since negative selection is imperfect and low affinity clones can escape this process (179, 180), we sought to determine whether Bim deficiency influenced the affinity of the T cell clones that escape negative selection. To address this question, we assessed CD69 induction in response to increasing concentrations of smcy peptide, a male-specific antigen to which Vβ8^{+} F cells respond (180). At 24 h post-stimulation in HYβ F mice, a population of CD8SP thymocytes began to induce CD69 at a peptide concentration of 10^{-8} M, while CD8SP thymocytes from HYβ M and HYβ Bim$^{-/-}$ M mice did not begin to induce CD69 until a peptide concentration of 10^{-6} M (**Fig. 3-2C**). This indicates that the CD8SP thymocytes that escape negative selection in male mice have a 100-fold reduction in affinity for smcy compared to female mice. Furthermore, Bim deficiency did not influence the affinity of the escapees, supporting the conclusion that UbA-mediated negative selection does not require Bim.

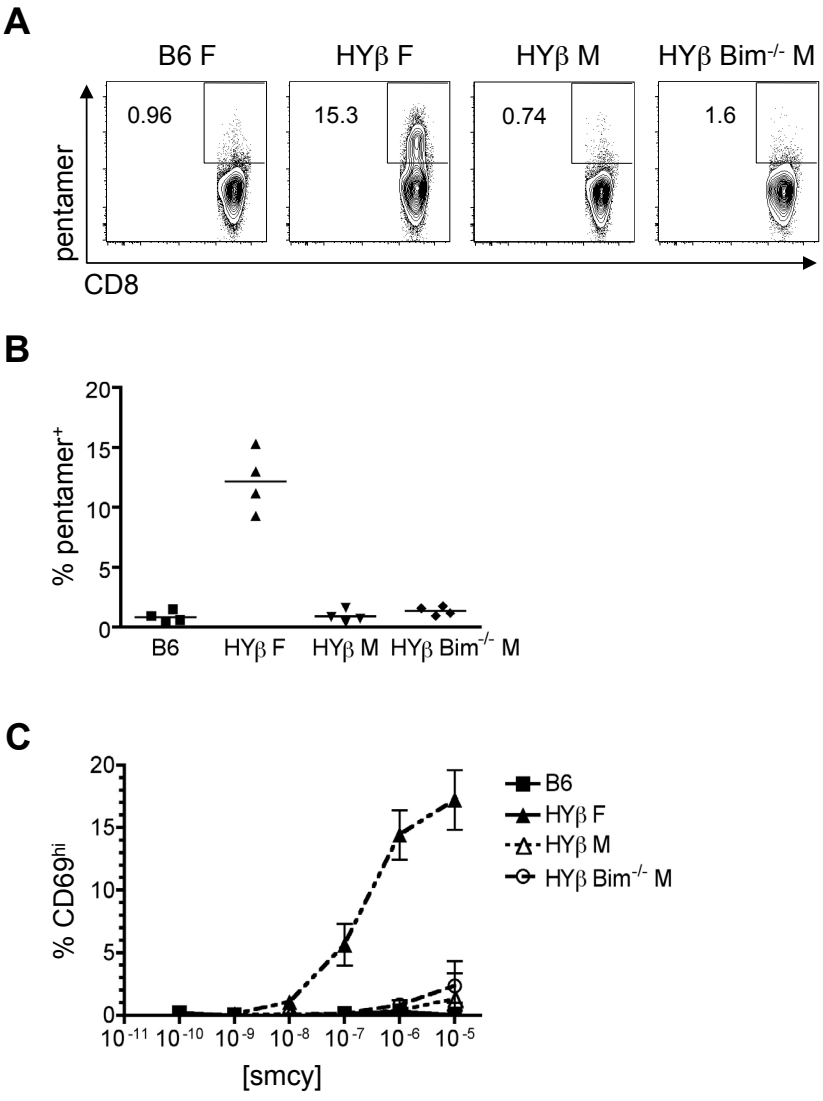


Figure 3-2. Bim is not required for UbA-mediated clonal deletion in an oligoclonal repertoire. (A) CD8 by D^b/smcy pentamer profiles of *ex vivo* splenocytes from indicated strains. **(B)** Compilation of percentage of pentamer-binding $CD8^+$ splenocytes from 4 mice in 4 independent experiments. **(C)** CFSE-labeled thymocytes from the indicated mice were cultured with B6 female splenocytes and the indicated concentration of smcy peptide for 24 h. The cells were harvested and induction of CD69 on CD8SP thymocytes was measured by flow cytometry. The percentage of CD8SP thymocytes that induced CD69 is depicted in the graph as the mean ± S.D. Data are compiled from at least 2 mice/strain in 3 independent experiments.

Accumulation of high-affinity signaled thymocytes in HY^{cd4} $Bim^{-/-}$ M mice

Since $T3.70^{+}$ DP thymocyte numbers suggested that deletion may be delayed when Bim is absent (**Fig. 3-1C**), we looked for differences in the phenotype of DP thymocytes from HY^{cd4} M vs. HY^{cd4} $Bim^{-/-}$ M mice. The maximum amount of CD69 and CD5 induced on $T3.70^{+}$ DP thymocytes was not different with or without Bim (**Fig. 3-3A**), indicating that thymocytes from both mice experienced similar TCR signal strength. However, the frequencies of $CD69^{+}$ and $CD5^{+}$ $T3.70^{+}$ DP thymocytes were modestly higher in HY^{cd4} $Bim^{-/-}$ M mice, consistent with delayed deletion of TCR-signaled thymocytes. In further support of those being longer-lived thymocytes, HY^{cd4} $Bim^{-/-}$ M mice showed a higher frequency of $T3.70^{+}$ DP expressing PD-1 (**Fig. 3-3A**), a marker that is maximally induced 48 h post-TCR stimulation (64). Because these markers can be induced under conditions other than negative selection (176, 232, 233), we also examined induction of Helios, a protein specifically induced by high affinity but not low affinity TCR signaling (19). We found that the proportion of $T3.70^{+}$ DP that are $Helios^{+}$ to those that are $Helios^{-}$ is approximately 2:1 in HY^{cd4} $Bim^{-/-}$ M mice compared to 1:1 in HY^{cd4} M mice (**Fig. 3-3B**). Similarly, a greater proportion of $T3.70^{+}$ CD8SP was $Helios^{+}$ in the absence of Bim. Collectively, these data indicate an accumulation of high affinity TCR-signaled DP thymocytes in HY^{cd4} $Bim^{-/-}$ M mice, consistent with delayed clonal deletion in the absence of Bim.

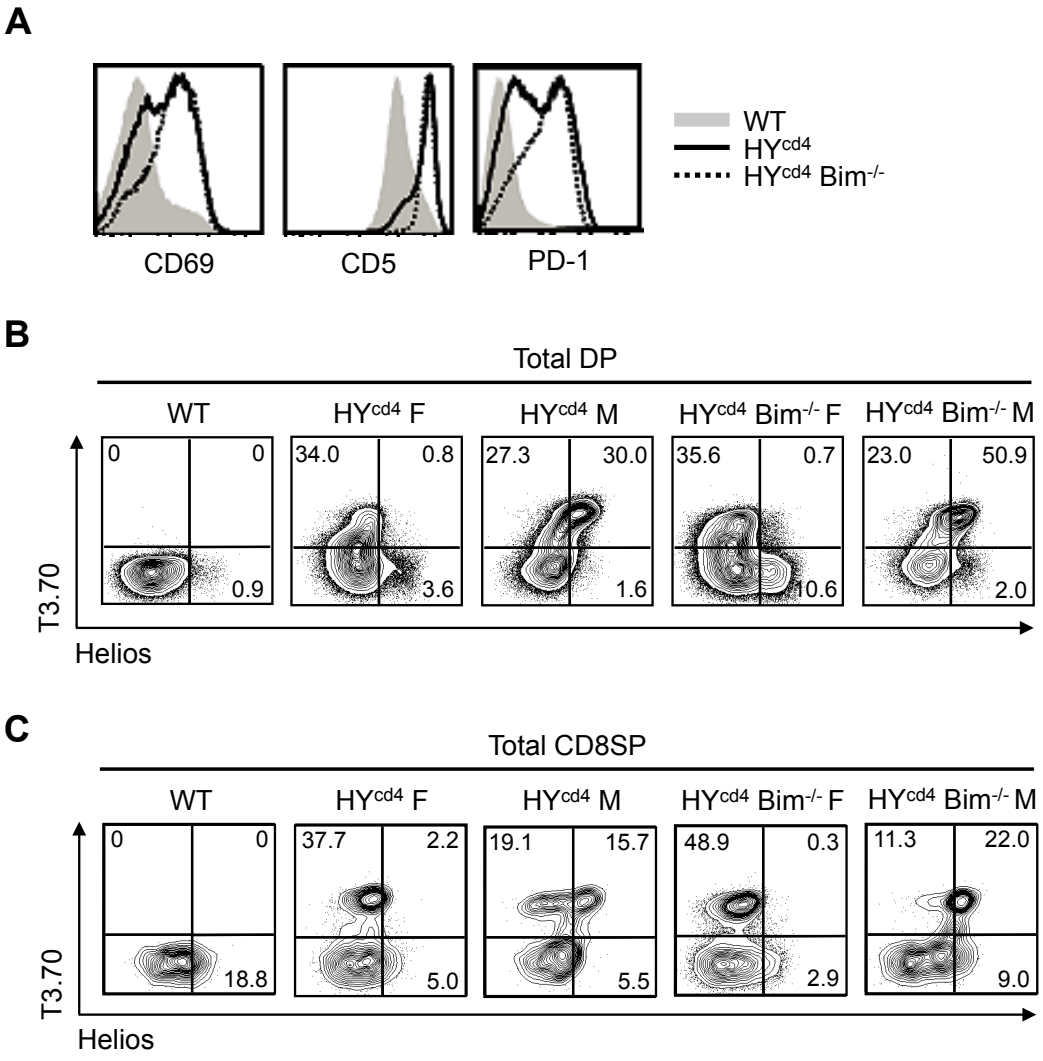


Figure 3-3. Accumulation of high affinity TCR-signaled thymocytes in the absence of Bim. **(A)** Expression of CD69, CD5, and PD-1 on total (WT) or T3.70^{+} (HY^{cd4}) DP thymocytes from indicated strains. Data are representative of 4 independent experiments. **(B)** Expression of Helios and T3.70 among total DP thymocytes and **(C)** total CD8SP thymocytes. Data represents a minimum of 3 mice from each strain from independent experiments.

Caspase-3-independent cell death during Bim-independent clonal deletion *in vivo*

Despite evidence that Bim-independent deletion is delayed, clonal deletion still likely occurred in HY^{cd4} $Bim^{-/-}$ M mice given the paucity of antigen-specific CD8SP thymocytes. To gain insight into the mechanism of Bim-independent deletion, we measured various features of cell death in DP thymocytes. As expected, we found that the frequency of $T3.70^{+}$ DP thymocytes with cleaved caspase-3 was significantly higher during negative selection in HY^{cd4} M mice compared to positive selection in HY^{cd4} F mice (**Fig. 3-4A and 3-4B**). However, baseline and high affinity TCR-induced caspase-3 activation were nearly abrogated in HY^{cd4} $Bim^{-/-}$ F and M mice, respectively. Despite the lack of caspase-3 activation, phosphatidylserine exposure still occurred during negative selection *in vivo*, as evidenced by an increased frequency of AV^{+} $T3.70^{+}$ DP in HY^{cd4} M and HY^{cd4} $Bim^{-/-}$ M mice compared to female counterparts (**Fig. 3-4A and 3-4C**). Since studies suggest that caspase-3 is the predominant executioner caspase in thymocytes (96, 98, 234), these data support a caspase-independent mechanism of UbA-mediated clonal deletion. Abrogation of caspase-mediated death may account for the delayed deletion of DP thymocytes. The modest increase in the frequency of AV^{+} $T3.70^{+}$ DP in HY^{cd4} $Bim^{-/-}$ M over HY^{cd4} M may also reflect accumulation of thymocytes destined to die (**Fig. 3-4A and 3-4C**).

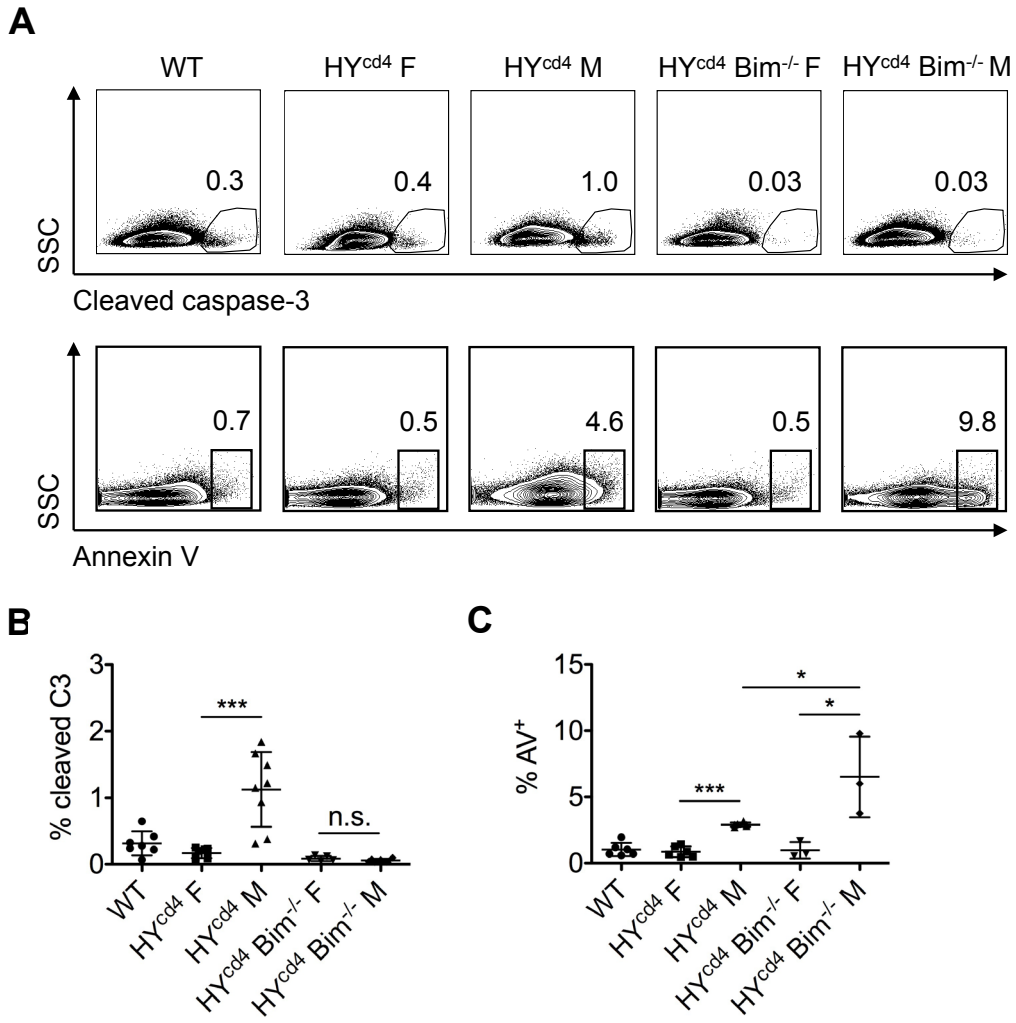


Figure 3-4. Caspase-3-independent cell death during Bim-independent clonal deletion *in vivo*. (A) Representative cleaved caspase 3 (C3) by side scatter (SSC) and Annexin V (AV) by SSC profiles of total (WT) or T3.70^{+} (HY^{cd4}) DP thymocytes from indicated mice. The frequency of total or T3.70^{+} DP thymocytes with cleaved caspase 3 **(B)** and Annexin V binding **(C)** were compiled. Data are compiled from at least 6 mice per strain in 6 independent experiments for cleaved caspase 3 and from at least 3 mice per strain in 3 independent experiments for Annexin V. Data depicts the mean ± S.D. * $p < 0.05$; *** $p < 0.001$; n.s. not significant.

Bim is not required for Nur77 induction upon TCR signaling

Since Nur77 is thought to be a mediator of negative selection (235) as well as caspase-independent cell death (169-171), we investigated the potential role for Nur77 in mediating clonal deletion in the HY^{cd4} $Bim^{-/-}$ M mice. Consistent with microarray data from various models of negative selection including the HY^{cd4} model (81, 125-129), we found that Nur77 protein expression was higher in DP thymocytes from male mice undergoing negative selection than female mice undergoing positive selection (**Fig 3-5A and 3-5B**). Overall, Nur77 induction during negative selection in HY^{cd4} $Bim^{-/-}$ M DP thymocytes was mildly, but not significantly, lower than in HY^{cd4} M DP thymocytes (**Fig 3-5B**). In addition to *ex vivo* TCR transgenic thymocyte analysis, we utilized plate-bound antibodies to stimulate polyclonal thymocytes in order to synchronize the duration of TCR stimulation received by WT and $Bim^{-/-}$ thymocytes. Along with anti-CD3 and anti-CD28, we included anti-CD2 since it had previously been used to enhance TCR signaling *in vitro*, perhaps by increasing Lck association with the TCR signaling complex (236). Though robust induction of Nur77 is observed as early as 2 h post-stimulation (160, 236), we chose to characterize thymocytes 6 h post-stimulation because preliminary data suggested that this was an optimal time point for detection of TCR-specific phosphatidylserine exposure. After 6 h of TCR stimulation, Nur77 induction was modestly, but not significantly, lower among $Bim^{-/-}$ DP thymocytes compared to WT DP thymocytes (**Fig. 3-5C, left**). Though Nur77 expression decreased by 24 h post-stimulation, TCR-specific Nur77 induction was still evident, now being modestly higher among $Bim^{-/-}$ DP thymocytes (**Fig. 3-5C, right**). Collectively, these data show that Bim deficiency has no significant impact on Nur77 induction. We therefore proceeded to investigate Nur77 as a candidate for mediating Bim-independent clonal deletion.

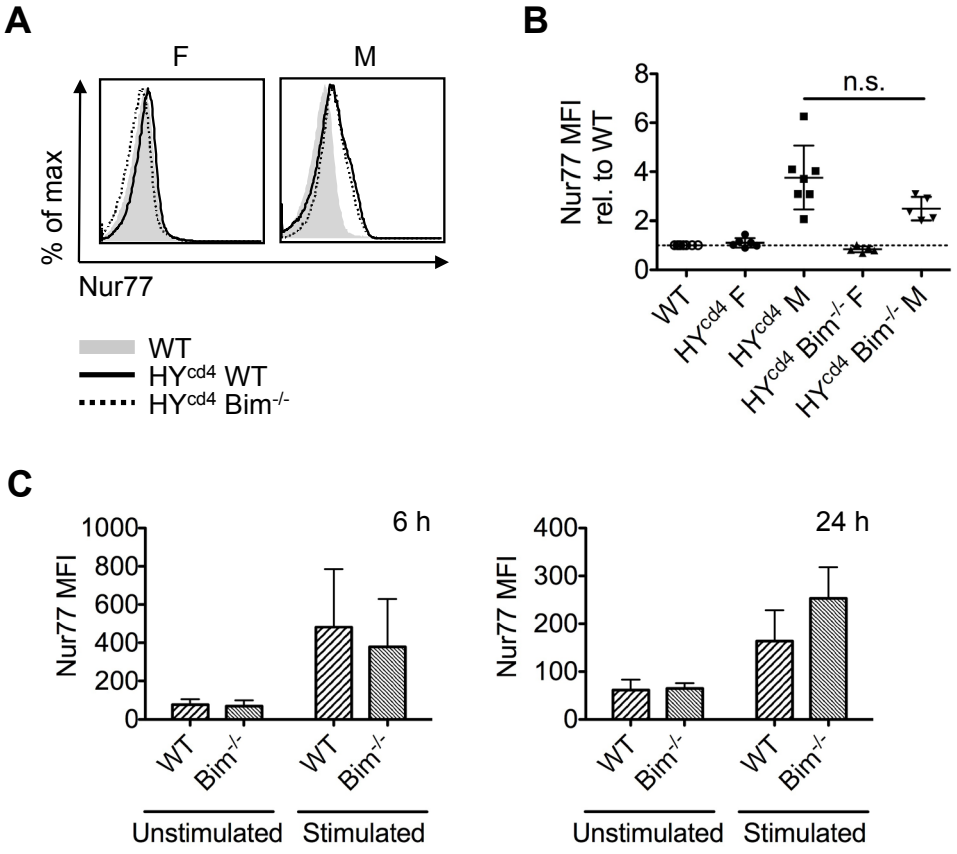


Figure 3-5. Bim is not required for Nur77 induction upon TCR signaling. Thymocytes were assessed for Nur77 protein expression by antibody staining. *Ex vivo* Nur77 expression in total (WT) or T3.70^{+} (HY^{cd4}) DP from indicated strains is depicted as representative histograms **(A)** or as fold change in MFI over that of WT **(B)**. Data represents a minimum of 5 mice per strain from at least 4 independent experiments. (**C)** *In vitro* Nur77 induction in DP thymocytes from non-TCR transgenic WT or Bim$^{-/-}$ mice stimulated with plate-bound anti-CD3ε (10 μg/mL), anti-CD28 (50 μg/mL), and anti-CD2 (10 μg/mL), or left unstimulated in PBS for indicated durations at 37°C. Data represents a minimum of 4 per strain from 4 independent experiments. Data is depicted as mean ± SD; n.s. not significant.

Bim but not Nur77 is important for TCR-induced caspase-mediated and caspase-independent death *in vitro*

Another advantage of the *in vitro* stimulation model was the ability to add the pan-caspase inhibitor Z-VAD-FMK to the cell culture. This allowed us to evaluate the contribution of Bim and Nur77 to TCR-induced caspase-mediated and caspase-independent thymocyte death. The presence of Z-VAD-FMK in the cell culture was effective at abrogating basal and TCR-induced caspase-3 activation even after 24 h (**Fig. 3-6**). Bim-deficient thymocytes showed no increase in caspase-3 activation upon TCR stimulation, supporting the conclusion that Bim is required for caspase-mediated clonal deletion *in vivo*. In contrast, Nur77 deficiency did not impair caspase-3 activation (**Fig. 3-6**). We next examined two features reported to occur during CICD: loss of mitochondrial membrane potential and phosphatidylserine exposure on the cell surface (114, 237), as indicated by inability of cells to retain TMRE and positive labeling with AV, respectively. Though both processes also occur during caspase-mediated apoptosis, measuring these features in the context of pan-caspase inhibition with Z-VAD-FMK would permit characterization of TCR-induced caspase-independent death. When caspase activity was inhibited, we observed a modest decrease in the frequency of $TMRE^{lo}$ WT DP after 6 h of stimulation (**Fig. 3-7A, left**), though levels were equivalent to control WT DP after 24 h (**Fig. 3-7A, right**). Similar trends were observed with AV labeling (**Fig. 3-7B**), consistent with phosphatidylserine exposure in the absence of caspase-3 activation *in vivo* (**Fig 3-4**) and supporting the conclusion that TCR-induced caspase-independent death is delayed. Surprisingly, we found that abrogation of caspase activity did not impair other features of cell death to the same degree as Bim deficiency. Specifically, while loss of mitochondrial membrane potential and phosphatidylserine exposure occurred in Z-VAD-FMK-treated WT DP, both of these

features were significantly reduced in $Bim^{-/-}$ DP, even when they were mediated by caspase-independent mechanisms (compare WT with Z-VAD-FMK and $Bim^{-/-}$ with Z-VAD-FMK) (**Fig. 3-7A and 3-7B**). Despite the role of Nur77 in caspase-independent death in other cell types (169-171), we found no reduction in the frequency of $TMRE^{lo}$ or AV^{+} DP in $Nur77^{-/-}$ Z-VAD-FMK-treated compared to WT Z-VAD-FMK-treated samples (**Fig. 3-7A and 3-7B**). These data indicate that Bim, but not Nur77, is a critical regulator of both caspase-mediated and caspase-independent death of thymocytes. Though reduced, increases in $TMRE^{lo}$ and AV^{+} DP were still observed upon TCR stimulation in the absence of Bim (1.4 and 1.5-fold over unstimulated, respectively, after 24 h), supporting the existence of a Bim- and caspase-independent cell death mechanism following TCR stimulation. Thus, these *in vitro* data strengthen our conclusion that clonal deletion in HY^{cd4} $Bim^{-/-}$ M mice was delayed but ultimately intact.

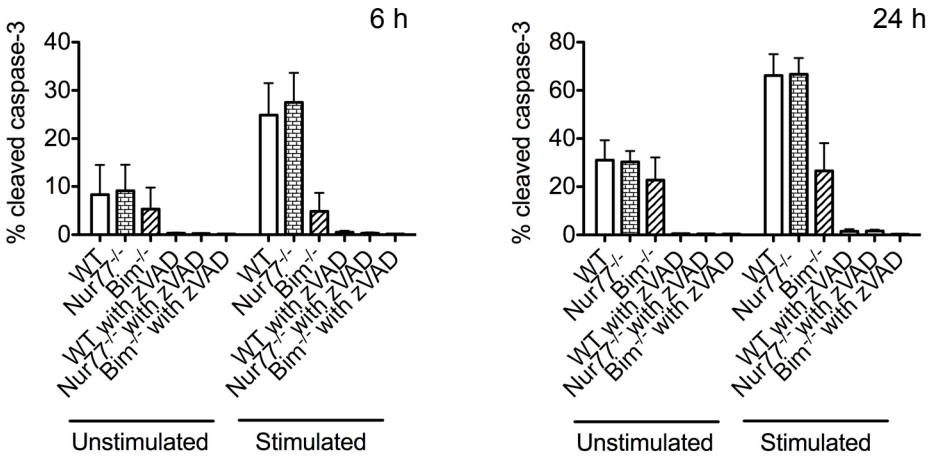


Figure 3-6. Bim is required for TCR-induced caspase-3 activation *in vitro*. Non-TCR transgenic WT, Nur77$^{-/-}$, or Bim$^{-/-}$ thymocytes were stimulated *in vitro* with plate-bound anti-CD3ε (10 μg/mL), anti-CD28 (50 μg/mL), and anti-CD2 (10 μg/mL) or left unstimulated, in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (zVAD), for the indicated durations at 37°C. After the stimulation period, cleaved caspase 3 was measured in DP thymocytes by flow cytometry. Data represents a minimum of 3 per strain from 3 independent experiments. Data is depicted as mean ± SD.

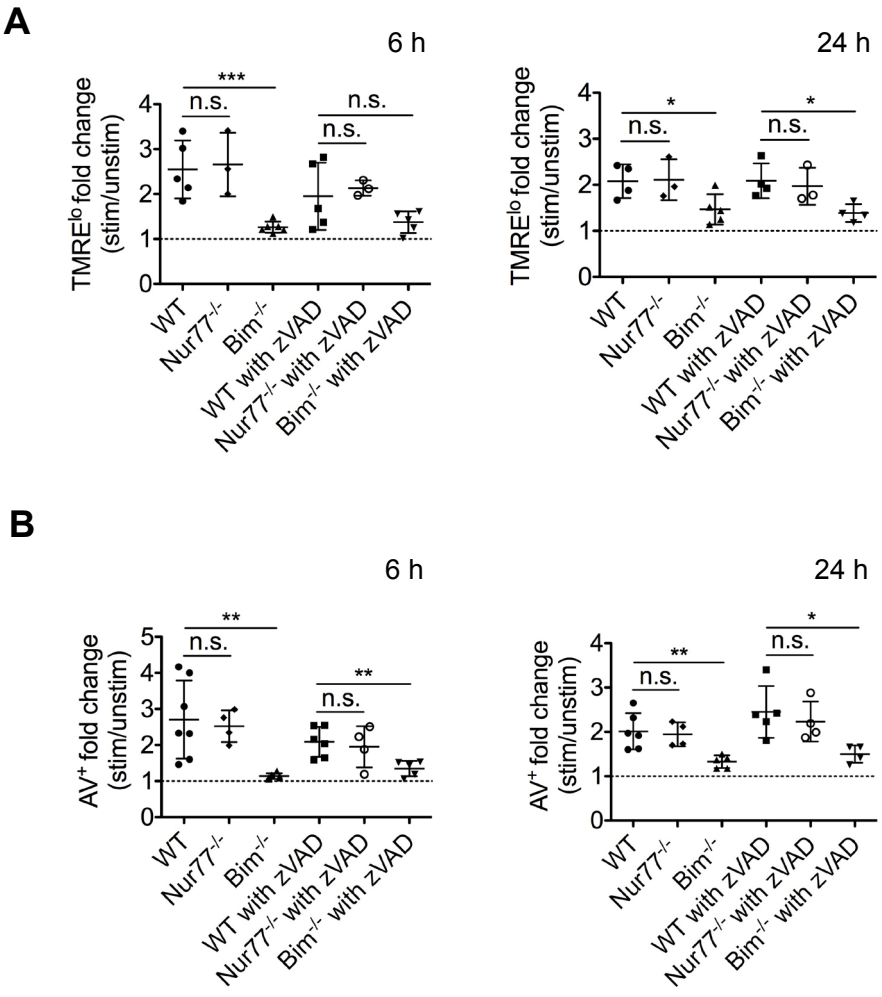


Figure 3-7. Bim but not Nur77 is important for TCR-induced caspase-mediated and caspase-independent death *in vitro*. Non-TCR transgenic WT, Nur77$^{-/-}$, or Bim$^{-/-}$ thymocytes were stimulated *in vitro* with plate-bound anti-CD3ε (10 μg/mL), anti-CD28 (50 μg/mL), and anti-CD2 (10 μg/mL) or left unstimulated, in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (zVAD), for the indicated durations at 37°C. After the stimulation period, DP thymocytes were assessed by flow cytometry for tetramethylrhodamine ethyl ester (TMRE) retention **(A)** and Annexin V binding (**B)**. Graphs depict the fold change in the frequency of TMRE^{lo} and AV^{+} DP thymocytes in stimulated over unstimulated samples. Data is depicted as mean ± SD and represents a minimum of 3 replicates from each condition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. not significant.

No conversion of Bcl-2 during negative selection in HY^{cd4} mice

While Nur77 was induced during negative selection *in vivo* and following TCR stimulation *in vitro* (**Fig. 3-5**), it was not able to compensate for Bim deficiency in activating caspase-3 (**Fig. 3-4 and 3-6**). Observations from the *in vitro* stimulation model further suggested that Nur77 is dispensable for TCR-induced death (**Fig. 3-7**), though Bim expression may have precluded revelation of Nur77's contribution. Though long associated with mediating T cell death (155, 156), the mechanism of action of Nur77 remains controversial. Two distinct mechanisms of Nur77-mediated thymocyte death have been proposed: induction of pro-apoptotic genes through its role as a transcription factor in the nucleus and conversion of Bcl-2 to a pro-apoptotic form via exposure of its Bcl-2 homology domain 3 (BH3) at mitochondria (159, 160, 163, 165). Seeing little evidence of a role for Nur77 in UbA-mediated clonal deletion thus far, we investigated its reported function in converting Bcl-2 in the HY^{cd4} model. Importantly, the conclusion that Bcl-2 is converted rested on data demonstrating that total Bcl-2 expression is unchanged (163). While we found a modest increase in binding of the Bcl-2 BH3 domain-specific antibody in HY^{cd4} WT and Bim$^{-/-}$ M DP over female counterparts, we also found increased total Bcl-2 expression (**Fig. 3-8A**). This is consistent with previous studies showing increased Bcl-2 mRNA following high affinity TCR stimulation (81, 125, 128). Therefore, in our hands, detection of the BH3 domain of Bcl-2 correlates with increased Bcl-2 expression rather than a bona fide 'conversion' event. When we calculated BH3 Bcl-2 mean fluorescence intensity (MFI) relative to total Bcl-2 MFI (depicted as arbitrary units), there was no significant difference between male and female DP, regardless of Bim deficiency (**Fig. 3-8B**). In fact, this ratio tended to be lower in male mice, supporting the conclusion that Bcl-2 conversion did not occur during

negative selection. These data are consistent with a lack of caspase-3 activation in HY^{cd4} $Bim^{-/-}$ M DP and argue against Nur77 as a mediator of Bim-independent clonal deletion.

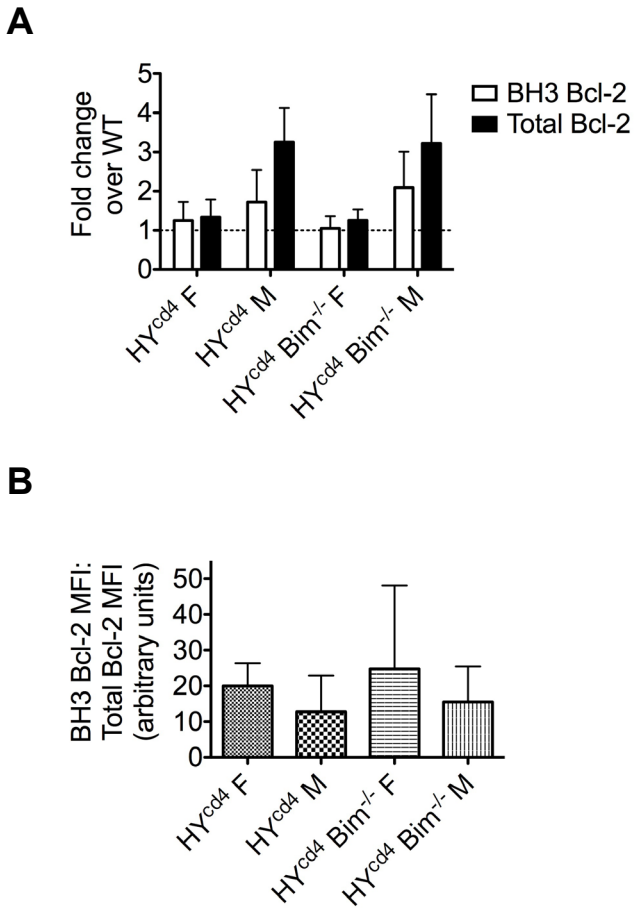


Figure 3-8. No conversion of Bcl-2 during negative selection in HY^{cd4} mice. Antibodies specific for the BH3 domain of Bcl-2 or another part of Bcl-2 ('total Bcl2') were used to label thymocytes *ex vivo*. **(A)** MFI for BH3 Bcl-2 or total Bcl-2 in T3.70^{+} DP from HY^{cd4} strains is depicted as fold change over MFI in total DP from WT mice. **(B)** The same data is depicted as a ratio of the MFI for BH3 Bcl-2 to the MFI of total Bcl-2 within each mouse. Data depicts as mean ± SD and represents a minimum of 4 mice per strain from 4 independent experiments.

Transgenic Nur77 mediates caspase-3 activation independently of Bim

We next investigated the possibility that Nur77 induction does not result in caspase-3 activation in HY^{cd4} $Bim^{-/-}$ M mice because Nur77 may require Bim as a downstream mediator. For example, it was found that Bim induction was reduced in Nur77-deficient thymocytes (160), which may speak to the transcription factor role ascribed to Nur77. To characterize the functional relationship between Nur77 and Bim, we utilized mice expressing a full-length Nur77 transgene (Nur77-FL) on Bim-sufficient and -deficient backgrounds. In Nur77-FL mice, the Nur77 transgene is driven by the $p56^{lck}$ promoter at the DN2/DN3 transition (238). By examining the ratio of WT vs. Nur77-FL-derived cells at various stages of T cell development in mixed bone marrow chimeras, we found that thymocytes first became sensitive to Nur77-mediated death between the DN3 and DN4 stages (**Fig. 3-9A**). Consequently, Nur77-FL mice exhibited severe reductions in the frequency and number of DP and SP thymocytes, regardless of Bim expression (**Fig. 3-9B and 3-9C**). Direct examination of caspase-3 activation in DP thymocytes showed that transgenic Nur77 expression was sufficient to activate caspase-3 independently of Bim (**Fig. 3-9D**). Therefore, dependency on Bim cannot explain the lack of Nur77-mediated caspase-3 activation during negative selection in HY^{cd4} $Bim^{-/-}$ M mice.

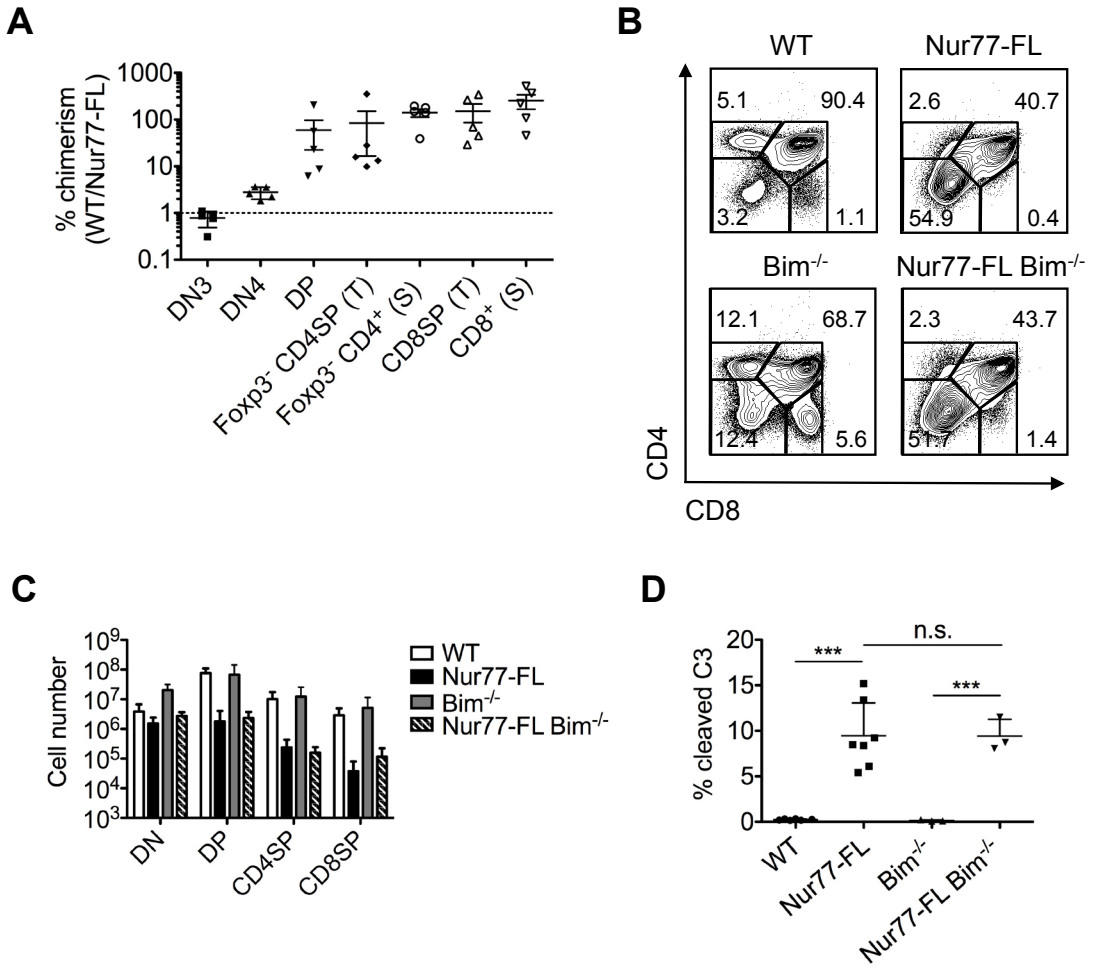


Figure 3-9. Transgenic Nur77 mediates caspase-3 activation independently of Bim. (A) Non-TCR transgenic CD45.1$^+$ WT and CD45.2$^+$ Nur77 transgenic (Nur77-FL) bone marrow were mixed at a ratio of 50:50 and transferred into lethally irradiated WT recipients. The contribution of WT vs. Nur77-FL bone marrow (% chimerism) in each T cell subset was calculated by dividing the ratio of %CD45.1$^+$ cells to %CD45.2$^+$ cells by the %CD45.1$^+$/%CD45.2$^+$ ratio from the CD19$^+$ B cell reference population. Data represent 5 chimeras generated from 2 independent donors. T, thymus; S, spleen. **(B)** CD8 by CD4 profiles of thymi from Nur77-FL mice on Bim$^{+/+}$ or Bim$^{-/-}$ backgrounds and their non-Nur77-transgenic counterparts. **(C)** The absolute number of cells in each thymocyte subset from indicated strains. **(D)** The frequency of DP thymocytes with cleaved caspase-3 from indicated strains. Data was obtained from a minimum of 3 mice per strain from 3 independent experiments and is depicted as mean ± SD; *** $p < 0.001$; n.s. not significant.

Transgenic Nur77-mediated caspase-3 activation is reduced in the context of TCR signaling

The ability of transgenic Nur77 to induce robust caspase-3 activation in DP thymocytes sharply contrasts the inability of endogenous Nur77 to activate caspase-3 in HY^{cd4} $Bim^{-/-}$ M DP thymocytes during negative selection. One key difference between the two settings is that transgenic Nur77 activity in Nur77-FL mice is generally not subject to regulation by TCR signaling, since expression of the Nur77 transgene precedes that of the TCR at the DP stage, and because many DP thymocytes do not experience productive TCR signaling in a polyclonal repertoire. In order to determine whether TCR signaling affects the function of Nur77, we crossed Nur77-FL mice onto the OT-I and HY^{cd4} TCR transgenic backgrounds. The OT-I TCR is expressed at the DN stage concurrent with the Nur77 transgene, whereas the HY TCR is expressed at the DP stage in the HY^{cd4} model. Transgenic Nur77 expression in polyclonal mice resulted in severely reduced thymic cellularity (**Fig. 3-10A**). Compared to polyclonal Nur77-FL mice, thymic cellularity was modestly but significantly rescued in HY^{cd4} Nur77-FL F and M mice and was approximately 10-fold higher in OT-I Nur77-FL mice. Consistent with increased cellularity, we found significantly decreased frequencies of cleaved caspase-3^{+} DP thymocytes in TCR transgenic Nur77-FL mice compared to polyclonal Nur77-FL mice (**Fig. 3-10B**). The decrease in Nur77-mediated death was not due to reduced Nur77 transgene expression, as evidenced by similar or higher expression of Nur77 in TCR transgenic DP thymocytes compared to polyclonal Nur77-FL DP thymocytes, while caspase-3 activation was disproportionately low. This observation was most dramatic in OT-I Nur77-FL mice, suggesting that earlier TCR expression, around the same time as the Nur77 transgene specifically, resulted in increased protection from apoptosis and rescue of thymocyte numbers. Despite the significant reduction in caspase-3 activation in OT-I Nur77-FL DP, progression to SP stages was still largely blocked by

transgenic Nur77 expression (**Fig. 3-10C**). This may be due to Nur77-mediated caspase-3 activation, which was still higher than in non-Nur77-transgenic mice, and/or unknown functions unrelated to apoptosis. Nevertheless, our data suggested that provision of productive TCR signaling reduced the potency of Nur77-mediated apoptosis. As an internal control, we compared apoptosis among *ex vivo* pre-selection ($CD69^-$) and post-selection ($CD69^+$) polyclonal Nur77-FL DP thymocytes. Consistent with the TCR transgenic data, we found that the frequency of caspase-3 activation was significantly lower among $CD69^+$ Nur77-FL DP thymocytes despite having similar Nur77 expression as $CD69^-$ Nur77-FL DP thymocytes (**Fig. 3-10D**). Whether through direct or indirect mechanisms, these data suggest that TCR signaling inhibits the pro-apoptotic function of Nur77, providing a potential explanation for the lack of caspase-3 activation during Bim-independent negative selection *in vivo*.

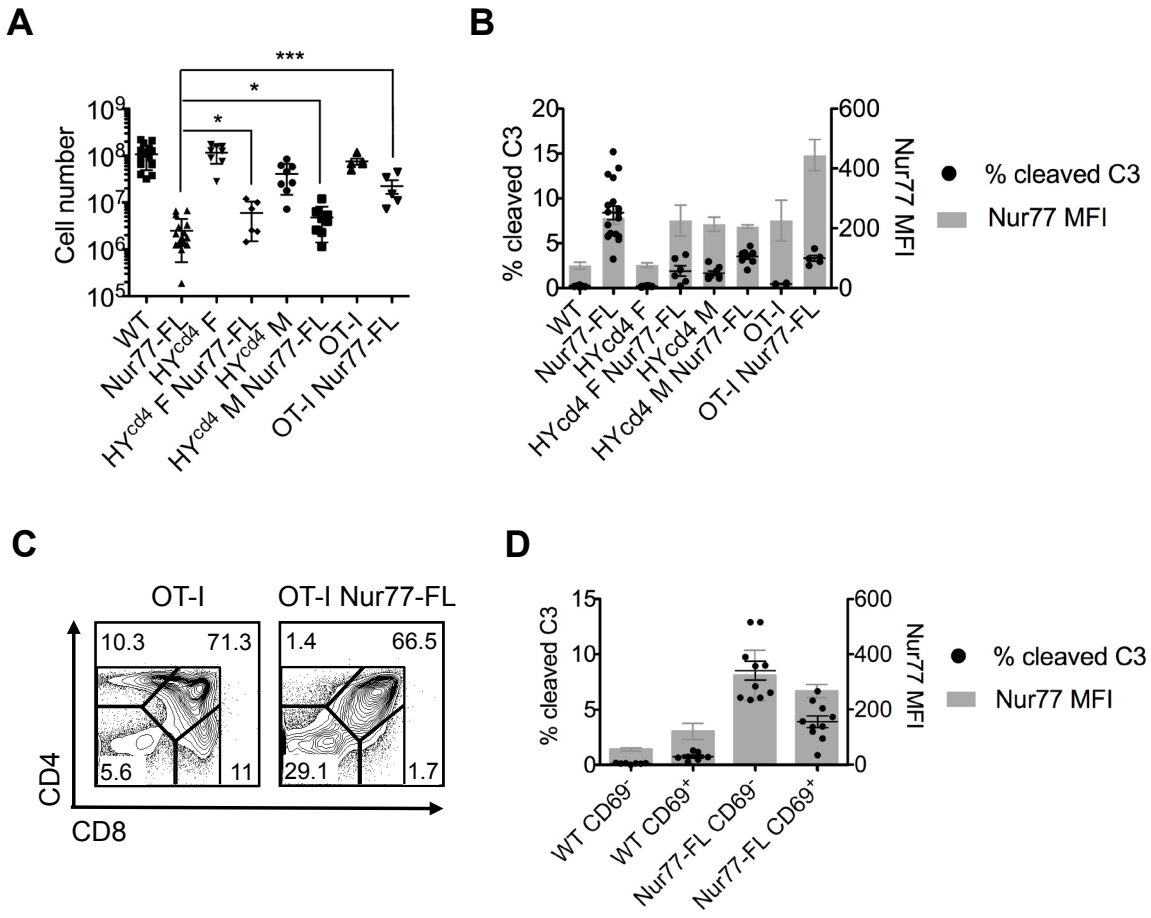


Figure 3-10. Transgenic Nur77-mediated caspase-3 activation is reduced in the context of TCR signaling. (**A**) Thymic cellularity of mice from indicated strains. Data depicts a minimum of 5 mice per strain from at least 2 independent experiments; mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (**B**) The frequency of cleaved caspase-3 in total DP (non-TCR transgenic), T3.70$^+$ DP (HY^{cd4}), and Vα2$^+$ DP (OT-I) was overlaid on Nur77 MFI from the same populations. Cleaved caspase-3 (but not Nur77 MFI) is significantly different between Nur77-FL and TCR transgenic Nur77-FL strains: $p < 0.01$ for HY^{cd4} F Nur77-FL and OT-I Nur77-FL; $p < 0.001$ for HY^{cd4} M Nur77-FL. Data depicts a minimum of 4 mice per strain from at least 2 independent experiments. **(C)** Representative phenotypes of OT-I and OT-I Nur77-FL thymi. (**D**) The frequency of cleaved caspase-3 in CD69$^+$ and CD69$^-$ DP from non-TCR transgenic mice was overlaid on Nur77 MFI from the same populations. Cleaved caspase-3 (but not Nur77 MFI) is significantly different ($p < 0.001$) between CD69$^+$ and CD69$^-$ Nur77-FL DP. Data was obtained from a minimum of 5 mice per strain from 3 independent experiments.

Nur77 is not required for, but can modulate, negative selection against UbA

Since several lines of evidence thus far suggest that Nur77 plays a minimal role in clonal deletion against UbA, we tested this hypothesis directly by examining Nur77 deficiency *in vivo*. To this end, we transplanted HY^{cd4} F or HY^{cd4} $Nur77^{-/-}$ F bone marrow mixed with WT F or M bone marrow into irradiated WT F or M recipients to model positive and negative selection, respectively (64). Chimeras were established using a 40:60 ratio of HY^{cd4} F or HY^{cd4} $Nur77^{-/-}$ F bone marrow to WT bone marrow in order to reduce the frequency of TCR transgenic thymocytes, thereby providing a more physiological environment for selection. Nur77 deficiency did not affect positive selection, as both HY^{cd4} → F and HY^{cd4} $Nur77^{-/-}$ → F chimeras contained a large population of $T3.70^{+}$ CD8SP (**Fig. 3-11A**). The vast majority of $T3.70^{+}$ CD8SP thymocytes were of the mature $CD24^{lo}$ phenotype as expected (**Fig. 3-11B**). Negative selection was also intact in the absence of Nur77, as evidenced by the lack of $T3.70^{+}$ CD8SP in HY^{cd4} → M and HY^{cd4} $Nur77^{-/-}$ → M chimeras. Of the very few $T3.70^{+}$ CD8SP thymocytes that were generated in M recipients, a smaller proportion was mature compared to those in F recipients (**Fig. 3-11B**). Furthermore, HY^{cd4} $Nur77^{-/-}$ →M chimeras tended to have an even lower proportion of $CD24^{lo}$ $T3.70^{+}$ CD8SP compared to HY^{cd4} → M. Lastly, we accounted for potential differences in precursor frequency by comparing the number of $T3.70^{+}$ DP to $T3.70^{+}$ CD8SP within each chimera (**Fig. 3-11C**) as well as across all experiments (**Fig. 3-11D**). With both types of analyses, we found no significant difference in positive or negative selection efficiency in the absence of Nur77, consistent with studies performed in classical TCR transgenic models (172, 177, 178).

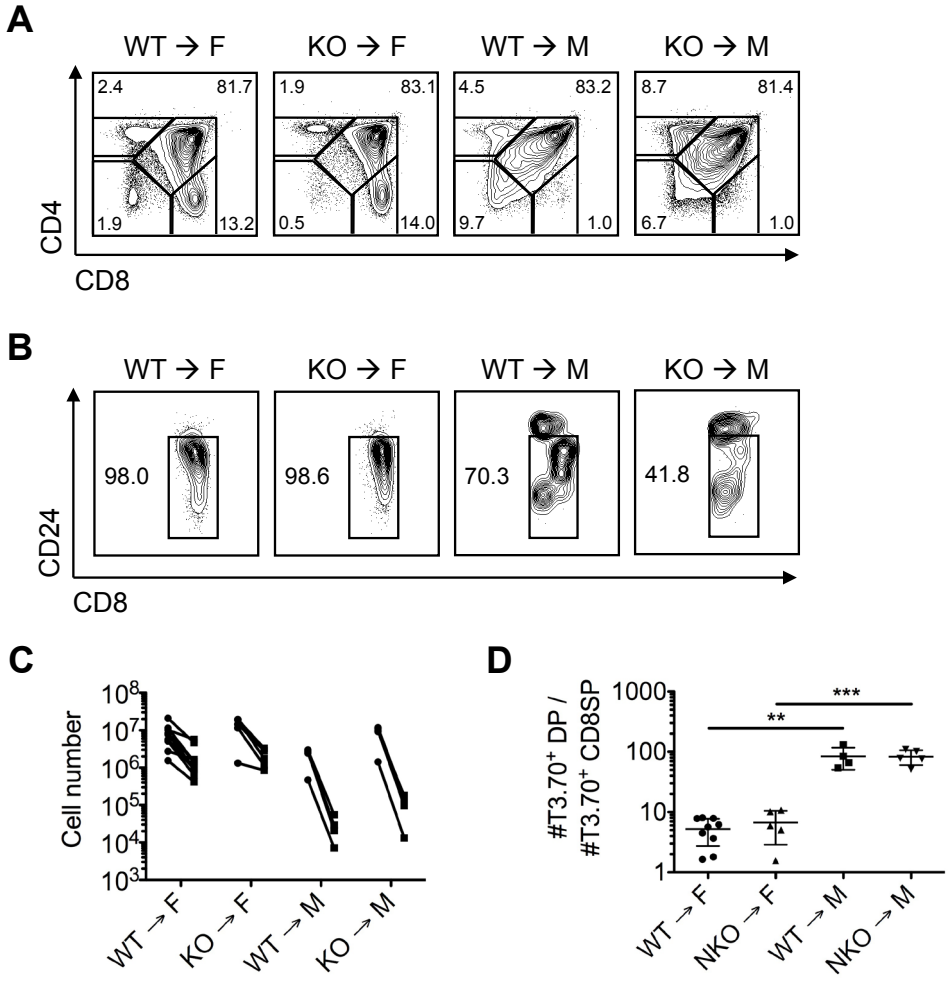


Figure 3-11. Nur77 is not required for UbA-mediated clonal deletion *in vivo*. A 40:60 mixture of HY^{cd4} F or HY^{cd4} $Nur77^{-/-}$ F bone marrow plus B6 bone marrow was transplanted into B6 F or M recipients. The resulting chimeras are labeled WT → F, KO → F, WT → M, KO → M, where WT or KO refers to the Nur77 status of the HY^{cd4} donor bone marrow. **(A)** CD8 by CD4 profiles of $T3.70^+$ thymocytes from indicated chimeras. **(B)** CD8 by CD24 profiles of $T3.70^+$ CD8SP thymocytes. The absolute numbers of $T3.70^+$ DP and $T3.70^+$ CD8SP were calculated and depicted as before-and-after **(C)** and scatter dot **(D)** plots. For WT → M chimeras, data represent 4 chimeras derived from 2 independent donors. For all other chimeras, data represent 5 chimeras derived from 3 independent donors. Data was collected over a minimum of 3 independent experiments. Data is depicted as mean ± SD; ** $p < 0.01$; *** $p < 0.001$.

Though Nur77 was not required for clonal deletion, changes in high affinity TCR signaling markers were observed during Nur77-independent negative selection. CD69, PD-1, and Helios are among the proteins induced during negative selection in the HY^{cd4} model (**Fig. 3-3**). As in native HY^{cd4} M mice, we observed Helios and PD-1 induction in $T3.70^{+}$ DP thymocytes from HY^{cd4} and HY^{cd4} $Nur77^{-/-}$ → M chimeras (**Fig. 3-12A**). Notably, the frequency of $Helios^{+}$ cells and the MFI of Helios were decreased in the absence of Nur77 (**Fig. 3-12A and 3-12B**). We also found that the frequency of $T3.70^{+}$ DP thymocytes expressing CD69 was increased in HY^{cd4} $Nur77^{-/-}$ → M compared to HY^{cd4} → M chimeras (**Fig. 3-12C and 3-12D**). It is worthwhile to note that the altered TCR signaling phenotype in HY^{cd4} $Nur77^{-/-}$ → M chimeras is distinct from that of HY^{cd4} $Bim^{-/-}$ M mice (**Fig. 3-3**). Specifically, the frequency of $CD69^{+}$ $T3.70^{+}$ DP was largely unaffected, while the frequency of $Helios^{+}$ $T3.70^{+}$ DP was increased in HY^{cd4} $Bim^{-/-}$ M compared to HY^{cd4} M mice. While Bim is important for TCR-induced apoptosis (**Fig. 3-4, 3-6, and 3-7)**, Nur77 deficiency did not impair caspase-3 activation *in vivo* (**Fig. 3-12E**), consistent with *in vitro* stimulation results. Therefore, the altered TCR signaling phenotype in HY^{cd4} $Nur77^{-/-}$ M chimeras suggests a role for Nur77 in negative selection that is distinct from clonal deletion.

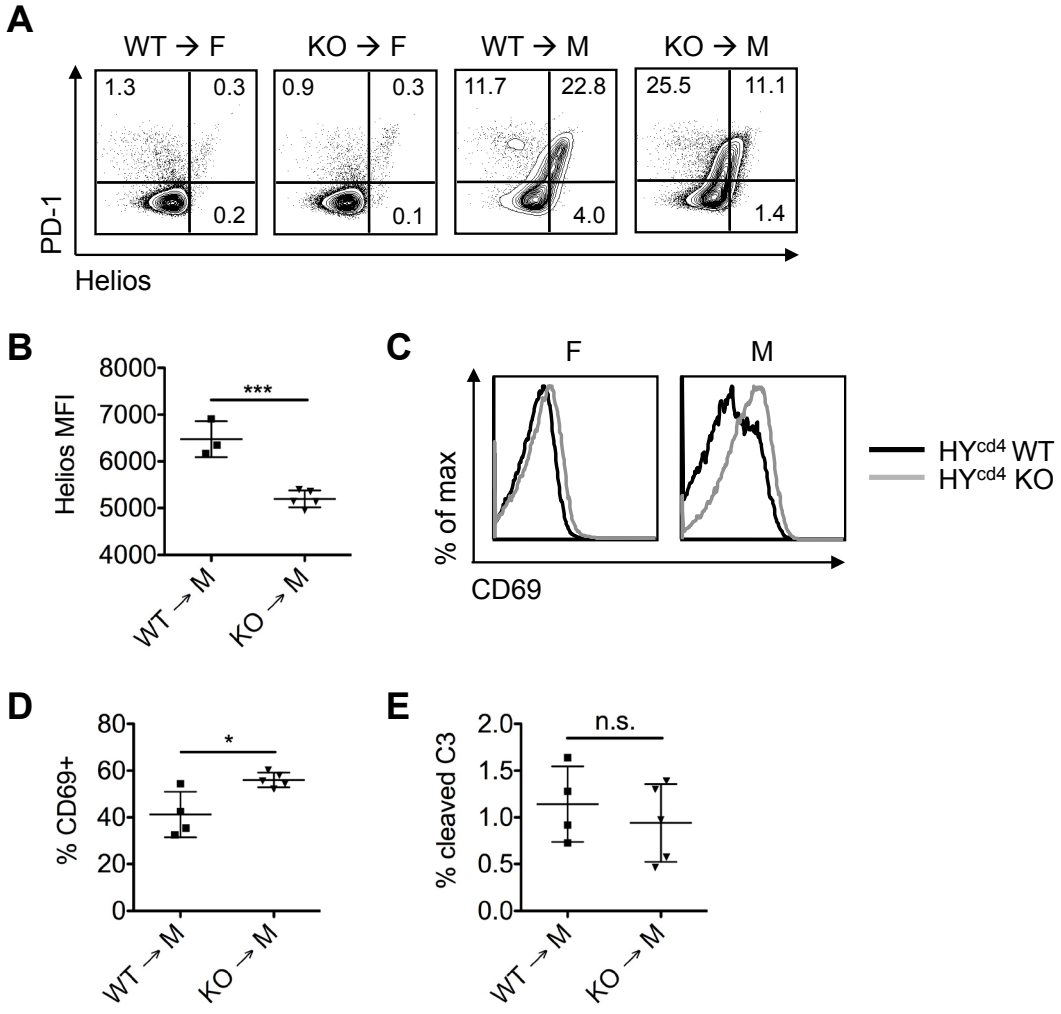


Figure 3-12. TCR signaling phenotype during UbA-mediated negative selection in the absence of Nur77. Mixed bone marrow chimeras were generated as in **Figure 3-11. (A)** Helios by PD-1 profiles of T3.70$^+$ DP thymocytes from indicated chimeras. **(B)** Helios MFI from Helios$^+$ T3.70$^+$ DP thymocytes from indicated chimeras. **(C)** CD69 expression on T3.70$^+$ DP thymocytes from indicated chimeras. **(D)** The frequency of CD69$^+$ T3.70$^+$ DP and **(E)** cleaved caspase 3$^+$ T3.70$^+$ DP from indicated chimeras. For WT → M chimeras, data represent 4 chimeras derived from 2 independent donors. For all other chimeras, data represent 5 chimeras derived from 3 independent donors. Data was collected over a minimum of 3 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; *** $p < 0.001$; n.s. not significant.

Combined deficiency in Bim and Nur77 results in impaired negative selection

Given that Bim is an important mediator of TCR-induced apoptosis, Bim expression may have precluded detection of a contribution by Nur77 to clonal deletion in the Nur77 single knockout experiments. To examine this possibility, we established chimeras using bone marrow from a Bim and Nur77 double-deficient donor. Specifically, we transferred a mix of CD45 congenic bone marrow from HY^{cd4} F, HY^{cd4} $Bim^{-/-}$ $Nur77^{-/-}$ (DKO) F, and WT F or M donors in a ratio of 20:20:60 into WT F or M recipients. Among the four M chimeras generated, there was variability in the frequency of $T3.70^{+}$ CD8SP thymocytes; two of the chimeras are depicted in **Fig. 3-13A**. In three of four M chimeras, the frequency of DKO-derived $T3.70^{+}$ CD8SP thymocytes was higher than those of WT origin. To determine whether clonal deletion was impaired in the absence of Bim and Nur77, we examined the number mature $CD24^{lo}$ $T3.70^{+}$ CD8SP, in particular, relative to the number of $T3.70^{+}$ DP precursors. Despite variability in the frequency of $T3.70^{+}$ CD8SP, by this more stringent criteria, we found that clonal deletion of DKO-derived thymocytes was consistently 3-4 fold less efficient compared to clonal deletion of WT-derived thymocytes (**Fig. 3-13B**). As with Bim-independent clonal deletion (**Fig. 3-3B**), the proportion of $T3.70^{+}$ DP expressing Helios was higher among cells of HY^{cd4} DKO origin compared to HY^{cd4} WT origin (**Fig. 3-13C**). However, the difference between WT and DKO populations in these HY^{cd4} mixed chimeras was less dramatic than between native HY^{cd4} M and HY^{cd4} $Bim^{-/-}$ M mice. This may be due to a lower $T3.70^{+}$ precursor frequency and consequently more efficient selection in chimeras. Importantly, the relative increase in $Helios^{+}$ $T3.70^{+}$ DP thymocytes of DKO origin was observed regardless of the frequency of $T3.70^{+}$ DKO CD8SP thymocytes in the chimeras, supporting the conclusion of delayed clonal deletion of DKO thymocytes. Though these data are consistent with impaired clonal deletion in the absence of

Bim and Nur77, the frequency of HY^{cd4} WT-derived $T3.70^{+}$ CD8SP in three of four HY^{cd4} WT + DKO mixed M chimeras was also higher than what we observe in HY^{cd4} M, HY^{c4} $Bim^{-/-}$, or HY^{cd4} $Nur77^{-/-}$ M mice. This raises the possibility that thymocyte development was also affected by thymocyte-extrinsic factors owing to Bim and Nur77 deficiency in other hematopoietic cells.

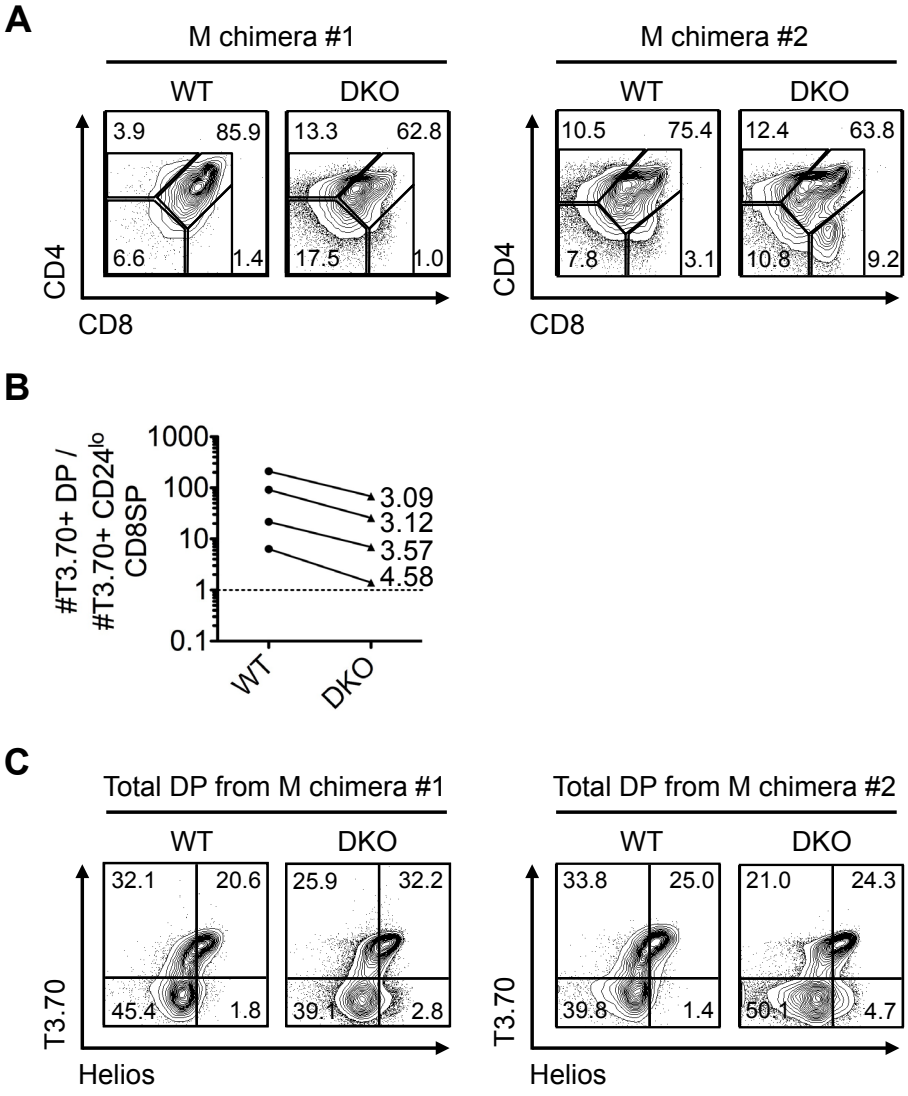


Figure 3-13. Bim and Nur77 double deficiency mildly impairs UbA-mediated clonal deletion. CD45 congenic HY^{cd4} F (WT) and HY^{cd4} Bim$^{-/-}$Nur77$^{-/-}$ (DKO) F bone marrow were mixed with B6 bone marrow in a ratio of 20:20:60 and transferred into lethally irradiated B6 M recipients. **(A)** CD8 by CD4 profiles of T3.70^{+} thymocytes of each donor origin within two M chimeras. **(B)** Ratio of the number of T3.70^{+} DP to T3.70^{+} CD8SP of each donor origin across all M chimeras. **(C)** Expression of Helios and T3.70 among total DP thymocytes of HY^{cd4} WT or HY^{cd4} DKO origin from the two M chimeras depicted in (A). Data represent 4 M chimeras generated from 1 bone marrow donor. Data was collected over 3 independent experiments.

We next examined the phenotype of WT vs. DKO-derived T3.70^{+} CD8SP in M chimeras. Similar to what was observed with native HY^{cd4} M CD8SP (**Fig. 3-3C**), when clonal deletion was efficient (i.e. in M chimera #1), about half of the few remaining WT T3.70^{+} CD8SP were Helios^{+} (**Fig. 3-14A**). However, in chimeras with a higher frequency of T3.70^{+} CD8SP (e.g. M chimera #2), we observed a decreased proportion of Helios^{+} WT-derived T3.70^{+} CD8SP. Similarly, we found increased downregulation of CD24 among WT-derived T3.70^{+} CD8SP in chimeras with a higher frequency of T3.70^{+} CD8SP (**Fig. 3-14B**). These trends also extend to thymocytes of DKO origin, as evidenced by a decreased proportion of Helios^{+} and an increased proportion of CD24^{lo} T3.70^{+} DKO-derived CD8SP in M chimera #2 compared to M chimera #1 (**Fig. 3-14A and 3-14B**). These trends were also true for the other M chimeras that are not depicted. These phenotypic variations may indicate progressive maturation of CD8SP thymocytes when they are not deleted efficiently. Regardless of the frequency of T3.70^{+} CD8SP present in a given chimera, the DKO-derived CD8SP population consistently exhibited a higher frequency of Helios downregulation than WT-derived counterparts (**Fig. 3-14A**). Similarly, unlike cells of WT origin, DKO-derived T3.70^{+} CD8SP were uniformly CD24^{lo} (**Fig. 3-14B**). Therefore, though there may be thymocyte-extrinsic factors at play affecting both WT and DKO-derived populations, these data reveal intrinsic differences in thymocytes that have undergone negative selection in the absence of Bim and Nur77. Since there appeared to be less efficient deletion of DKO thymocytes (**Fig. 3-13**), these observations may reflect progressive maturation of accumulated CD8SP in the thymus.

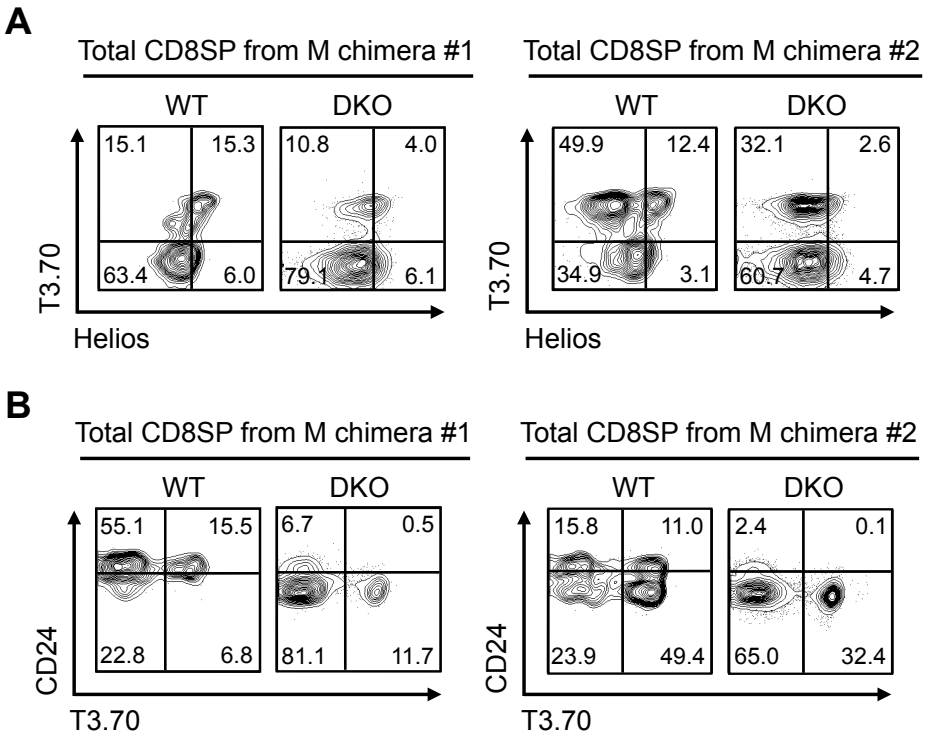


Figure 3-14. Phenotype of Bim and Nur77 double-deficient T3.70^{+} CD8SP. Mixed bone marrow chimeras were generated as in **Figure 3-13. (A)** Helios by T3.70 profiles and **(B)** T3.70 by CD24 profiles of total CD8SP thymocytes of HY^{cd4} WT or HY^{cd4} DKO origin from the two M chimeras depicted in Fig. 3-13A.

Combined Bim and Nur77 deficiency results in enhanced positive selection

Collectively, our data points to delayed and impaired UbA-mediated clonal deletion of HY^{cd4} $Bim^{-/-}$ (**Fig. 3-1 and Fig. 3-3**) and HY^{cd4} DKO thymocytes (**Fig. 3-13**), respectively. As proteins with pro-apoptotic potential, loss of Bim and Nur77 may result in impaired mechanisms of programmed cell death. This is more pronounced in the case of Bim deficiency than Nur77 deficiency (**Fig. 3-4, 3-6, and 3-7**). We also wondered if loss of Bim and/or Nur77 might affect positive selection efficiency by extending the lifespan of DP precursors or by altering TCR signaling programs. To this end, we generated chimeras using F recipients and HY^{cd4} bone marrow donors deficient in Bim and/or Nur77 as in **Fig. 3-11 and Fig. 3-13**. Consistent with observations in native HY^{cd4} $Bim^{-/-}$ F mice (**Fig. 3-1**), HY^{cd4} $Bim^{-/-}$ → F chimeras had an increased frequency of $T3.70^{+}$ CD8SP thymocytes compared to HY^{cd4} WT → F chimeras (**Fig. 3-15A**). There was also a trend towards a decreased ratio of $T3.70^{+}$ DP to $T3.70^{+}$ CD8SP in HY^{cd4} $Bim^{-/-}$ → F compared to HY^{cd4} WT → F chimeras (**Fig. 3-15B**). While HY^{cd4} $Nur77^{-/-}$ → F chimeras did not exhibit altered positive selection, the frequency of DKO-derived $T3.70^{+}$ CD8SP and the number of DKO $T3.70^{+}$ CD8SP relative to DKO $T3.70^{+}$ DP precursors were dramatically higher compared to when only Bim was absent. Transcriptional analysis of HY^{cd4} mice previously indicated that Nur77 is upregulated during positive selection, though to a lesser degree than negative selection (81). These data suggest that Nur77 may normally play an inhibitory role during positive selection, albeit a minor one since an effect was only apparent when Bim was additionally deleted. Along with data showing differences in TCR signaling marker expression during negative selection in the absence of Nur77 (**Fig. 3-12**), these observations on positive selection may point to a general role for Nur77 in modulating TCR signaling during thymic selection.

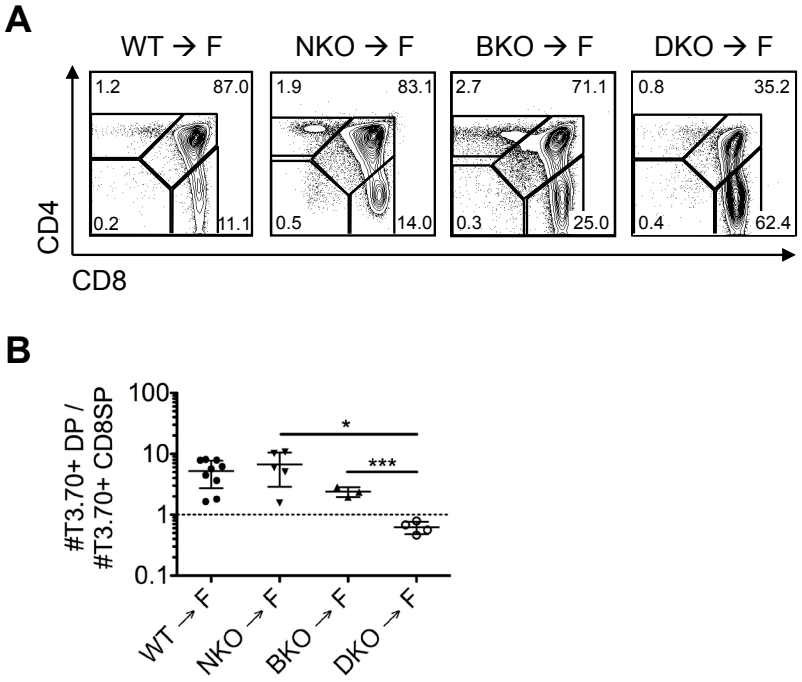


Figure 3-15. Combined Bim and Nur77 deficiency results in enhanced positive selection. HY^{cd4} F (WT), HY^{cd4} $Nur77^{-/-}$ F (NKO), or HY^{cd4} $Bim^{-/-}$ F (BKO) bone marrow was mixed with B6 bone marrow in a ratio of 40:60 and transferred into lethally irradiated B6 F recipients. DKO chimeras were generated by transplanting a mixture of CD45 congenic HY^{cd4} F (WT), HY^{cd4} $Bim^{-/-}Nur77^{-/-}$ (DKO) F bone marrow, and B6 bone marrow in a ratio of 20:20:60 into lethally irradiated B6 F recipients. **(A)** CD8 by CD4 profiles of $T3.70^{+}$ thymocytes of indicated donor origin. **(B)** Ratio of the number of $T3.70^{+}$ DP thymocytes to $T3.70^{+}$ CD8SP thymocytes of each donor origin. For HY^{cd4} WT + HY^{cd4} DKO → F mixed chimeras, data depicts 4 chimeras of each type derived from 1 BM donor. For single knockout chimeras, data depicts a minimum of 5 chimeras of each type derived from a minimum of 2 independent BM donors. Data is depicted as mean ± SD; * $p < 0.05$; *** $p < 0.001$.

In summary, our investigation into the roles of Bim and Nur77 in thymocyte development in this chapter highlights the importance of using physiological models like HY^{cd4} over classical TCR transgenic models. Reconciling previous conclusions (141, 142), we have shown that Bim is critical for TCR-induced apoptosis in DP thymocytes, but that Bim is ultimately not required for UbA-mediated clonal deletion. In contrast to Bim, loss of Nur77 did not result in impaired DP apoptosis *in vitro* or *in vivo*. The lack of a role for Nur77 in apoptosis cannot wholly be explained by redundancy by other proteins such as Nor-1, as Nur77 was induced during Bim-independent clonal deletion *in vivo* but did not activate caspase-3. While we agree with the literature that Nur77 has pro-apoptotic potential, our data suggests that this function of Nur77 is limited during physiological negative selection. Along with changes in TCR signaling markers, this may indicate a previously unrecognized reciprocal regulation between Nur77 and TCR signaling pathways. While Bim deficiency had a more discernible impact than Nur77 deficiency on positive and negative selection, studies in double knockout chimeras have revealed a contribution by Nur77 to both processes. It is possible that the effect of Nur77 deficiency in positive and negative selection has more to do with altering the selection threshold than thymocyte survival as previously thought. In the case of UbA-mediated negative selection, combined Bim and Nur77 deficiency also appeared to affect clonal deletion efficiency by thymocyte-extrinsic means. Collectively, our studies in the HY^{cd4} model have shed light on the complex mechanisms behind thymocyte selection and suggest that past paradigms be revisited.

CHAPTER 4: RESULTS - The role of Bim and Nur77 in MHC class I-restricted TRA-mediated negative selection

In addition to UbA-mediated negative selection in the cortex, multiple lines of evidence have established the thymic medulla as a key site of self-tolerance induction (239). One important reason is that TRA expression driven by the transcription factor Aire is exclusive to mTECs (66). Because thymocyte migration to the medulla occurs at a later $CD4^{+}CD8^{int}$ stage following positive selection (240-242), gene expression both within the thymocyte and its surrounding environment are substantially different from UbA-mediated negative selection. It therefore follows that the molecular mechanisms of negative selection may differ depending on context. Indeed, following our finding that Bim is not required for UbA-mediated clonal deletion in the HY^{cd4} model, other work from our group showed that Bim is required for TRA-mediated deletion of CD8SP thymocytes in the OT-I Rip-mOva model (143). Others have since corroborated the critical role of Bim in MHC class I-restricted TRA-mediated deletion, as well as demonstrating its importance in MHC class II-restricted TRA-mediated deletion using the OT-II Rip-mOva model (144). In the OT-II Rip-mOva model, Nur77 was implicated as a driver of Bim expression (160). In fact, combined Nur77 and Bim deficiency did not further impair deletion of CD4SP thymocytes over Nur77 single deficiency. The observation that Nur77 deficiency can impair TRA-mediated deletion of CD4SP thymocytes is a notable contrast to the minimal role of Nur77 in UbA-mediated deletion. In light of these findings, we sought to characterize the role of Nur77 and its relationship with Bim in TRA-mediated deletion of CD8SP thymocytes and the consequent effects on tolerance.

Thymocyte development in OT-I chimeras in the absence of Bim and Nur77

Since our group has previously used the well-established OT-I Rip-mOva model to study the impact of Bim deficiency on TRA-mediated deletion, we proceeded to use this TCR transgenic model to study the role of Nur77. In order to examine the effect of Nur77 single deficiency as well as combined Nur77 and Bim deficiency, we generated full bone marrow chimeras from four types of donors: OT-I $Bim^{+/-}Nur77^{+/-}$ (henceforth referred to as Het/Het), OT-I $Bim^{+/-}Nur77^{-/-}$ (NKO), OT-I $Bim^{-/-}Nur77^{+/-}$ (BKO), and OT-I $Bim^{-/-}Nur77^{-/-}$ (DKO). Unlike in HY^{cd4} mice, expression of the transgenic OT-I TCRα chain (Vα2) occurs at the DN stage. As a consequence of premature TCR formation and signaling compared to endogenous TCRs, the majority of thymocytes in OT-I native and chimeric mice are $V\alpha2^{+}$. Distinct developmental stages of $V\alpha2^{+}$ thymocytes were more readily observed on CD8 by CD4 profiles than in HY^{cd4} mice (**Fig. 4-1**). Though the OT-I NKO → Rip-mOva mouse depicted in this particular set of data appears to uniquely exhibit an increased frequency of $V\alpha2^{+}$ DN thymocytes, alterations in the DN subset were not consistently associated with any specific chimera. In fact, an increased frequency of $V\alpha2^{+}$ DN thymocytes was also occasionally observed in WT recipients, indicating that they do not result from self-antigen-induced selection. Because this thesis is primarily concerned with mechanisms of negative selection, we will not delve further into the nature of DN thymocytes in the OT-I model system.

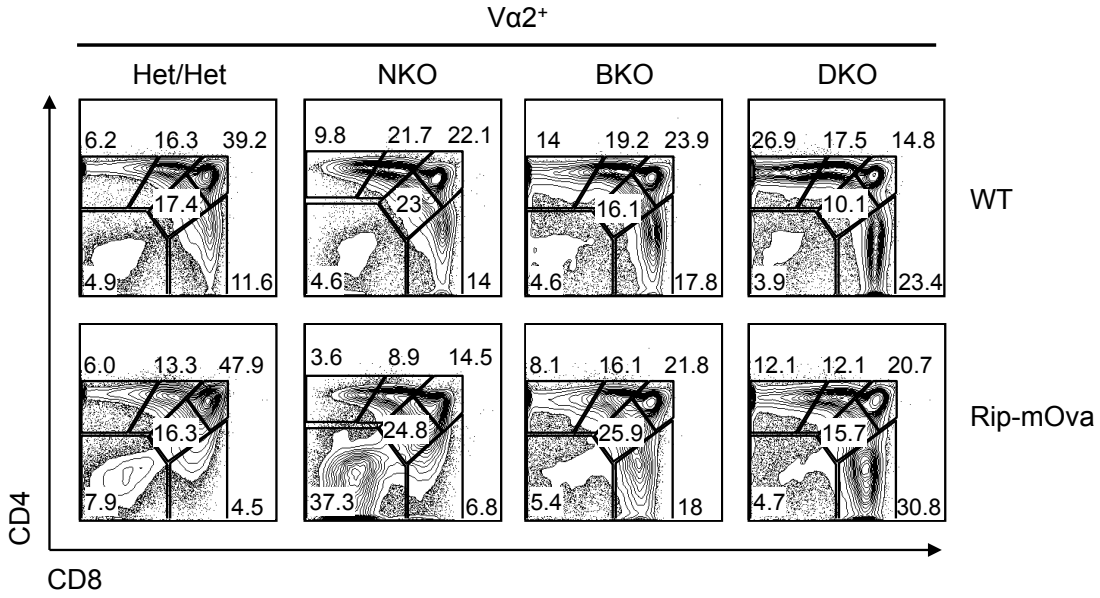


Figure 4-1. Thymocyte development in OT-I chimeras. OT-I $Bim^{+/-}Nur77^{+/-}$ (Het/Het), OT-I $Bim^{+/+}Nur77^{-/-}$ (NKO), OT-I $Bim^{-/-}Nur77^{+/+}$ (BKO), or OT-I $Bim^{-/-}Nur77^{-/-}$ (DKO) bone marrow was transferred into lethally irradiated WT or Rip-mOva recipients. Thymocytes were analyzed *ex vivo* by flow cytometry. A complete set of CD8 by CD4 profiles of $V\alpha2^{+}$ thymocytes from one experiment is depicted here. Data is generally representative of a minimum of 7 chimeras of each type, generated from 3 independent donors in each case, except OT-I BKO → Rip-mOva (5 from 2 donors) and OT-I DKO → Rip-mOva (1 from 1 donor).

We did observe a tendency towards higher frequencies of CD4SP and CD8SP among $V\alpha2^+$ thymocytes in OT-I → WT chimeras when Bim, Nur77, or both molecules were deficient (**Fig. 4-1**). As in non-transgenic mice, DP^{bright} thymocytes have low TCR expression and represent the pre-selection population, while DP^{dull} and $CD4^+CD8^{int}$ thymocytes represent post-TCR-signaled stages (243, 244). In OT-I → WT chimeras, in which positive selection occurs, a portion of $V\alpha2^+$ thymocytes fell into the CD4SP gate post-selection. Compared to pre-selection DP^{bright} cells, many $V\alpha2^+$ CD4SP thymocytes have upregulated TCR expression to levels comparable to $V\alpha2^+$ CD8SP, but consistently expressed higher CD24 than $V\alpha2^+$ CD8SP (**Fig. 4-2A**). This suggests that $V\alpha2^+$ CD4SP may not become mature SP thymocytes or are delayed in their maturation compared to $V\alpha2^+$ CD8SP thymocytes. Because the DP^{dull} and $CD4^+CD8^{int}$ stages may become CD4SP thymocytes, whose ultimate fate and function in the OT-I model is unclear, we calculated the ratio of pre-selection $V\alpha2^+$ DP^{bright} thymocytes to progeny $V\alpha2^+$ CD8SP thymocytes as a measure of positive selection efficiency. Consistent with a general increase in the frequency of CD8SP thymocytes, selection was more efficient in OT-I NKO → WT, OT-I BKO → WT and OT-I DKO → WT compared to OT-I → Het/Het chimeras (**Fig. 4-2B**). These results are reminiscent of the increased positive selection efficiency in the absence of Bim and Nur77 in HY^{cd4} chimeras (**Fig. 3-15**). However, unlike in the HY^{cd4} model, combined Bim and Nur77 deficiency did not result in a substantial enhancement over either single knockout. Another notable difference is that Nur77 deficiency was sufficient to alter positive selection in the OT-I model, indicating that Nur77 has a greater role in this context.

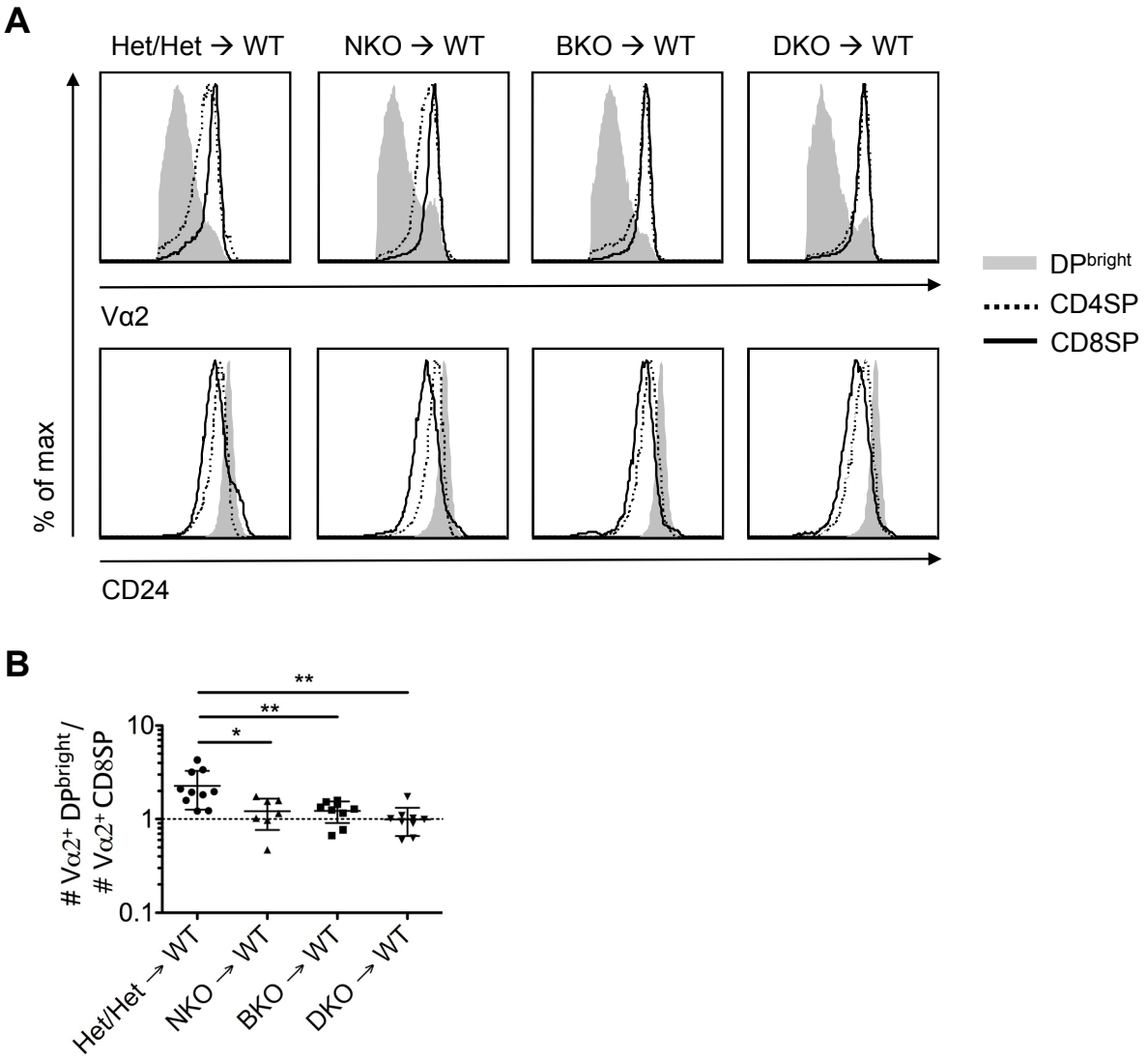


Figure 4-2. Increased positive selection is associated with Bim or Nur77 deficiency. (A) Expression of Vα2 (top) and CD24 (bottom) among $V\alpha2^+$ thymocytes from indicated subsets in WT recipients. **(B)** Ratio of the number of $V\alpha2^+$ DP^{bright} thymocytes to the number of $V\alpha2^+$ CD8SP thymocytes in WT recipients. Data represent a minimum of 7 chimeras of each type generated from 3 independent donors in each case. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$.

Nur77 deficiency mildly impairs TRA-mediated deletion of CD8SP thymocytes

Negative selection in the presence of Bim and Nur77 is predominantly mediated through clonal deletion, as evidenced by a reduction in the frequency of $V\alpha2^+$ CD8SP in OT-I Het/Het → Rip-mOva chimeras compared to OT-I Het/Het → WT controls (**Fig. 4-1**). Nur77 deficiency tended to result in a mildly elevated frequency of CD8SP, though this parameter was not significantly different from OT-I Het/Het → Rip-mOva chimeras overall. However, examination of the maturity of CD8SP thymocytes revealed that a greater proportion of $V\alpha2^+$ CD8SP were $CD24^{lo}$ in OT-I NKO → Rip-mOva compared to OT-I Het/Het → Rip-mOva chimeras (**Fig. 4-3A**). This increased frequency also translated into a higher number of mature antigen-specific CD8SP thymocytes (**Fig. 4-3B**), suggesting that clonal deletion was modestly but significantly impaired in the absence of Nur77. Therefore, unlike its minimal role in UbA-mediated deletion of DP thymocytes, these data suggest that Nur77 plays a more important role in TRA-mediated deletion of CD8SP, as was the case for CD4SP in OT-II Rip-mOva mice (160). As previously shown (143), Bim deficiency nearly abrogated TRA-mediated deletion of CD8SP, as evidenced by a similar frequency and absolute number of antigen-specific CD8SP in OT-I BKO Rip-mOva and WT recipients (**Fig. 4-1, 4-3B**). We currently only have data for one OT-I DKO → Rip-mOva chimera, but the number of $V\alpha2^+$ $CD24^{lo}$ CD8SP there was higher than in any OT-I BKO → Rip-mOva, perhaps indicative of Nur77 deficiency further impairing deletion even on a Bim-deficient background.

It is also interesting to note that CD8SP in the $V\alpha2^+$ $CD24^{lo}$ quadrant did not have uniform expression of CD24 in OT-I BKO and DKO → WT chimeras, in which there were a significant proportion of cells expressing lower CD24 levels than in OT-I Het/Het or NKO → WT chimeras

(**Fig 4-3A**). Furthermore, in OT-I BKO and DKO Rip-mOva chimeras, a lower proportion of $V\alpha2^{+}$ $CD24^{lo}$ CD8SP were of the CD24 "ultra-low" population compared to WT recipient counterparts. Since CD24 downregulation is associated with thymocyte maturation, one possible interpretation of these observations is that despite escaping deletion in the absence of Bim, many antigen-specific CD8SP may have experienced some degree of non-deletional tolerance induction in the thymus.

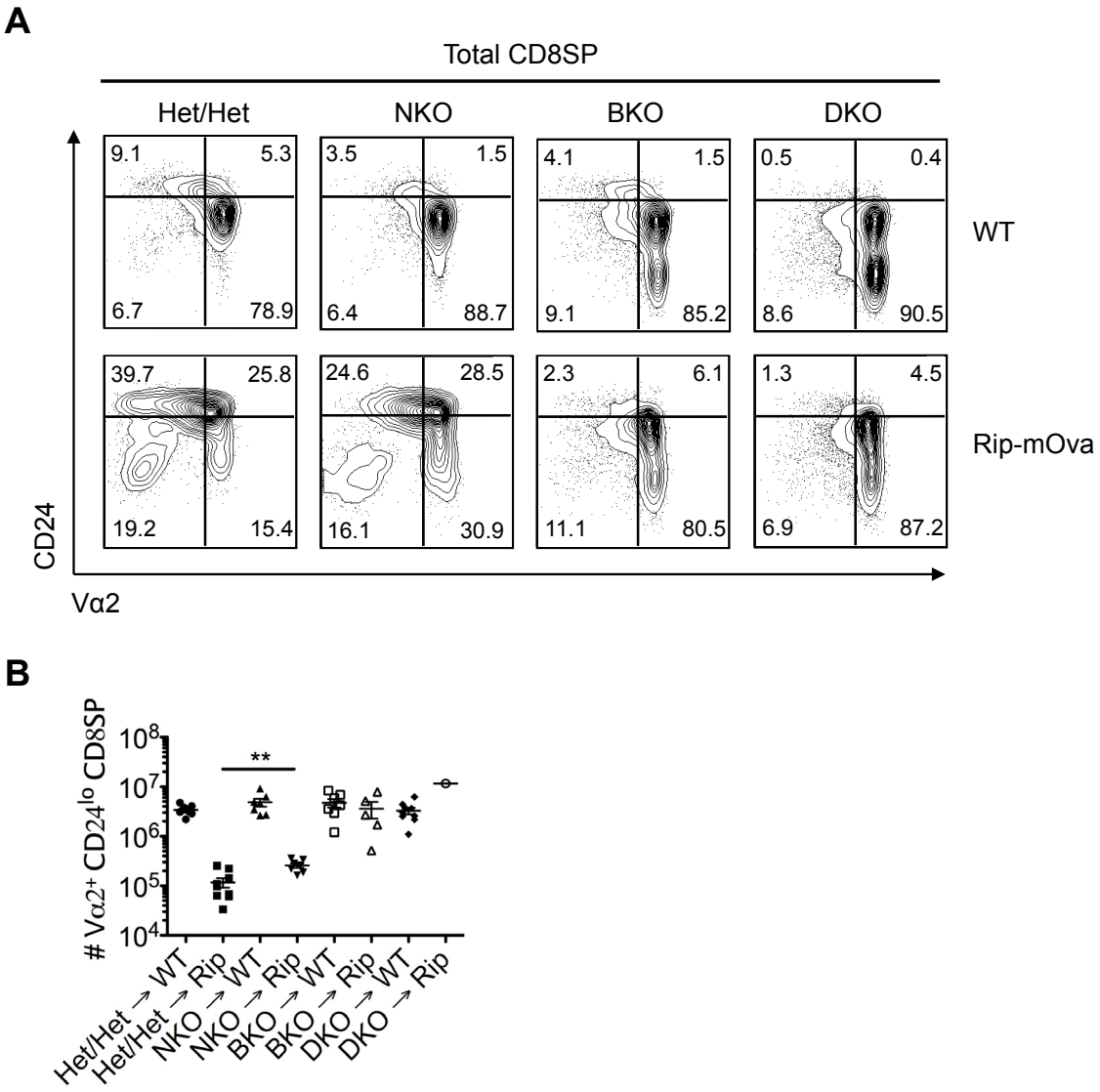


Figure 4-3. Nur77 deficiency mildly impairs TRA-mediated deletion of CD8SP thymocytes. **(A)** Representative Vα2 by CD24 profiles of total CD8SP thymocytes from each chimera. **(B)** The absolute number of $V\alpha2^{+}CD24^{lo}$ CD8SP thymocytes from each chimera. Thymocytes were analyzed *ex vivo* by flow cytometry. Data is representative of a minimum of 7 chimeras of each type, generated from 3 independent donors in each case, except OT-I BKO → Rip-mOva (5 from 2 donors) and OT-I DKO → Rip-mOva (1 from 1 donor). Data is depicted as mean ± SD; ** $p < 0.01$.

Bim induction during negative selection is intact in the absence of Nur77

Though a correlation between the transactivation function and the pro-apoptotic activity of Nur77 has been shown, target genes known to be relevant to negative selection remained elusive (159), fueling the argument that Nur77 may function via a transcription-independent mechanism. However, a more recent study showed that Nur77-deficient thymocytes from OT-II Rip-mOva mice expressed a decreased amount of Bim transcripts than Nur77-sufficient counterparts, which was one reason given for why Nur77 deficiency impaired clonal deletion in this model (160). Given that Bim is also a key mediator of deletion in the OT-I Rip-mOva model, we examined by flow cytometry whether decreased expression of Bim protein could be a factor in modestly impairing deletion of CD8SP thymocytes. Compared to counterparts from WT recipients, Bim induction was apparent in $V\alpha2^+$ CD8SP from OT-I Het/Het and OT-I NKO → Rip-mOva chimeras (**Fig. 4-4A**). All OT-I Het/Het → Rip-mOva chimeras exhibited bimodal distribution of Bim into Bim-expressing and non-expressing populations, whereas all of the OT-I NKO → Rip-mOva chimeras exhibited a whole population shift compared to OT-I NKO → WT controls. However, the frequency of CD8SP from Rip-mOva recipients that induced Bim to a higher level than in control CD8SP thymocytes from WT recipients was similar between OT-I Het/Het and OT-I NKO. This suggests that high affinity-induced Bim expression was comparable in the presence and absence of Nur77. Of the population of CD8SP expressing a high amount of Bim, the amount of Bim protein was mildly higher in OT-I Het/Het → Rip-mOva chimeras, though baseline Bim was higher among OT-I Het/Het → WT CD8SP thymocytes as well (**Fig. 4-4B**). These results are consistent with the approximately 1.5-fold lower expression of Bim in OT-II NKO Rip-mOva CD4SP compared to OT-II Rip-mOva CD4SP thymocytes (160). Our difference of 1.2-fold in Bim MFI was even milder, which may be due to using a Bim

heterozygous control and a different assay (Bim protein by flow cytometry in our hands as opposed to Bim transcripts by microarray). Because the absolute amount of Bim was only mildly lower, and fold induction over WT recipient CD8SP was similar, decreased Bim expression is likely only a small factor in explaining the impaired clonal deletion in OT-I NKO → Rip-mOva chimeras.

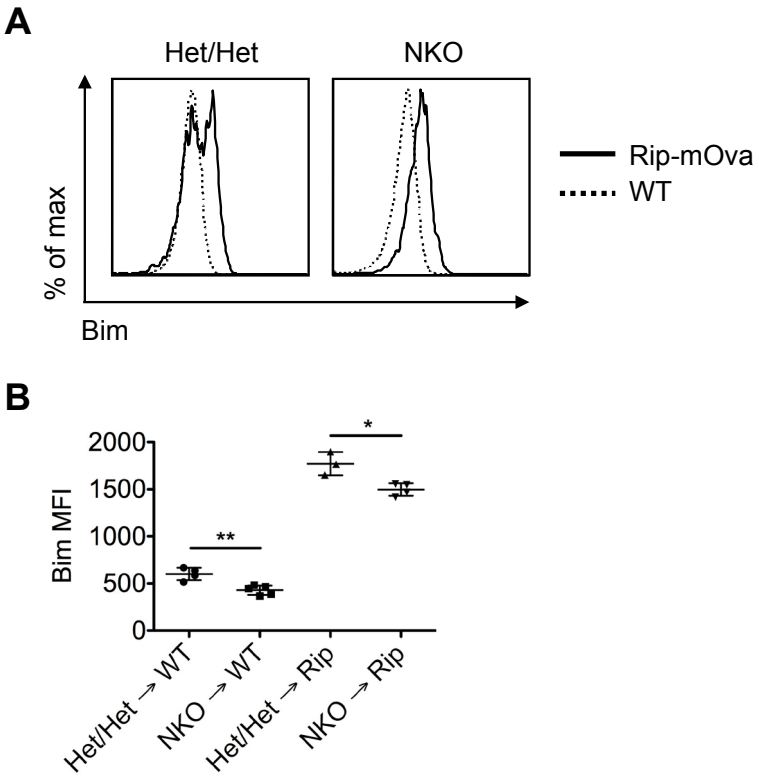


Figure 4-4. Bim induction during negative selection is intact in the absence of Nur77. *Ex vivo* thymocytes were fixed and permeabilized and stained with anti-Bim antibody. **(A)** Bim expression in Vα2$^+$ CD8SP thymocytes from indicated chimeras. **(B)** MFI of Bim in Vα2$^+$ CD8SP thymocytes from indicated chimeras was quantified and compiled. Thymocytes were analyzed *ex vivo* by flow cytometry. Data represent a minimum of 3 chimeras from 2 independent donors. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$.

Induction of Helios and PD-1 in Rip-mOva CD8SP during negative selection

In addition to Bim, we examined the expression of a variety of other proteins induced during negative selection. Among these are Helios and PD-1, which we found in OT-I Het/Het and NKO → Rip-mOva Vα2^{+} CD8SP to be largely co-expressed (**Fig. 4-5A**). As with Bim induction, there was a tendency for OT-I Het/Het → Rip-mOva chimeras to exhibit bimodal upregulation of Helios and PD-1 (7 of 9) compared to OT-I NKO → Rip-mOva chimeras (2 of 7). When each population was examined further, we found that Helios^{hi}PD-1^{hi} CD8SP thymocytes had the highest Vα2 expression, though the Helios^{lo}PD-1^{lo} fraction also included CD8SP with maximal Vα2 expression (**Fig. 4-5B**). These results demonstrate a correlation between the amount of TCR expression and induction of TCR-regulated proteins. Unlike UbA-mediated negative selection in the HY^{cd4} model when Nur77 was deficient (**Fig. 3-12A and 3-12B**), we did not find any significant differences in the amount of Helios or PD-1 expression in CD8SP thymocytes from OT-I NKO → Rip-mOva compared to OT-I Het/Het → Rip-mOva chimeras. While Helios induction was normal, we noted impaired induction of PD-1 in the absence of Bim (**Fig. 4-5A**), which was also different from HY^{cd4} mice (**Fig. 3-3A**). One possible explanation for this difference is that Vα2^{+} CD8SP in OT-I BKO → Rip-mOva chimeras are not deleted and may be downregulating PD-1. In contrast, T3.70^{+} DP in HY^{cd4} Bim$^{-/-}$ M mice are ultimately deleted, such that the majority of DP thymocytes observed at any given time may be young. In the one OT-I DKO → Rip-mOva chimera we analyzed, we also observed impaired induction of PD-1, though there may have been a partial rescue compared to OT-I BKO → Rip-mOva (**Fig. 4-5A**). Though we do not know if the one OT-I DKO → Rip-mOva chimera is an accurate representation, it may be another subtle change in TCR signaling markers resulting from Nur77 deficiency.

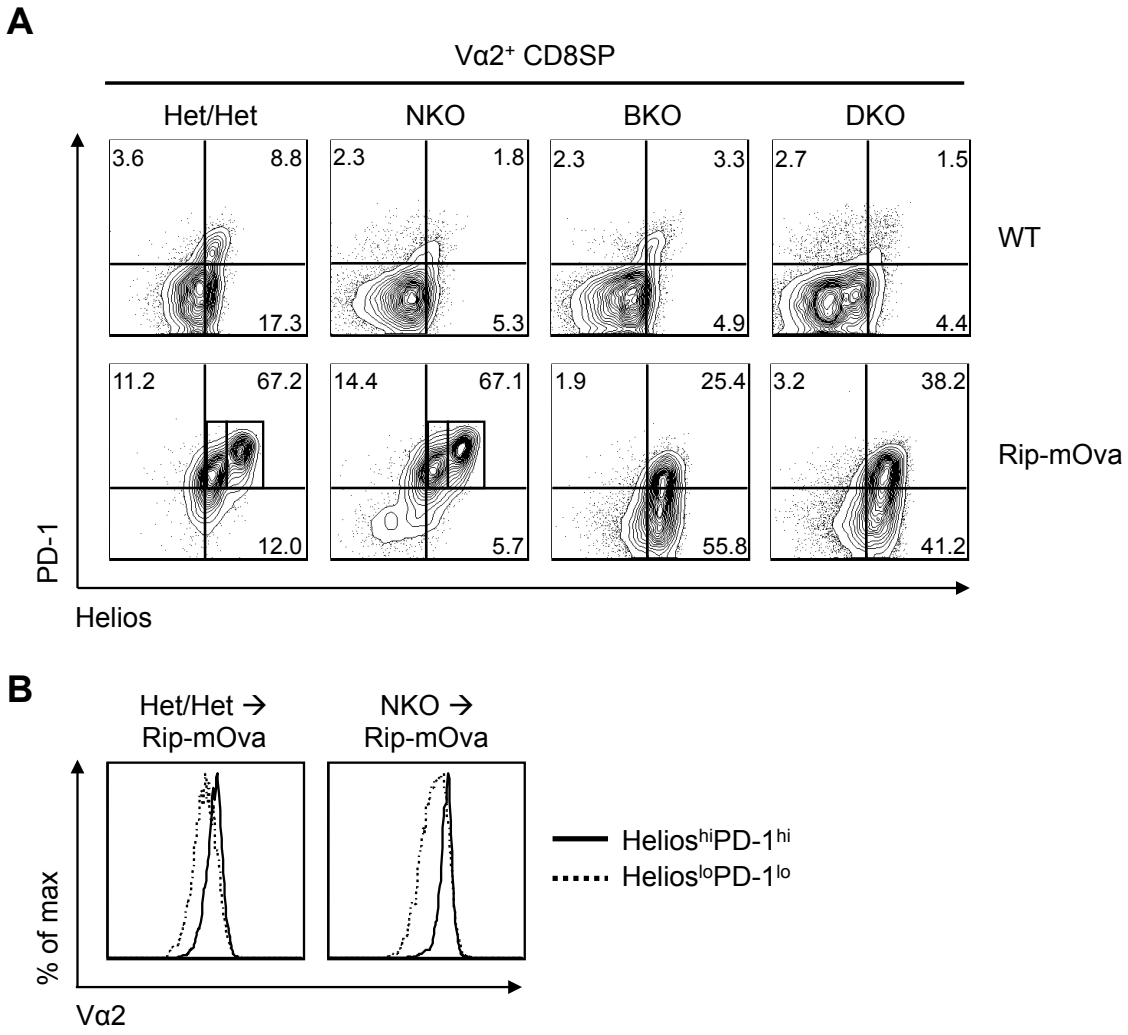


Figure 4-5. Induction of Helios and PD-1 in Rip-mOva CD8SP during negative selection. Thymocytes were analyzed *ex vivo* by flow cytometry. **(A)** Helios by PD-1 profiles of Vα2$^+$ CD8SP thymocytes from all chimeras. Data was collected from a minimum of 7 chimeras generated from 3 independent donors, except in the case of OT-I BKO → Rip-mOva (5 from 2 donors) and OT-I DKO → Rip-mOva (1 from 1 donor). Distinct Helios^{hi}PD-1^{hi} and Helios^{lo} PD-1^{lo} populations were observed among Vα2$^+$ CD8SP thymocytes from OT-I Het/Het → Rip-mOva (7 of 9) and OT-I NKO → Rip-mOva (2 of 7) chimeras. **(B)** Vα2 expression in Helios^{hi}PD-1^{hi} and Helios^{lo}PD-1^{lo} Vα2$^+$ CD8SP (from gates in A) from indicated chimeras.

Other phenotypic changes in Rip-mOva CD8SP during negative selection

CD44 is a protein that is well established as a marker of mature T cell activation, but it is unknown whether CD44 induction occurs in TCR-signaled thymocytes. We found that CD44 was upregulated on a fraction of $V\alpha2^+$ CD8SP during negative selection in OT-I Het/Het → Rip-mOva chimeras (**Fig. 4-6A**). Reminiscent of the bimodal expression of Bim, Helios, and PD-1 induction in OT-I Het/Het → Rip-mOva mice, there was also a population of CD8SP that did not upregulate CD44. The amount of CD44 expressed by this $CD44^-$ fraction was even lower than on $V\alpha2^+$ CD8SP from OT-I Het/Het → WT mice, suggesting that CD44 is also induced to a low degree in the course of thymocyte maturation. Similar results were observed in OT-I NKO → Rip-mOva mice, though these chimeras tended to have a greater proportion of $CD44^+$ $V\alpha2^+$ CD8SP compared to OT-I Het/Het → Rip-mOva chimeras (observed in 4 of 6 sets of paired mice) (**Fig. 4-6A**). In both OT-I Het/Het and NKO → Rip-mOva chimeras, the $CD44^+$ CD8SP had the highest Vα2 expression (**Fig. 4-6B**), similar to what we observed with Helios and PD-1 (**Fig. 4-5B**). Surprisingly, the amount of CD44 induced on thymocytes during negative selection was as high as on $CD44^+$ peripheral $V\alpha2^+$ $CD8^+$ T cells (**Fig. 4-6A**). This supports the induction of CD44 as a bona fide result of TCR signaling in thymocytes. Despite expression of CD44 equivalent to activated T cells, preliminary data indicated that the majority of $CD44^+$ $V\alpha2^+$ CD8SP thymocytes had high CD24 expression, suggesting that they were mostly immature thymocytes and not of peripheral origin (**Fig. 4-6C**). In comparison, $V\alpha2^+$ CD8SP thymocytes from WT recipients destined to survive were mostly $CD24^{lo}CD44^-$. Collectively, these data show that CD44 is another marker that correlates with TCR expression and signaling strength. Furthermore, similar to the Helios induction patterns, the higher proportion of $CD44^+$ thymocytes in OT-I NKO → Rip-mOva chimeras may indicate altered TCR signaling in the

absence of Nur77. Despite the higher frequency of high affinity-signaled thymocytes, clonal deletion was not enhanced, but rather impaired in OT-I NKO → Rip-mOva compared to OT-I Het/Het → Rip-mOva chimeras (**Fig. 4-3**). Thus, the phenotype of Nur77-deficient negative selection in this model system may result from alterations in apoptosis as well as other uncharacterized functions of Nur77.

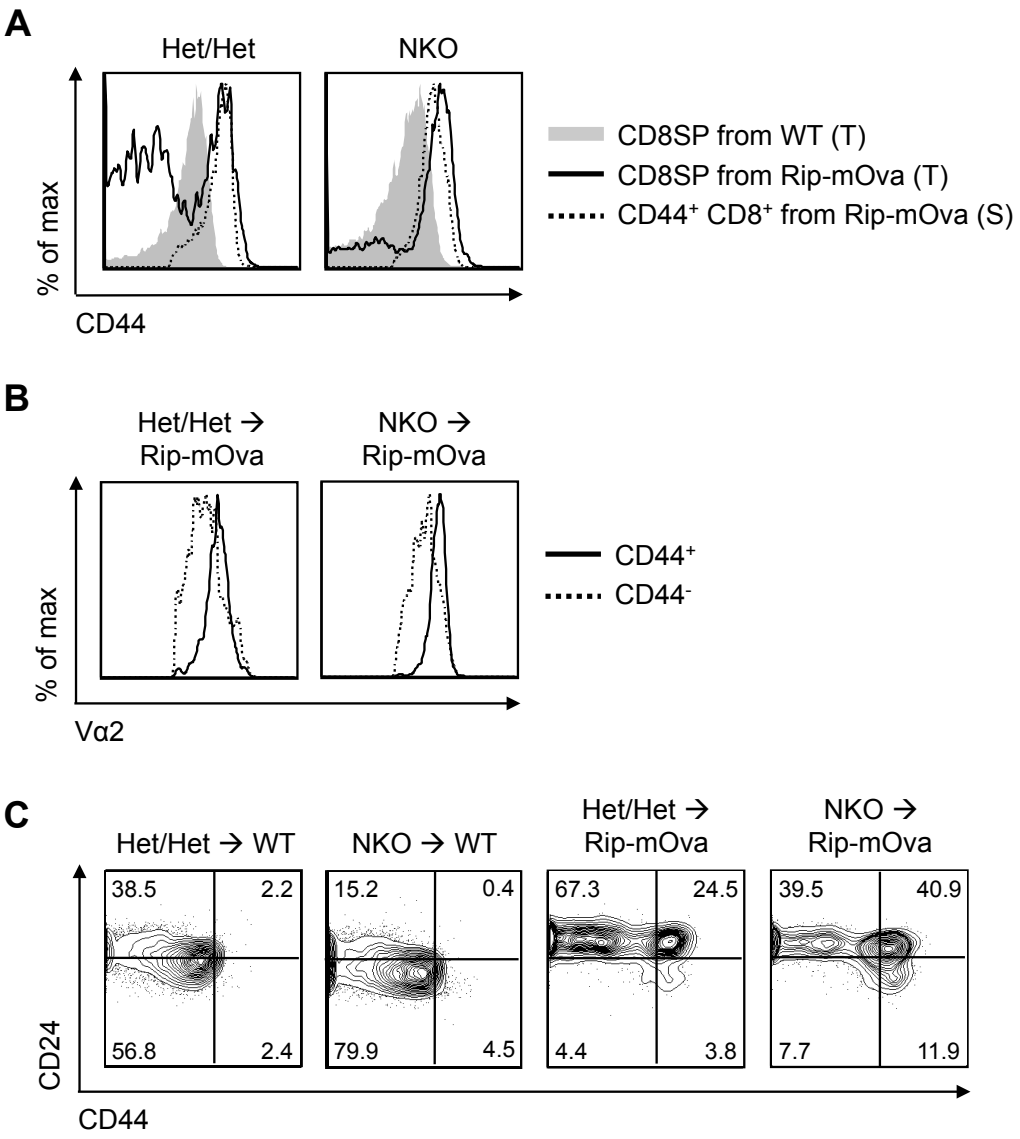


Figure 4-6. CD44 is induced during negative selection. Thymocytes or splenocytes were analyzed *ex vivo* by flow cytometry. **(A)** CD44 expression on $V\alpha2^+$ CD8SP thymocytes (T) or $CD44^+$ $V\alpha2^+$ $CD8^+$ splenocytes (S) from indicated chimeras. The comparison in CD44 induction between OT-I Het/Het → Rip-mOva and OT-I NKO →Rip-mOva chimeras is representative of 4 pairs of mice, each genotype generated from 2 independent donors. **(B)** Vα2expression on $CD44^+$ and $CD44^-$ $V\alpha2^+$ CD8SP thymocytes from indicated chimeras. **(C)** CD44 by CD24 profiles of $V\alpha2^+$ CD8SP thymocytes from indicated chimeras. Data is representative of 5 OT-I Het/Het → WT (2 donors), 4 OT-I Het/Het → Rip-mOva (2 donors), 3 OT-I NKO → WT (2 donors), and 2 OT-I NKO → Rip-mOva (2 donors).

Lastly, we found that CD8SP thymocytes from Rip-mOva chimeras expressed a lower amount of IL-7Rα (CD127) than those in WT recipients, regardless of Nur77 expression (**Fig. 4-7**). Since CD127 was induced at the $CD4^+CD8^{int}$ stage to a higher level than that found at the CD8SP stage in Rip-mOva mice, these data indicate that CD127 downregulation occurred during negative selection. Given the importance of IL-7 signaling in homeostatic proliferation and survival of peripheral T cells (245), this suggests that the small number of $V\alpha2^+$ CD8SP thymocytes in OT-I Het/Het and NKO → Rip-mOva chimeras that escaped clonal deletion may have impaired survival in the periphery. Therefore, downregulation of CD127 in the thymus may represent an alternative mechanism of negative selection.

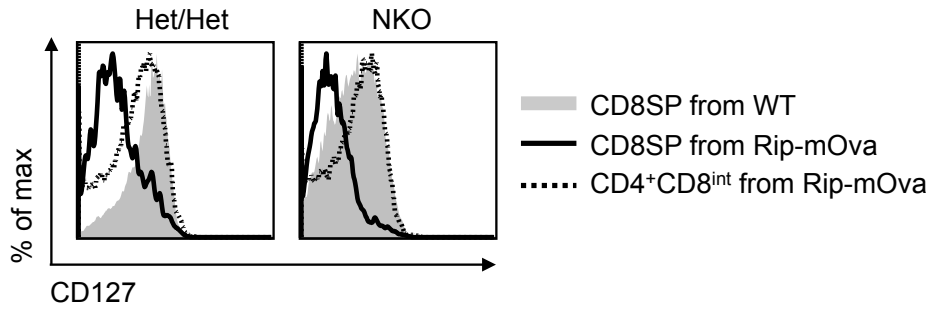


Figure 4-7. CD127 is downregulated during negative selection. CD127 expression on Vα2^{+} thymocytes from the indicated subsets in indicated chimeras. Thymocytes were analyzed *ex vivo* by flow cytometry. Data is representative of a minimum of 3 mice of each chimera type generated from at least 2 independent donors.

Impaired clonal deletion results in an increased number of antigen-specific $CD8^+$ T cells in spleen

Though most antigen-specific clones were deleted in the thymi of OT-I Het/Het and NKO → Rip-mOva chimeras (**Fig. 4-3**), we did find a small but distinct population of $V\alpha2^+$ $CD8^+$ cells in their spleens (**Fig. 4-8A**). Consistent with an increased number of $V\alpha2^+$ $CD24^{lo}$ CD8SP thymocytes, OT-I NKO → Rip-mOva chimeras had a modest but significant increase in the number of splenic antigen-specific T cells compared to Het/Het (**Fig. 4-8B**). The block in clonal deletion due to Bim deficiency in OT-I BKO and DKO → Rip-mOva chimeras also translated into a dramatic increase in the frequency and number of $V\alpha2^+$ $CD8^+$ cells compared to Bim-sufficient Rip-mOva chimeras. The increased number of $V\alpha2^+$ $CD24^{lo}$ CD8SP in the OT-I DKO → Rip-mOva chimera compared to OT-I BKO→ Rip-mOva chimeras that we previously noted (**Fig. 4-3B**) did not translate into a higher number of $V\alpha2^+$ $CD8^+$ splenocytes. This may be due the peripheral niche already being maximally occupied due to enhanced survival of cells when Bim is deficient.

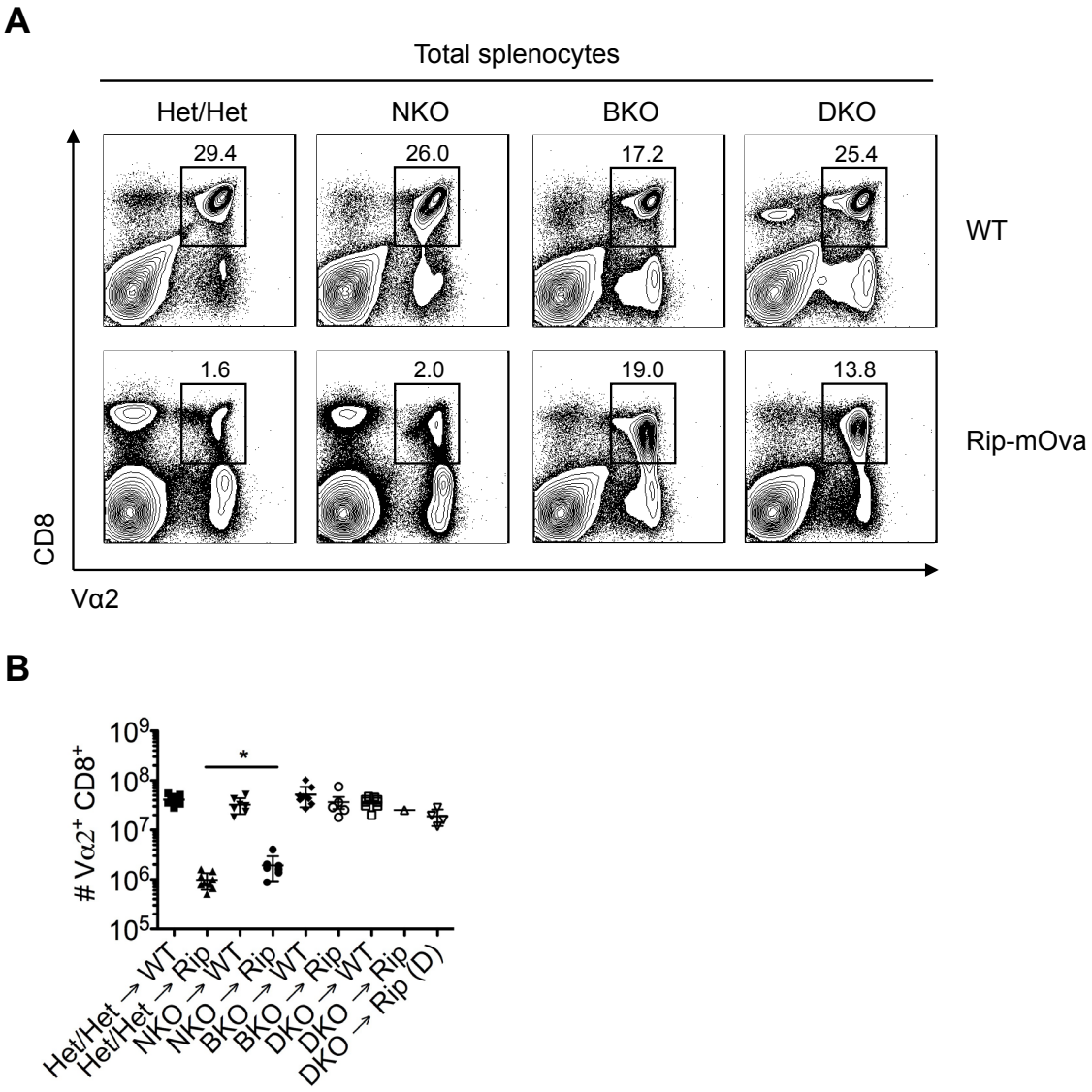


Figure 4-8. Impaired clonal deletion results in an increased number of antigen-specific CD8$^+$ T cells in the spleen. (A) Representative Vα2 by CD8 profiles of splenocytes from all chimeras. **(B)** The absolute number of Vα2$^+$ CD8$^+$ cells in the spleens of each chimera. Splenocytes were analyzed *ex vivo* by flow cytometry. Data is representative of a minimum of 7 chimeras from 3 independent donors, except in the case of OT-I BKO → Rip-mOva (5 from 2 donors) and OT-I DKO → Rip-mOva (1 from 1 donor). Data is depicted as mean ± SD; * $p < 0.05$.

Differences in the phenotype of peripheral antigen-specific CD8$^+$ T cells due to Nur77 or Bim deficiency

We next examined the activation and memory phenotypes of the splenic Vα2$^+$ CD8$^+$ T cells in each chimera. We found that the frequencies of central memory (CD44$^+$CD62L^{hi}) and effector memory (CD44$^+$CD62L^{lo}) subsets were significantly higher in OT-I NKO → WT compared to OT-I Het/Het → WT chimeras (**Fig. 4-9A-C**). However, the frequency of each memory subset was not significantly different between OT-I BKO and OT-I DKO → WT mice. These data may indicate that generation of memory T cells is increased in the absence of Nur77 but may be opposed by Bim deficiency. Compared to their respective WT recipient controls, we observed increased frequencies of central and effector memory Vα2$^+$ CD8$^+$ T cells in OT-I Het/Het → Rip-mOva and OT-I NKO → Rip-mOva chimeras. The only exception is the OT-I NKO → WT and OT-I NKO → Rip-mOva pair depicted in **Fig. 4-9A**; these plots were chosen because this particular experiment had the only complete set of chimeras thus far. Unlike in WT recipients, in the context of cognate antigen expression, we did not find any significant differences in the frequencies of memory subsets due to Nur77 deficiency. This suggests that efficient clonal deletion in both OT-I Het/Het → Rip-mOva and OT-I NKO → Rip-mOva chimeras resulted in a paucity of peripheral Vα2$^+$ CD8$^+$ T cells (**Fig. 4-8B**), which permitted escapees to have less competition for activation or memory signals. Conversely, in contrast to OT-I Het/Het or NKO → Rip-mOva chimeras, a very low proportion of Vα2$^+$ CD8$^+$ T cells in OT-I BKO and DKO → Rip-mOva mice expressed CD44 (**Fig. 4-9A**). This is consistent with decreased opportunity for T cells to encounter antigen due to the large number of T cells present in the spleen. Furthermore, the high number of Vα2$^+$ CD8$^+$ T cells in the periphery of OT-I BKO and DKO → Rip-mOva mice could mean that the niche for memory cells has been filled, resulting in a low proportion of

memory subsets. However, this is unlikely to be the sole explanation since there is also a large number of $V\alpha2^+$ $CD8^+$ splenocytes in OT-I BKO and DKO → WT mice, yet WT recipients exhibit a higher proportion of $CD44^+CD62L^{hi}$ cells, which are also more distinctly CD44-expressing than in Rip-mOva recipients (**Fig. 4-8A**). Another potential explanation is that Bim-deficient T cells have an intrinsic impairment in cognate antigen-induced activation.

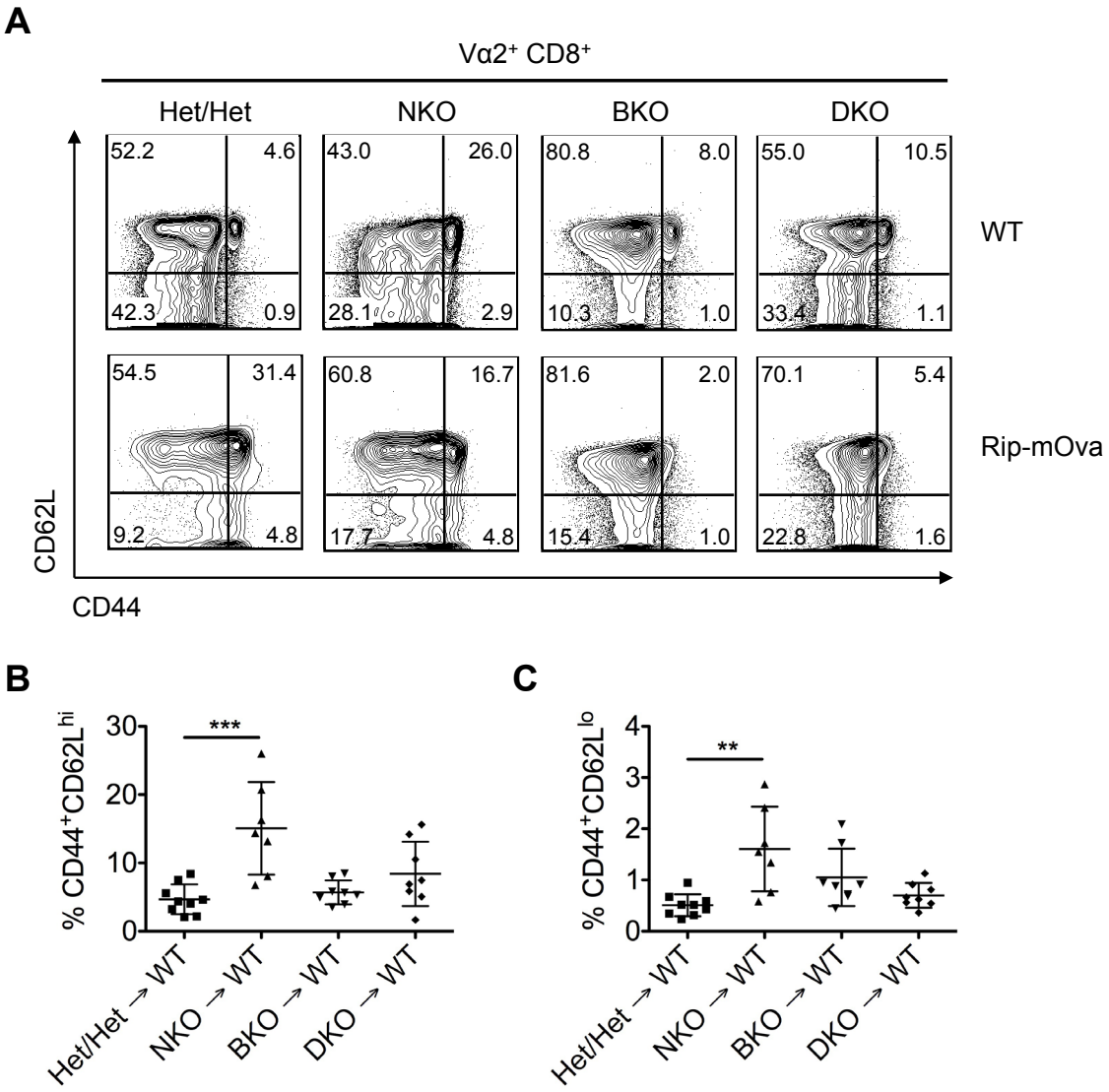


Figure 4-9. Differences in the phenotype of peripheral antigen-specific CD8$^+$ T cells due to Bim or Nur77 deficiency. (A) CD44 by CD62L profiles of Vα2$^+$ CD8$^+$ splenocytes from each chimera. **(B)** Compilation of the frequency of CD44$^+$CD62L^{hi} and CD44$^+$CD62L^{lo} cells within the Vα2$^+$ CD8$^+$ splenocyte population in WT recipients. Splenocytes were analyzed *ex vivo* by flow cytometry. Data is representative of a minimum of 7 chimeras from 3 independent donors, except in the case of OT-I BKO → Rip-mOva (5 from 2 donors) and OT-I DKO → Rip-mOva (1 from 1 donor). Data is depicted as mean ± SD; $^{**}p < 0.01$; $^{***}p < 0.001$.

We previously showed downregulation of CD127 on Vα2$^+$ CD8SP thymocytes from OT-I Het/Het and NKO → Rip-mOva chimeras relative to WT recipient counterparts (**Fig. 4-7**), and speculated on whether this would impair the fitness of those T cells in the periphery. We found that despite downregulation of CD127 on thymocytes, the majority of Vα2$^+$ CD8$^+$ splenocytes from OT-I Het/Het and NKO → Rip-mOva expressed CD127 to a similar level as Vα2$^+$ CD8$^+$ splenocytes from OT-I Het/Het and NKO → WT controls (**Fig. 4-10**). This suggests that the T cells have recovered CD127 expression in the periphery or the minority of CD127$^+$ thymocytes have outcompeted the CD127$^-$ thymocytes. Though CD127 expression on peripheral T cells from both OT-I Het/Het and NKO → Rip-mOva chimeras was high compared to thymocytes, we noted that CD127 expression on Vα2$^+$ CD8$^+$ splenocytes was consistently different between OT-I Het/Het and NKO → Rip-mOva chimeras. Specifically, while Vα2$^+$ CD8$^+$ T cells from OT-I Het/Het → Rip-mOva chimeras recovered CD127 expression to levels equivalent to T cells from OT-I Het/Het → WT controls, Vα2$^+$ CD8$^+$ T cells from OT-I NKO → Rip-mOva chimeras expressed a lower amount of CD127 than those from OT-I NKO → WT controls (**Fig. 4-10**). CD127 is downregulated on effector T cells but is critical for development of authentic memory T cells (246, 247). Therefore, despite comparable generation of CD44$^+$CD62L^{hi} and CD44$^+$CD62L^{lo} subsets in OT-I Het/Het and OT-I NKO → Rip-mOva chimeras, these data may indicate that while the number of antigen-specific T cells is higher in the absence of Nur77 (and correspondingly, the number of CD44$^+$ cells), maintenance of memory populations may be impaired. Since CD127 is downregulated upon IL-7 signaling (248), an alternative interpretation of the modest decrease in CD127 expression is that Vα2$^+$ CD8$^+$ T cells from OT-I NKO → Rip-mOva chimeras have experienced more IL-7 signaling. This could have implications on their ability to survive and proliferate.

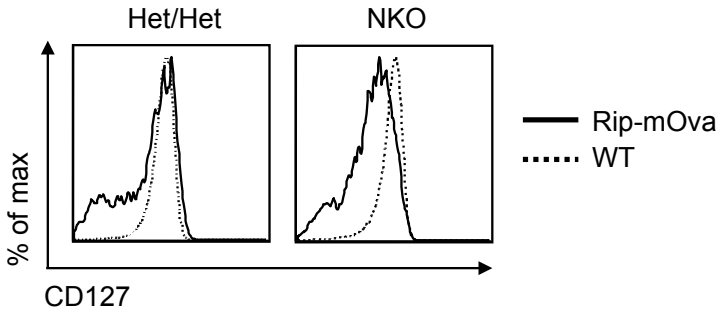


Figure 4-10. Decreased CD127 expression on T cells from OT-I NKO → Rip-mOva chimeras. CD127 expression on *ex vivo* $V\alpha2^+ CD8^+$ splenocytes from indicated chimeras. Data is representative of a minimum of 3 mice of each chimera type generated from at least 2 independent donors.

Increased CD69 induction on T cells from Rip-mOva chimeras deficient in Nur77 or Bim in response to antigen stimulation *in vitro*

OT-I NKO and OT-I BKO → Rip-mOva mice did not develop diabetes despite an increased number of $V\alpha2^+ CD8^+$ peripheral T cells **(Table 1**). This was particularly astonishing for OT-I BKO → Rip-mOva chimeras, which contained approximately 27-fold higher numbers of antigen-specific T cells compared to OT-I Het/Het → Rip-mOva chimeras (**Fig. 4-8B**). Our group previously reported that OT-I BKO → Rip-mOva T cells showed impaired proliferation and cytokine production in response to Ova peptide stimulation *in vitro* (143). Consequently, we examined whether T cells that escaped thymic deletion in any of the other chimeras exhibited differences in function. To this end, we stimulated bulk splenocytes from each chimera with B6 splenocytes pulsed with 100 nM of Ova for various durations. We first characterized CD69 induction after 24 h of stimulation to assess TCR signaling potential. In all WT recipients, the vast majority of $V\alpha2^+ CD8^+$ T cells upregulated CD69 to a similar degree (**Fig. 4-11A**). In OT-I Het/Het → Rip-mOva chimeras, a large fraction of $V\alpha2^+ CD8^+$ T cells did not respond to antigen. In comparison, the frequency of $V\alpha2^+ CD8^+$ T cells from OT-I NKO → Rip-mOva chimeras that upregulated CD69 upon antigen stimulation tended to be higher (**Fig 4-11A and 4-11B**). Furthermore, there was a trend towards an increased amount of CD69 induced in $V\alpha2^+ CD8^+$ T cells from OT-I NKO → Rip-mOva chimeras compared to $V\alpha2^+ CD8^+$ T cells from OT-I Het/Het→ Rip-mOva chimeras (**Fig. 4-11C**). Though there is variability between experiments, the increase in the frequency and magnitude of CD69 induction associated with Nur77 deficiency was observed in 5 of 6 pairs of OT-I Het/Het → Rip-mOva and OT-I NKO → Rip-mOva mice. Since CD69 is an early T cell activation marker, it is also possible that examination

at a time point earlier than 24 h would have showed more significant differences between OT-I Het/Het and OT-I NKO → Rip-mOva thymocytes.

Despite the fact that OT-I BKO → Rip-mOva $V\alpha2^+$ $CD8^+$ cells exhibit limited proliferation and cytokine production (143), we found that most of them, as well as those from the OT-I DKO→ Rip-mOva chimera, were able to induce CD69 to a similar degree. This indicates that some aspects of T cell function (e.g. TCR signaling or early activation) are less affected than others. Furthermore, these data validate the conditions of the stimulation assay since the majority of T cells were able to interact with antigen. However, while T cells from all Rip-mOva chimeras can respond to antigen, they did not upregulate CD69 to the same degree as in WT recipients (**Fig. 4-11A**). Therefore, antigen-specific T cells from all Rip-mOva chimeras showed evidence of being tolerized to some degree.

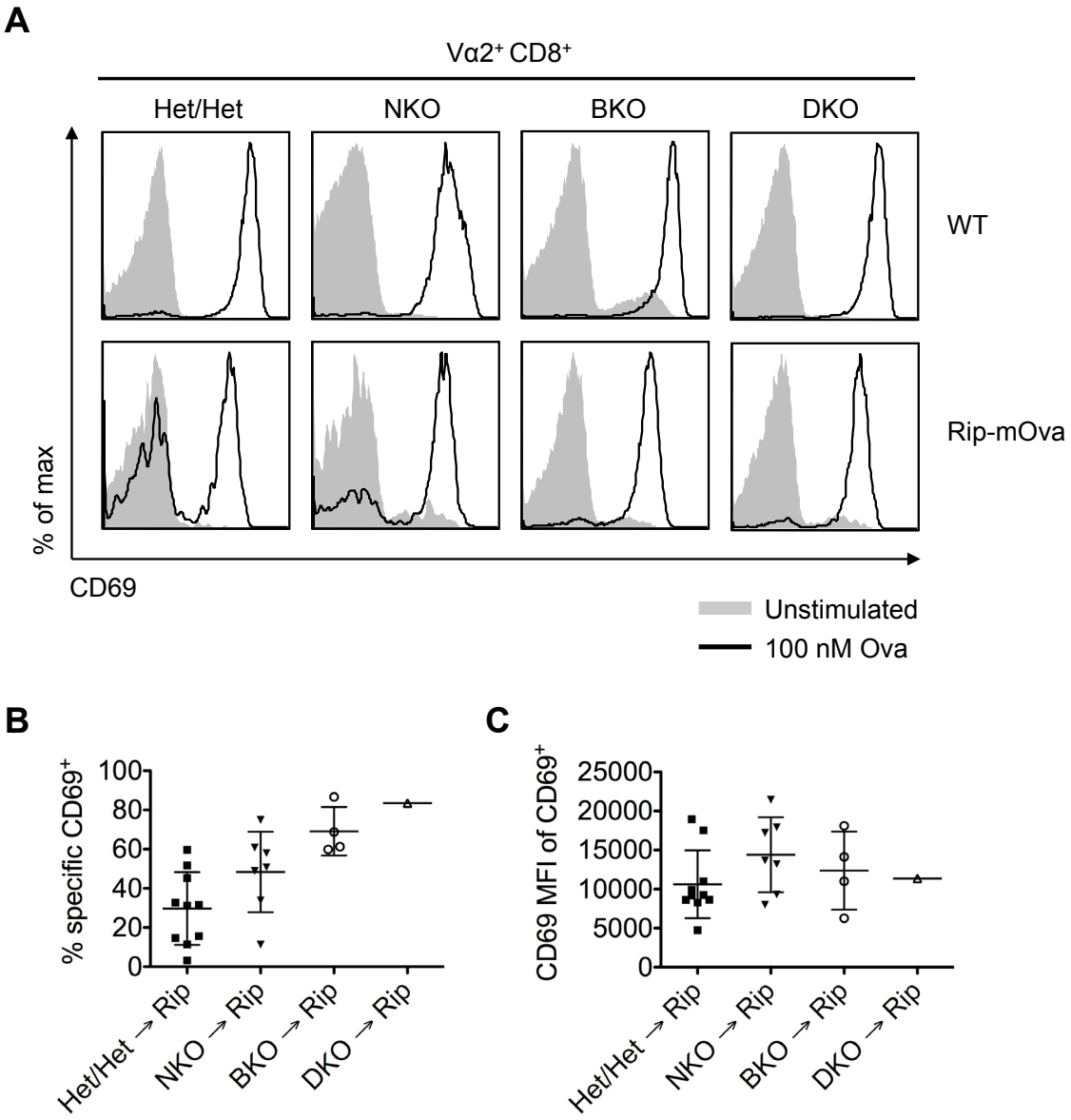


Figure 4-11. T cells from Rip-mOva chimeras have reduced CD69 induction upon antigen stimulation *in vitro*. Bulk chimeric splenocytes were cultured with unloaded B6 splenocytes (unstimulated) or B6 splenocytes pulsed with 100 nM of Ova peptide at a ratio of 4:1. Cells were analyzed after 24 h by flow cytometry. **(A)** CD69 expression on $V\alpha2^+$ $CD8^+$ splenocytes. **(B)** The amount of antigen-specific CD69 induction on $V\alpha2^+$ $CD8^+$ cells was calculated by subtracting the frequency of $CD69^+$ cells in unstimulated samples from the frequency of $CD69^+$ cells in Ova-stimulated samples. **(C)** The MFI of CD69 within the $CD69^+$ $V\alpha2^+$ $CD8^+$ population. Data is representative of a minimum of 4 chimeras from at least 2 independent donors, except OT-I DKO → Rip-mOva (1 from 1 donor). Data is depicted as mean ± SD.

Nur77 deficiency relieves impairment in proliferation of T cells from Rip-mOva chimeras

We went on to examine the ability of T cells from each chimera to proliferate in response to Ova by measuring dilution of CFSE. After 48 h of stimulation, $V\alpha2^+$ $CD8^+$ cells from all WT recipients underwent a similar degree of proliferation (**Fig. 4-12A**). Consistent with a low frequency of CD69 upregulation, most OT-I Het/Het → Rip-mOva T cells did not proliferate. A fraction of cells did proliferate to some degree but went through a fewer number of cell divisions than most OT-I Het/Het → WT T cells. As shown previously, proliferation of OT-I BKO → Rip-mOva T cells was inhibited (143). We consistently found that $V\alpha2^+$ $CD8^+$ cells from OT-I NKO → Rip-mOva experienced a greater amount of proliferation in response to antigen than those from OT-I Het/Het → Rip-mOva chimeras (**Fig. 4-12A**). Furthermore, in the one experiment involving the OT-I DKO → Rip-mOva chimera, $V\alpha2^+$ $CD8^+$ cells from the OT-I DKO → Rip-mOva mouse proliferated more than those from the OT-I BKO → Rip-mOva mouse. These differences between the Rip-mOva chimeras were also observed after 72 h of stimulation, while proliferation in WT recipients remained comparable (**Fig. 4-12B**). These data suggest that Nur77 deficiency may relieve some of the inhibition of proliferation, and are consistent with the trend towards increased CD69 induction after 24 h (**Fig. 4-11**). Again, it is important to point out that the degree of proliferation exhibited by $V\alpha2^+$ $CD8^+$ T cells from any of Rip-mOva recipients did not match that of WT recipient controls, even at the later time point. This provides further evidence that antigen-specific T cells in Rip-mOva chimeras are not fully functional.

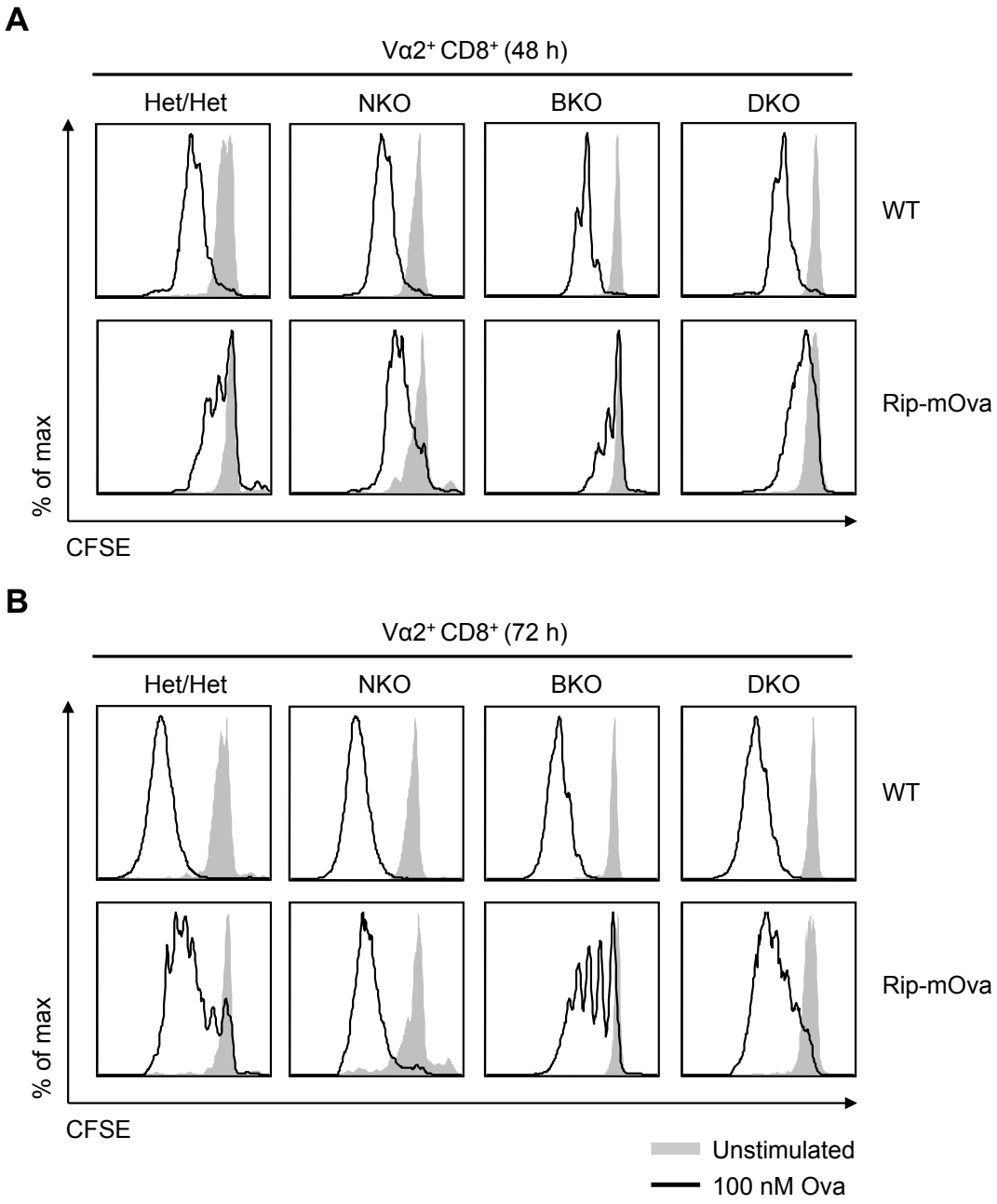


Figure 4-12. Nur77 deficiency relieves impairment in proliferation of T cells from Rip-mOva chimeras. Bulk splenocytes from each chimera were labeled with carboxyfluorescein succinimidyl ester (CFSE) and then cultured with unloaded B6 splenocytes or B6 splenocytes pulsed with 100 nM of Ova at a ratio of 4:1. Cells were collected after 48 h **(A)** and 72 h **(B)** of culture and CFSE was measured in $V\alpha2^+$ $CD8^+$ cells. Comparisons between OT-I NKO → Rip-mOva and OT-I BKO → Rip-mOva to OT-I Het/Het → Rip-mOva are representative of 6 and 3 pairs of mice, respectively.

T cells from Rip-mOva chimeras have impaired cytokine production

The last aspect of T cell function that we assayed was production of IFNγ and TNFα upon restimulation of splenocytes with Ova after 5 days of prior stimulation. Without restimulation, cytokine production in WT recipient T cells was not detected. Upon restimulation, $V\alpha2^+$ $CD8^+$ cells from all WT recipients showed a comparable degree of cytokine production, with most cells producing both IFNγ and TNFα or just TNFα (**Fig. 4-13A**). When we examined $V\alpha2^+$ $CD8^+$ cells from Rip-mOva chimeras that were not restimulated, we detected some degree of cytokine staining (**Fig. 4-13B**). If this is not a staining artifact, it may indicate that Ova encounter in Rip-mOva chimeras *in vivo* induced a low level of cytokine production that could be detected after 5 days of *in vitro* culture. Upon restimulation of Rip-mOva T cells with Ova peptide *in vitro*, we did not observe much increase in cytokine production. Compared to OT-I Het/Het → Rip-mOva mice, T cells from OT-I NKO → Rip-mOva cells sometimes produced greater amounts of IFNγ and TNFα when restimulated (**Fig. 4-13B**); this occurred in 3 of 6 pairs of mice. This would be in agreement with increased activation and proliferation of OT-I NKO → Rip-mOva T cells, which were more consistently observed. However, the main point illustrated by this experiment is that cytokine production by T cells from any of the Rip-mOva chimeras was greatly reduced compared to WT chimeras (**Fig. 4-13A**). Therefore, high affinity antigen encounter in Rip-mOva mice imposed tolerance on different aspects of T cell function to different degrees.

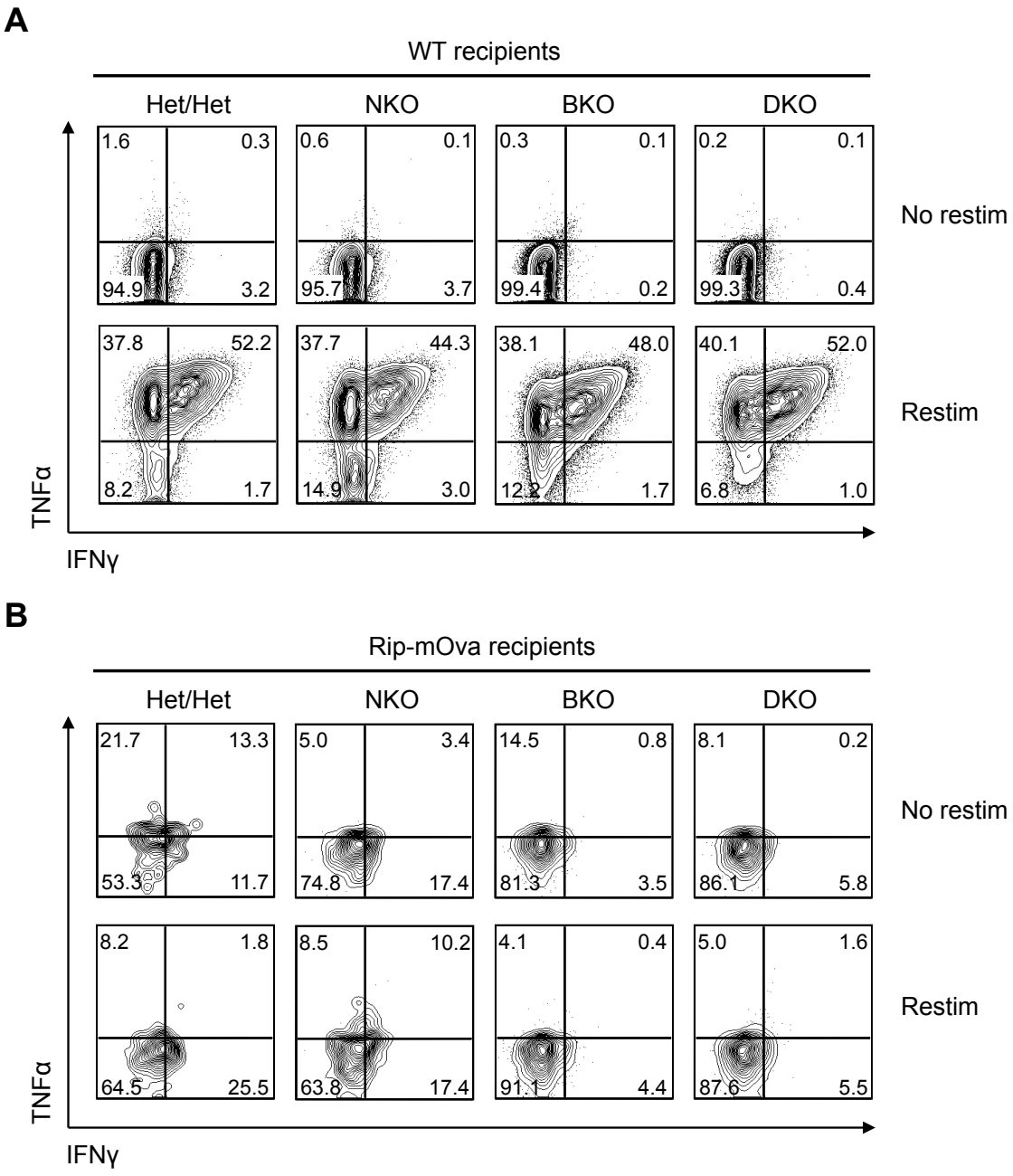


Figure 4-13. T cells from Rip-mOva chimeras have impaired cytokine production. Bulk splenocytes from each chimera were cultured with B6 splenocytes pulsed with 100 nM Ova peptide at a ratio of 4:1. After 5 days of culture, cells were restimulated with 100 nM of Ova in the presence of brefeldin A for 4 h or were not restimulated. Intracellular IFNγ and TNFα were measured in $V\alpha2^{+}$ $CD8^{+}$ cells from WT **(A)** and Rip-mOva **(B)** chimeras by flow cytometry.

High incidence of diabetes in OT-I DKO → Rip-mOva chimeras

The ultimate measure of autoimmunity in the OT-I Rip-mOva model is development of diabetes. As per criteria given in the literature, diabetes was defined as two consecutive blood glucose measurements of more than 15 mmol/L (~270 mg/dL) (249) taken at least one day apart. OT-I DKO → Rip-mOva chimeras were the only type of chimera to develop diabetes (**Table 1**). Furthermore, the incidence of diabetes was high (4 of 5OT-I DKO → Rip-mOva chimeras harvested), with each mouse exhibiting blood glucose readings well above the diagnostic threshold. These data demonstrate that while Bim deficiency was sufficient to impair clonal deletion and produce a large population of antigen-specific T cells (**Fig. 4-3 and 4-8**), additional deficiency in Nur77 was required for autoimmunity. The effect of Nur77 deficiency may be related to a higher propensity for the T cells to undergo activation (**Fig. 4-11**) and proliferation (**Fig. 4-12**) compared to Nur77-sufficient T cells. It should be noted that diabetic OT-I DKO →Rip-mOva chimeras were not included in the phenotypic and functional experiments depicted in this chapter up to this point. However, preliminary assessment indicates no great differences in the composition or function of the antigen-specific T cell population in diabetic mice compared to the non-diabetic OT-I DKO → Rip-mOva chimera. This would suggest that $V\alpha2^+$ $CD8^+$ cells utilize mechanisms other than secretion of IFNγ and TNFα to mediate pancreatic islet destruction. Though pathology would have ultimately been T cell-mediated, given that diabetes only developed in the context of TCR transgenic and cognate antigen expression (i.e. not in OT-I DKO → WT chimeras), it is possible that Bim and Nur77 deficiency in other immune cells also contributed to autoimmunity. This would be reminiscent of the thymocyte-extrinsic factors resulting in an increased $T3.70^+$ CD8SP population in HY^{cd4} DKO → M chimeras (**Fig. 3-14**).

Chimera	Diabetes incidence	Average terminal blood glucose (mmol/L)
OT-I Het/Het → WT	0/9	6.3
OT-I NKO → WT	0/7	6.7
OT-I BKO → WT	0/8	9.0
OT-I DKO → WT	0/9	7.8
OT-I Het/Het → Rip-mOva	0/9	6.6
OT-I NKO → Rip-mOva	0/7	7.2
OT-I BKO → Rip-mOva	0/5	7.9
OT-I DKO → Rip-mOva	4/5	13.1 in non-diabetic (1/5) 25.0 in diabetic (4/5)

Table 1. High incidence of diabetes in OT-I DKO → Rip-mOva chimeras.

Blood glucose was monitored on a weekly basis using commercial products starting 2 weeks and up to 14 weeks after bone marrow reconstitution. Mice with a blood glucose reading of over 15 mmol/L would be tested again 1 day later. Mice with two consecutive blood glucose measurements over 15 mmol/L were considered diabetic. Terminal blood glucose refers to the last reading taken before harvesting the mice – to a maximum of 1 week prior in the case of non-diabetic mice and 1 day prior in the case of diabetic mice. Each type of chimera was generated from at least 2 independent bone marrow donors.

In summary, the results presented in this chapter provide insight into the roles of Bim and Nur77 in clonal deletion and other tolerance mechanisms in a TRA model. In comparison to UbA-mediated deletion of DP thymocytes, Nur77 and Bim play more important roles in TRA-mediated deletion of CD8SP thymocytes. As in DP thymocytes, Bim deficiency had a greater effect on deletion efficiency than Nur77 deficiency. Nur77 may have a minor contribution to Bim induction in CD8SP thymocytes, which may explain why Nur77 deficiency resulted in a modest impairment in clonal deletion. While others showed that Nur77 partly regulates Bim induction in the OT-II Rip-mOva model as well, combined Bim and Nur77 deficiency had the same phenotype as Nur77 single deficiency in that system (160), whereas combined Bim and Nur77 deficiency had the same phenotype as Bim single deficiency in our OT-I Rip-mOva model. Together, these data highlight additional differences between MHC class I and class II-restricted negative selection, with Nur77 playing a greater role in the deletion of CD4SP thymocytes. Despite some thymocytes surviving clonal deletion, antigen-specific T cells in the periphery of Rip-mOva chimeras were functionally impaired compared to T cells from WT recipients. However, some aspects of T cell function were increased in the absence of Nur77. The effect of Nur77 deficiency was only observed in Rip-mOva recipients, in which primary antigen encounter has already occurred, pointing to a potential role for Nur77 in anergy induction. Along with data from the HY^{cd4} model, these results suggest that Nur77 has previously unappreciated roles in T cell biology apart from its pro-apoptotic function. This effect of Nur77 deficiency, combined with impaired clonal deletion due to Bim deficiency, resulted in most OT-I DKO → Rip-mOva chimeras developing diabetes.

CHAPTER 5: RESULTS - The role of Bim and Nur77 in thymocyte development in a polyclonal repertoire

In the previous chapters, we examined negative selection against UbA and TRA using MHC class I-restricted TCR transgenic mouse models. Since MHC class II-restricted thymocytes are thought to experience stronger and/or longer TCR signaling, resulting in a transcriptional program distinct from that of MHC class I-restricted thymocytes (250), it is possible that they also undergo different molecular mechanisms of negative selection. Indeed, our studies in the OT-I Rip-mOva system show that the contributions of Bim and Nur77 in clonal deletion are different from what others have found in the OT-II Rip-mOva model (143, 144, 160). In addition to deletion, other forms of thymic tolerance may be particularly relevant to MHC class II-restricted thymocytes. For example, a subset of $FR4^{hi}CD73^{hi}$ $CD4^{+}$ T cells has been identified as anergic in an autoimmune model (224), but it is presently unclear whether this phenotype was induced in the thymus. In a related vein, natural Treg cells are thought to result from high affinity self-antigen encounter in the thymus and are critical for suppression of autoimmune disease (230, 231). The niche for Treg selection is much smaller than that of conventional T cells, and consequently, there are few thymic Treg cells in TCR transgenic mice (191, 251, 252). In addition to these alternative fates for CD4SP thymocytes, it is thought that downregulation of CD4 and CD8 following TCR signaling can occur (253). Post-selection TCR^{+} DN thymocytes have recently been implicated as precursors of $CD8\alpha\alpha^{+}$ IELs (207), and may also develop into other regulatory DN subsets (209). In order to study the role of Bim and Nur77 in clonal deletion and other forms of tolerance in the context of physiological TCR diversity, we transitioned from

antigen-specific TCR transgenic models to non-TCR-transgenic mice with polyclonal repertoires.

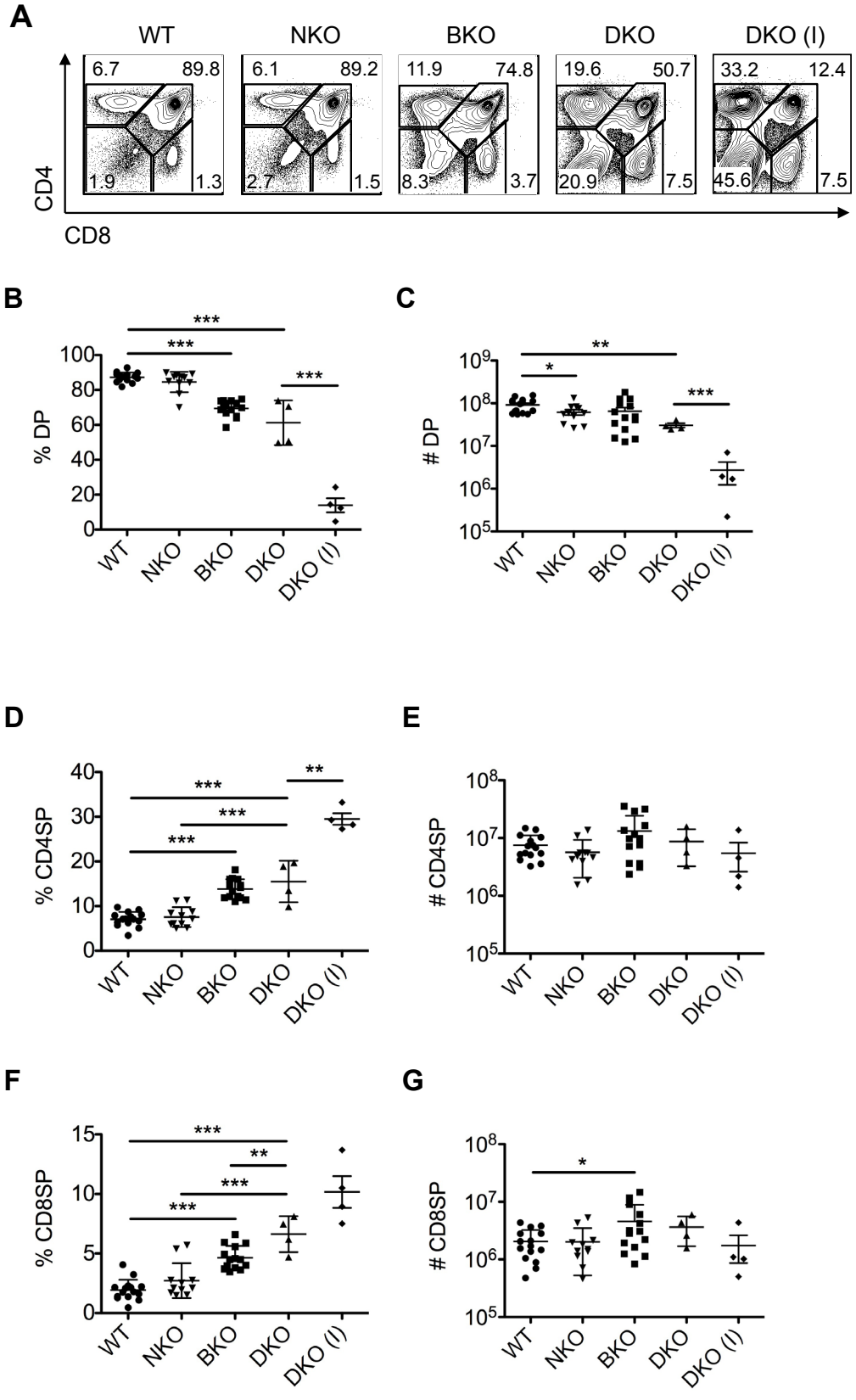
Altered development of thymocyte subsets in the absence of Bim and Nur77

In this chapter, we characterized non-TCR transgenic mice of the B6 background deficient for Bim (BKO), Nur77 (NKO), or both genes (DKO). Upon examining the thymi, we found that BKO and DKO mice exhibited altered proportions of DP to SP thymocytes compared to WT mice (**Fig. 5-1A**). Specifically, BKO mice had significantly lower frequencies of DP (**Fig. 5-1B**) and significantly higher frequencies of CD4SP (**Fig. 5-1D**) and CD8SP thymocytes (**Fig. 5-1F**) compared to WT mice. Similarly, DKO mice had significant increases in the frequencies of CD4SP and CD8SP thymocyte compared to NKO mice as well as WT mice. In contrast, NKO mice did not exhibit any differences in the proportion of thymocyte subsets compared to WT mice. Furthermore, DKO mice only differed significantly from BKO mice with respect to the frequency of CD8SP thymocytes, and there was still overlap in the two data sets (**Fig. 5-1F**). These results suggest that while Nur77 may have a minor contribution to clonal deletion and/or positive selection in a polyclonal repertoire, the increased proportion of SP thymocytes is mainly associated with Bim deficiency. Though we do not know how much of the increase is due to impaired clonal deletion in non-TCR transgenic mice, we found signs of both enhanced positive selection and impaired/delayed clonal deletion associated with Bim deficiency in HY^{cd4} and OT-I chimeras. Compared to the lower frequency of DP thymocytes in BKO and DKO mice, the difference in the absolute number of DP thymocytes between strains was subtle (**Fig. 5-1C**). Nur77 deficiency seemed to have an effect in decreasing DP numbers, resulting in mild but significant decreases in NKO and DKO mice compared to WT. Though Bim deficiency increases DN thymocyte survival, Bim-deficient DN thymocytes were found to be impaired in

proliferation, which was thought to limit the DP pool to a size comparable to WT (208). Despite a comparable or lower number of DP thymocytes, BKO and DKO mice exhibited mildly higher numbers of CD4SP and CD8SP thymocytes compared to WT (**Fig. 5-1E and 5-1G**). However, the only significant increase was found in the CD8SP subset in BKO mice. The disparity between dramatic differences in frequency but not absolute number of SP thymocytes in BKO and DKO mice may indicate a limited thymic niche able to support SP cells. Nevertheless, the altered ratio of DP to SP thymocytes suggests that deficiency in Bim, and to a lesser extent Nur77, affects thymocyte development following TCR-mediated selection.

In addition to observing that DKO mice exhibited a skewed DP to SP ratio, we found that four of eight DKO mice exhibited an even more extreme phenotype characterized by a severe loss in the frequency and number of DP thymocytes compared to the other four DKO mice (**Fig. 5-1A-C**). DP thymocytes are much more sensitive than SP cells to apoptosis induced by various stimuli including TCR stimulation (254), glucocorticoids (255), and inflammatory insult (256).Given that Bim and Nur77 participate in clonal deletion of thymocytes, and likely apoptosis of other immune cells, we speculate that the loss of DP thymocytes in some DKO mice is a result of excessive inflammation. On account of the putative inflammation, DKO mice that exhibit severe DP loss will be henceforth termed DKO (I) to distinguish them from DKO mice with the 'normal' phenotype. As a consequence of decreased frequency of DP thymocytes in DKO (I) mice, there were elevated frequencies of CD4SP (**Fig. 5-1D**) and CD8SP (**Fig. 5-1F**; $p = 0.06$) compared to normal DKO mice. However, absolute numbers of SP thymocytes were not higher than in the other DKO mice (**Fig. 5-1E and 5-1G**), likely due to the extreme loss of DP precursors. Though many characteristics were common to both types of DKO mice, their data

sets were not combined and figures labeled DKO involve those mice of the normal phenotype only.


A
WT
NKO
BKO
DKO
DKO (I)
6.7
89.8
1.9
1.3
6.1
89.2
2.7
1.5
11.9
74.8
8.3
3.7
19.6
50.7
20.9
7.5
33.2
12.4
45.6
7.5
CD4
CD8
B
% DP
C
DP
D
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E
CD4SP
F
% CD8SP
G
CD8SP

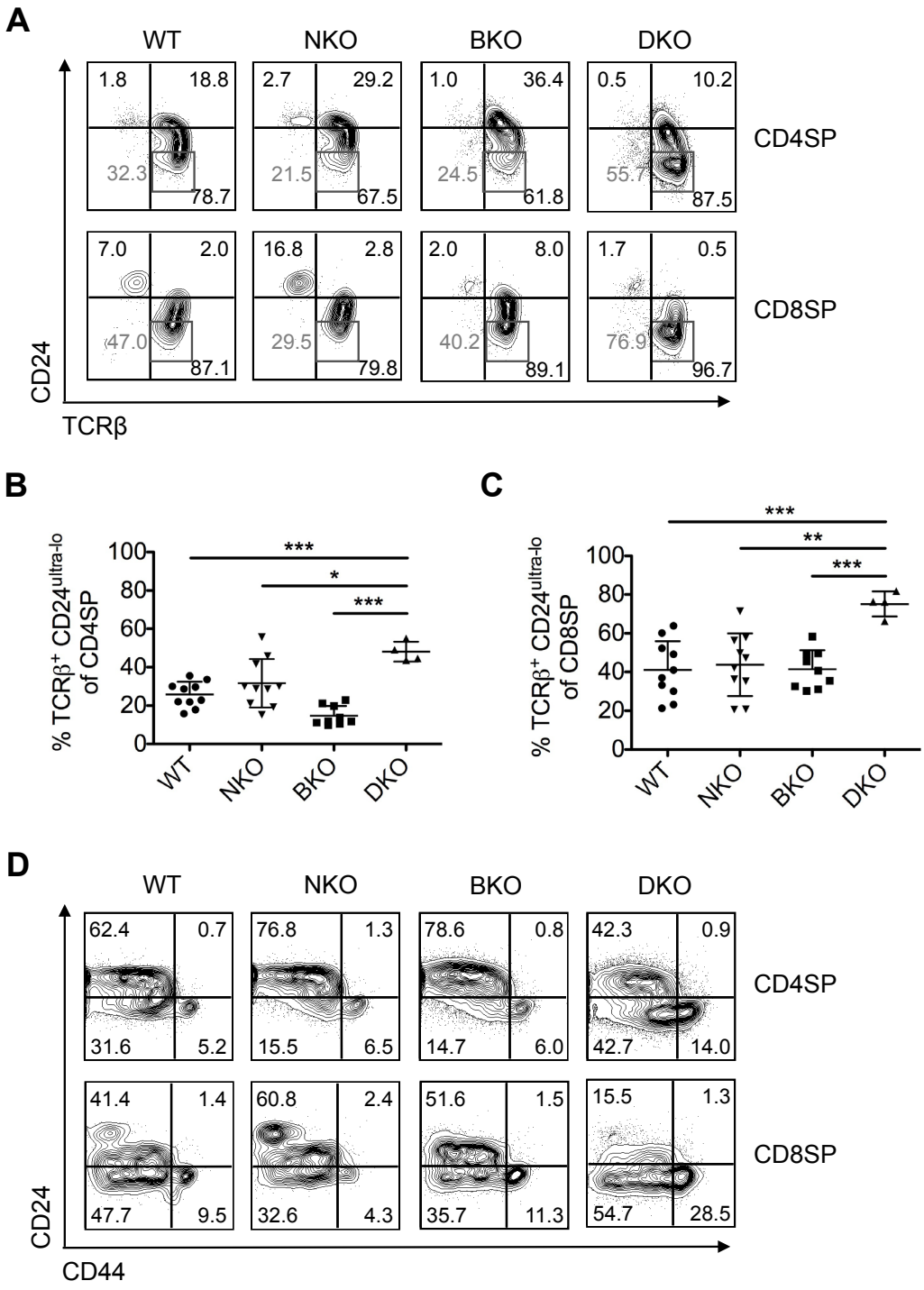
Figure 5-1. Altered development of thymocyte subsets in the absence of Bim and Nur77. Thymocytes from non-TCR transgenic WT, $Nur77^{-/-}$ (NKO), $Bim^{-/-}$ (BKO), and $Nur77^{-/-}Bim^{-/-}$ (DKO) mice were analyzed *ex vivo* by flow cytometry. DKO mice that exhibited a dramatic reduction in the DP thymocyte compartment are labeled as DKO (I) and depicted separately from other DKO mice. **(A)** Representative CD8 by CD4 profiles of thymi. **(B)** Frequency and **(C)** absolute number of DP thymocytes from indicated mice. **(D)** Frequency and **(E)** absolute number of CD4SP thymocytes from indicated mice. (**F)** Frequency and **(G)** absolute number of CD8SP thymocytes from indicated mice. Data represents a minimum of 4 mice of each strain from 4 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Increase in SP thymocytes of the most mature stage in DKO mice

While transition between the DP to SP stages appeared to be enhanced in BKO mice compared to WT or NKO mice, a similar proportion of CD4SP and CD8SP from BKO mice exhibited the mature TCRβ^{+}CD24^{lo} phenotype as in the other strains (**Fig. 5-2A**). This may indicate that Bim deficiency increases the proportion of DP thymocytes receiving a positive selection signal but is not sufficient to promote continued SP maturation. Within the TCRβ^{+}CD24^{lo} quadrant, SP thymocytes show different degrees of CD24 downregulation, with the most mature SPs expressing the lowest levels of CD24 (henceforth termed CD24$^{ultra\text{-}lo}$ and depicted as the smaller square gate on **Fig. 5-2A**). Not only was the frequency of TCRβ^{+}CD24^{lo} CD4SP or CD8SP highest in DKO mice, the proportion of CD24$^{ultra\text{-}lo}$ SP thymocytes was significantly higher in DKO mice than in WT mice and either single knockout strain (**Fig. 5-2B and 5-2C**). Although not previously quantified, these data are consistent with observations in HY^{cd4} (**Fig. 3-14**) and OT-I (**Fig. 4-3**) chimeras. The difference between DKO and the single knockouts suggests that a combination of Bim and Nur77 deficiency was necessary to produce an accumulation of mature SP thymocytes. This may indicate enhanced maturation of DKO SP thymocytes, conceivably through altered TCR signaling and increased survival.

In addition to enhanced maturation, other possible explanations for the increase in CD24$^{ultra\text{-}lo}$ SP thymocytes include increased retention in the thymus, impaired export, or recirculation of mature T cells. To investigate the question of recirculation, we examined CD44 expression on SP thymocytes since most T cells that recirculate to the thymus are CD44^{hi} (257). By plotting CD44 against CD24, we found that only a fraction of CD24$^{ultra\text{-}lo}$ CD4SP and CD8SP thymocytes were CD44^{hi} (**Fig. 5-2D**). In the CD4SP subset, a higher proportion of CD24$^{ultra\text{-}lo}$ cells were CD44^{hi} in

NKO, BKO, and DKO mice compared to WT mice. Similar trends were observed in the CD8SP subset, except the proportion of $CD44^{hi}$ $CD24^{ultra\text{-}lo}$ CD8SP thymocytes was not always different between WT and NKO mice. These data suggest that some of the $CD24^{ultra\text{-}lo}$ SP thymocytes were recirculating mature T cells, and that the frequency of recirculation was higher in NKO, BKO, and DKO mice compared to WT. However, the data also show that the majority of $CD24^{ultra\text{-}lo}$ CD4SP and CD8SP thymocytes in all mice were not $CD44^{hi}$. A sizeable fraction of $CD24^{ultra\text{-}lo}$ SP thymocytes were $CD44^{int}$, consistent with our proposal of CD44 upregulation during normal thymocyte maturation. Therefore, the accumulation of mature thymocytes in DKO mice is partially due to Bim and Nur77 deficiency affecting mechanisms other than recirculation. Whereas high induction of CD44 was apparent on immature $CD24^{lo}$ thymocytes undergoing negative selection in OT-I Rip-mOva chimeras (**Fig. 4-6A**), $CD44^{hi}$ thymocytes are almost exclusively $CD24^{ultra\text{-}lo}$ in polyclonal mice (**Fig. 5-2D**). This indicates that CD44 induction is not a suitable marker for thymocytes undergoing negative selection in polyclonal mice.


A
WT
NKO
BKO
DKO
1.8
18.8
32.3
78.7
2.7
29.2
21.5
67.5
1.0
36.4
24.5
61.8
0.5
10.2
55.7
87.5
CD4SP
7.0
2.0
47.0
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16.8
2.8
29.5
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8.0
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CD8SP
CD24
TCRβ
B
% TCRβ+ CD24ultra-lo of CD4SP
***
*
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WT
NKO
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DKO
C
% TCRβ+ CD24ultra-lo of CD8SP
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D
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5.2
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42.3
0.9
42.7
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CD4SP
41.4
1.4
47.7
9.5
60.8
2.4
32.6
4.3
51.6
1.5
35.7
11.3
15.5
1.3
54.7
28.5
CD8SP
CD44

Figure 5-2. Increase in SP thymocytes of the most mature stage in DKO mice. (A) TCRβ and CD24 expression among CD4SP and CD8SP thymocytes from each strain. Numbers in each corner depict the frequency in the indicated quadrant. Among total CD4SP and CD8SP thymocytes, those that have the lowest CD24 expression ($CD24^{ultra\text{-}lo}$) were additionally gated with a grey square. The frequency of $TCR\beta^{+}CD24^{ultra\text{-}lo}$ cells among total CD4SP and CD8SP thymocytes is depicted in grey to the left of the gate. **(B)** The frequency $TCR\beta^{+}CD24^{ultra\text{-}lo}$ CD4SP thymocytes and **(C)** $TCR\beta^{+}CD24^{ultra\text{-}lo}$ CD8SP thymocytes were quantified over all experiments. **(D)** CD44 by CD24 profiles of total CD4SP and CD8SP thymocytes from each strain. Data represents a minimum of 4 mice of each strain from 4 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Bim and Nur77 deficiency results in altered CD127 expression on SP thymocytes

In the previous chapter, we reported downregulation of CD127 on OT-ICD8SP thymocytes in chimeras undergoing negative selection, whereas CD127 expression remained high in chimeras that did not experience negative selection (**Fig. 4-7**). Given the importance of IL-7 signaling in mature T cell homeostasis, examining CD127 expression may provide further insight into the maturation of SP thymocytes. We found that the frequency and level of CD127 expression on polyclonal CD4SP and CD8SP were the same in WT and NKO mice, with the majority of SP thymocytes being $CD127^+$ (**Fig. 5-3A and 5-3B**). In contrast, most CD4SP and CD8SP thymocytes from BKO mice were $CD127^-$. The frequency of $CD127^+$ CD4SP or CD8SPthymocytes in DKO mice was also lower than in WT and NKO mice, but the difference was smaller in the CD8SP subset (**Fig. 5-3A and 5-3B**). These data suggest that while most SP thymocytes attained the mature $TCR\beta^+CD24^{lo}$ in BKO and DKO mice (**Fig. 5-2A**), most of them may not be as fit as SP thymocytes from WT or NKO mice. A body of literature has established that CD127 is downregulated upon strong TCR signaling (258). Thus, one possibility for the increased frequency of $CD127^-$ SP thymocytes is an accumulation of cells unable to be deleted in BKO and DKO mice. Another explanation is that many thymocytes failed to upregulate CD127 following positive selection due a limited thymic niche and increased frequencies of CD4SP and CD8SP thymocytes in BKO and DKO mice. Despite the stark difference in the thymus, the majority of splenic $CD4^+$ and $CD8^+$ T cells from all strains expressed a similar level of CD127, although a small fraction of BKO and DKO T cells remained $CD127^-$ (**Fig. 5-3C and Fig. 5-3D**). This suggests that BKO and DKO SP thymocytes eventually upregulated CD127 in the periphery or that the $CD127^+$ T cells outcompeted the $CD127^-$ T cells.

Another interesting observation is that despite DKO mice having a lower frequency of $CD127^+$ CD4SP or CD8SP than WT or NKO mice, the proportion of DKO SP expressing CD127 was higher than in BKO mice (**Fig. 5-3A and 5-3B**). This was especially exaggerated in the CD8SP subset, in which we consistently observed a fraction of DKO CD8SP that expressed a higher amount of CD127 than even WT and NKO CD8SP (**Fig. 5-3B**). The difference between BKO and DKO mice suggests that Nur77 deficiency played a role in increasing CD127 expression, though the effect was only apparent in combination with Bim deficiency. Along with exaggerated downregulation of CD24 on DKO SP (**Fig. 5-2**), these data support a role for Nur77 in thymocyte maturation.

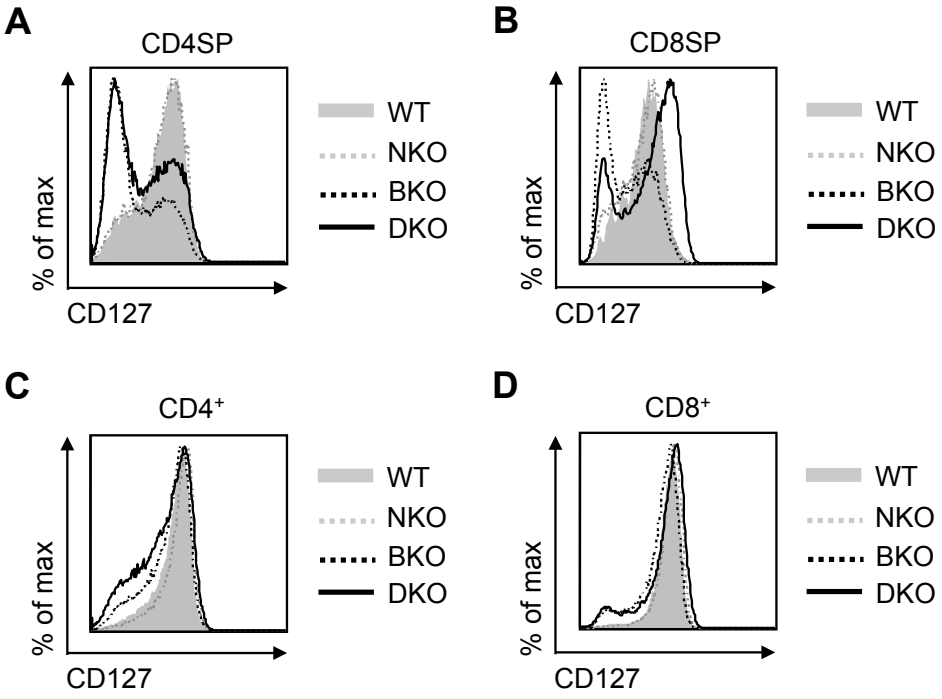


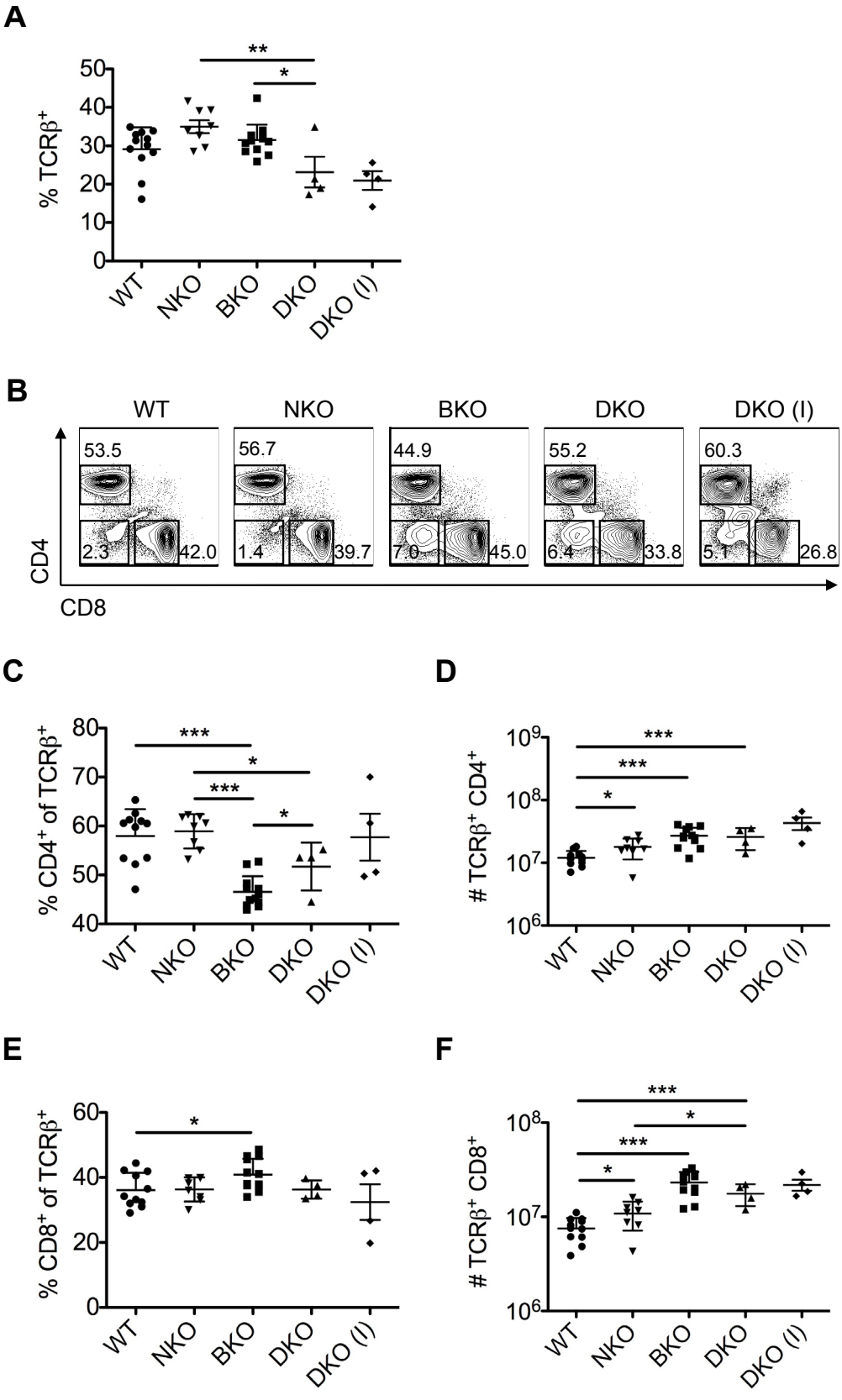
Figure 5-3. Bim and Nur77 deficiency results in altered CD127 expression on SP thymocytes. Representative histograms depicting CD127 expression on CD4SP thymocytes **(A)**, CD8SP thymocytes **(B)**, $CD4^+$ splenic T cells **(C)**, and $CD8^+$ splenic T cells **(D)** from each strain. Data represents a minimum of 4 mice of each strain from 4 independent experiments.

Bim and Nur77 deficiency increases the number of $CD4^+$ and $CD8^+$ T cells in the spleen

Given the results in the thymus, we next determined if any of the other thymic differences in Bim and/or Nur77 knockout mice extended to the peripheral T cell population, or whether homeostatic mechanisms negated them as in the case of CD127 expression. The frequency of $TCR\beta^+$ cells in the spleen was not significantly different between WT, NKO, and BKO mice, but was lower in both types of DKO mice (**Fig. 5-4A**). The decreased proportion of $TCR\beta^+$ T cells may indicate an increase in other immune cells, perhaps owing to increased survival in the absence of Bim and Nur77. Preliminary data suggests that the absence of Bim or Nur77 does not alter the development of $NK1.1^+$ $TCR\beta^+$ T cells. Among the $TCR\beta^+$ splenocytes, we found a modest but significant increase in the frequency of DN T cells in BKO and DKO mice (**Fig. 5-4B**), consistent with an increased frequency of DN thymocytes in these mice (**Fig. 5-1A**). The proportion of T cells that were DN was considerably lower in the DKO spleen compared to the DKO thymus (**Fig. 5-1A**). This is consistent with the idea that upon reaching the DP stage, DKO thymocytes experience enhanced positive selection and SP maturation or survival, resulting in a distribution of peripheral T cell subsets similar to WT.

The increased frequency of splenic DN correlated with a significant decrease in the frequency of $CD4^+$ T cells in the spleen, more so in BKO mice compared to DKO mice (**Fig. 5-4C**). In fact, the frequency of $CD4^+$ T cells in BKO mice was significantly lower than in WT or NKO mice. Furthermore, the frequency of $CD8^+$ T cells was actually higher in BKO mice compared to WT (**Fig. 5-4E**), emphasizing that the increased proportion of DN correlated with a decreased proportion of $CD4^+$ T cells. Despite this, Bim deficiency was associated with increased cell numbers of both $CD4^+$ (**Fig. 5-4D**) and $CD8^+$ (**Fig. 5-4F**) T cells in BKO and DKO mice

compared to WT. These data underscore the importance of Bim in peripheral T cell survival (137, 139, 259). Though Nur77 deficiency was insufficient to alter the number of SP thymocytes, NKO mice had elevated numbers of $CD4^+$ and $CD8^+$ T cells, which may indicate that pro-apoptotic role of Nur77 is greater in peripheral T cells than thymocytes. However, Bim still appeared to have a more dominant role, since BKO and DKO mice had higher cell numbers than NKO mice. Indeed, since the majority of BKO and DKO T cells had normal CD127 expression (**Fig. 5-3C and Fig. 5-3D**), we would not have expected a survival handicap.


A
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40
30
20
10
0
**
*
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NKO
BKO
DKO
DKO (I)
B
WT
NKO
BKO
DKO
DKO (I)
53.5
2.3
42.0
56.7
1.4
39.7
44.9
7.0
45.0
55.2
6.4
33.8
60.3
5.1
26.8
CD4
CD8
C
% CD4+ of TCRβ+
80
70
60
50
40
***
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*
D
TCRβ+ CD4+
10^9
10^8
10^7
10^6
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*
E
% CD8+ of TCRβ+
60
40
20
0
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F
TCRβ+ CD8+
10^8
10^7
10^6
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*

Figure 5-4. Bim and Nur77 deficiency increases the number of $CD4^+$ and $CD8^+$ T cells in the spleen. (A) Frequency of $TCR\beta^+$ cells in the spleen of indicated mice. **(B)** Representative CD8 by CD4 profiles of $TCR\beta^+$ splenocytes. **(C)** The frequency of $CD4^+$ cells among $TCR\beta^+$ splenocytes and **(D)** the absolute number of $TCR\beta^+$ $CD4^+$ splenocytes across all experiments. **(E)** The frequency of $CD8^+$ cells among $TCR\beta^+$ splenocytes and **(F)** the absolute number of $TCR\beta^+$ $CD8^+$ splenocytes across all experiments. Data represents a minimum of 4 mice of each strain from 4 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; *** $p < 0.001$.

Enhanced activation of DKO T cells

We next characterized the splenic T cells for their activation phenotype by assessing CD44 and CD62L expression (**Fig. 5-5A**). This is one aspect of T cell biology that varied considerably with age, and thus we incorporated data only from mice 6 months of age or younger. We found a modest but significant increase in the frequency of $CD44^{+}CD62L^{hi}$ central memory T cells from both $CD4^{+}$ (**Fig. 5-5B**) and $CD8^{+}$ (**Fig. 5-5C**) subsets in BKO mice. NKO mice tended to exhibit higher frequencies of central memory T cells as well, but the differences were not significant compared to WT. However, Nur77 deficiency did appear to have some impact, since the frequency of $CD44^{+}CD62L^{hi}$ $CD8^{+}$ T cells was significantly higher in DKO compared to BKO mice (**Fig. 5-5C**). The frequency of central memory T cells, particularly in the $CD8^{+}$ lineage, was even higher in DKO (I) mice than normal DKO mice, and may reflect increased activation in the putative inflammatory environment. For $CD44^{+}CD62L^{lo}$ cells, neither Bim nor Nur77 deficiency was sufficient to alter their frequency in the $CD4^{+}$ or $CD8^{+}$ lineages. However, combined Bim and Nur77 deficiency resulted in significantly a higher frequency of $CD44^{+}CD62L^{lo}$ $CD4^{+}$ (**Fig. 5-5D**) and $CD8^{+}$ (**Fig. 5-5E**) T cells compared to either single knockout strain. $CD44^{+}CD62L^{lo}$ $CD4^{+}$ T cells are of particular interest as Tfh cells are found within this subset, and an accumulation of these cells is linked to excessive B cell activation and autoimmunity (260-262). In accordance with the increase in $CD44^{+}CD62L^{lo}$ $CD4^{+}$ T cells found only in DKO mice, preliminary data suggests that DKO but not BKO or NKO mice have an elevated frequency of $CXCR5^{+}PD\text{-}1^{+}$ $CD4^{+}$ T cells. Overall, analysis of the peripheral T cell population extended what we observed in the thymus in that enhanced selection and maturation of SP thymocytes translated into increased numbers and activation of mature T cells in mice deficient in Bim and Nur77. The fact that combined deficiency produced the greatest differences

in most aspects is consistent with the unique propensity of DKO mice to develop a phenotype suggestive of inflammation.

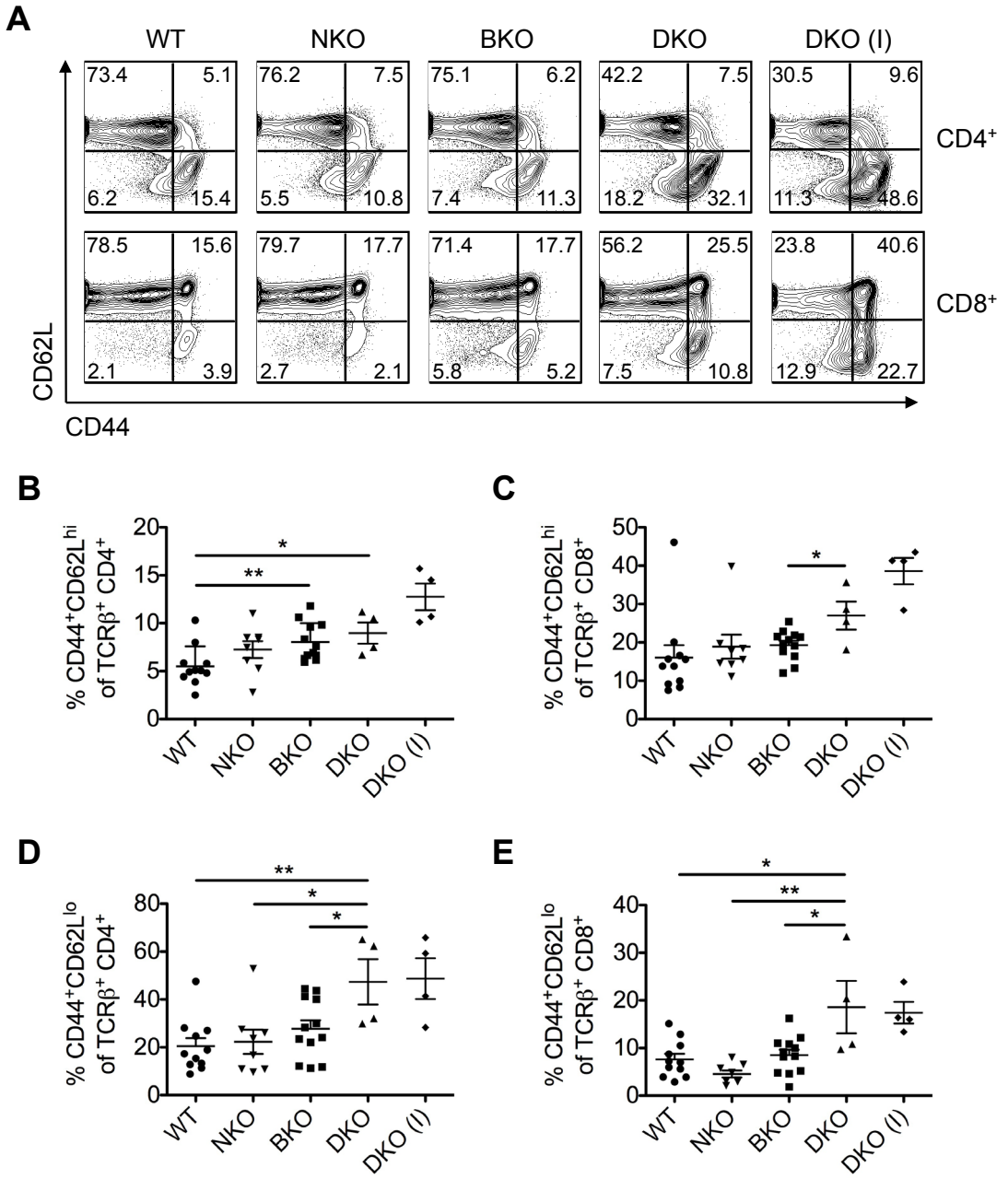

Figure 5-5. Enhanced activation of DKO T cells. (A) CD44 by CD62L profiles of TCRβ$^+$ CD4$^+$ and CD8$^+$ splenocytes. The frequency of central memory T cells within the CD4$^+$ **(B)** and CD8$^+$ **(C)** subsets, as well as the frequency of effector memory T cells within the CD4$^+$ **(D)** and CD8$^+$ **(E)** subsets was compiled over all experiments. Data represents a minimum of 4 mice of each strain from 4 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$.

Increase in high affinity TCR-signaled thymocytes in Bim-deficient mice

The ultimate phenotype of the peripheral T cells in Bim and Nur77-deficient polyclonal repertoires could have resulted from changes in positive or negative selection in the thymus and/or mature T cell biology. To gain insight on the role of Bim and Nur77 in negative selection in particular, we assessed induction of Helios and PD-1 in DP and SP thymocytes. We specifically looked at $Foxp3^-$ populations to exclude Treg cells, though this does not exclude any other high affinity antigen-selected fates such as anergy induction. Most DP thymocytes undergo death by neglect in a polyclonal repertoire and not negative selection. This was reflected by very low frequencies of $Helios^+PD\text{-}1^+$ cells in the DP, CD4SP, and CD8SP subsets in WT mice (**Fig. 5-6A**). There were no significant changes in the frequencies of these populations in NKO mice, consistent with Nur77 deficiency having limited impact on negative selection. In contrast, BKO and DKO mice had significantly higher frequencies of $Helios^+PD\text{-}1^+$ DP and CD4SP (**Fig. 5-6A, 5-6B, and 5-6C**). There was considerable variability within the CD8SP subset, but in BKO and DKO mice, the $Helios^+PD\text{-}1^+$ fraction contained CD8SP that expressed higher amounts of Helios and PD-1 than those found in WT or NKO mice (**Fig. 5-6A**). These results are in accordance with the original report in polyclonal BKO mice showing an accumulation of $Helios^+$ CD4SP (19), and are consistent with impaired clonal deletion due to Bim deficiency in TCR transgenic models.

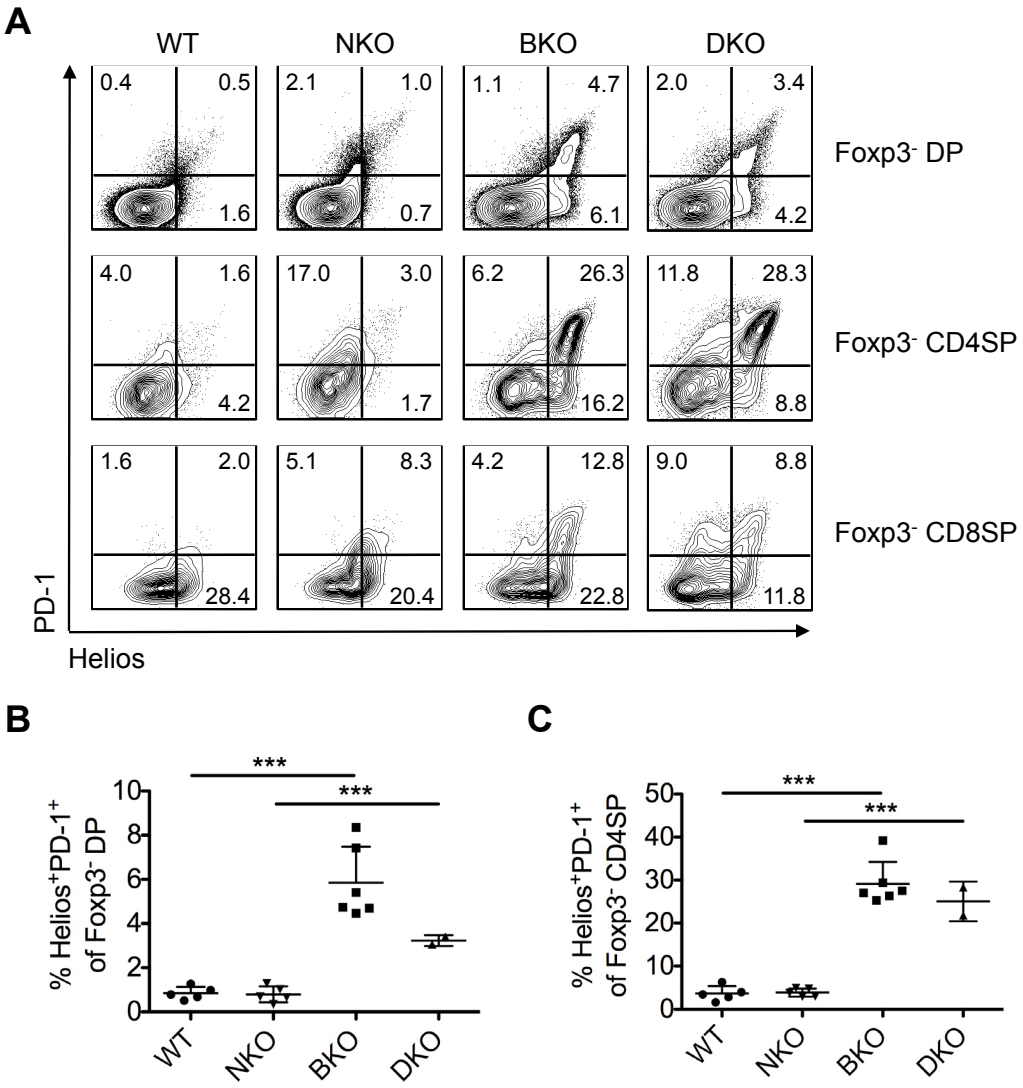

Figure 5-6. Increase in high affinity TCR-signaled thymocytes in Bim-deficient mice. (A) Helios and PD-1 expression among Foxp3$^-$ DP, CD4SP, and CD8SP thymocytes. Compilation of the frequency of Helios$^+$PD-1$^+$ cells among Foxp3$^-$ DP thymocytes **(B)** and Foxp3$^-$ CD4SP thymocytes **(C)**. Data represents a minimum of 5 mice of each strain from 4 independent experiments, except in the case of DKO mice representing 2 mice from 2 independent experiments. Data is depicted as mean ± SD; *** $p < 0.001$.

Increase in Treg cells in the absence of Bim and Nur77

We continued to investigate whether a deficiency in Bim and/or Nur77 resulted in an increase in self-reactive T cells by assessing $Foxp3^+$ Treg cells in the thymus and spleen. Selection of Treg cells in the thymus has been shown to result from higher self-reactivity. While recent studies found a correlation between the affinity of the TCR for self-antigen and the efficiency of Treg generation, it has become apparent that the range of antigen affinity for Treg selection is broad (263, 264). Theoretically, Treg selection efficiency could be increased by preventing high affinity-signaled thymocytes from undergoing clonal deletion or by lowering the TCR signaling threshold. Deficiency in Bim or Nur77 has the potential to meet these criteria, and in fact, other groups have reported that Bim (20) or Nur77 (148, 160) deficiency results in increased Treg cells in the thymus. In our hands, we also found that Bim or Nur77 single deficiency was sufficient to increase the frequency of Foxp3-expressing CD4SP thymocytes over WT (**Fig. 5-7A and 5-7B**). We additionally show that combined Bim and Nur77 deficiency resulted in a dramatic increase in the frequency of $Foxp3^+$ CD4SP over either single knockout strain. Furthermore, DKO (I) mice had a significantly higher frequency of thymic Treg compared to normal DKO. This may be due to the decreased DP pool, resulting in less intraclonal competition and increased efficiency of Treg selection (191, 252). Although BKO and NKO thymi contained an elevated frequency of $Foxp3^+$ CD4SP over WT, this difference was abrogated in the spleen (**Fig. 5-7A and 5-7D**). Only DKO mice continued to exhibit a significant increase in the proportion of $Foxp3^+$ $CD4^+$ splenic T cells. However, DKO (I) mice no longer had a dramatic increase compared to normal DKO mice. This may reflect a limited niche that can support Treg cells in the periphery. In terms of cell numbers, Bim deficiency was associated with increasing Treg cells in the thymi (**Fig. 5-7C**) and spleen (**Fig. 5-7E**) of BKO and DKO mice. In the spleen, Nur77

deficiency also had a minor effect on increasing Treg numbers, since DKO mice showed a significant increase over BKO mice.

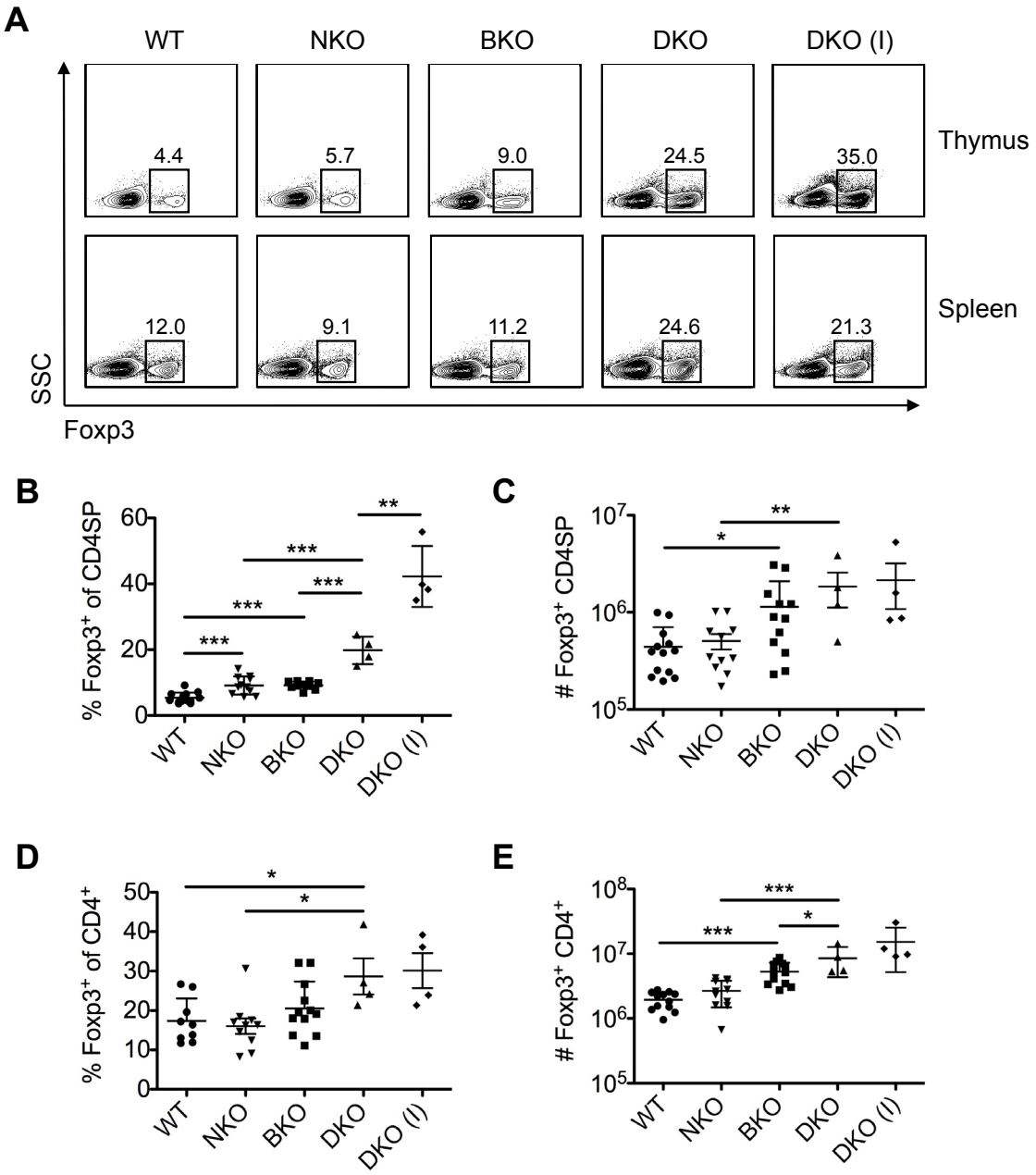

Figure 5-7. Increase in Treg cells in the absence of Bim and Nur77. (A) Representative plots showing the frequency of Foxp3$^+$ cells among CD4SP thymocytes and CD4$^+$ splenocytes from each strain. Compiled frequencies and absolute numbers of Foxp3$^+$ cells among CD4SP thymocytes **(B, C)** and CD4$^+$ splenocytes **(D, E)**. Data represents a minimum of 4 mice of each strain from 4 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Increase in anergic phenotype CD4$^+$ T cells in the absence of Bim and Nur77

In addition to selection of Treg, induction of anergy is another non-deletional form of tolerance. In fact, the two mechanisms cooperate as Treg were found to be critical for generation of a subset of peripheral anergic CD4$^+$ T cells marked by high expression of FR4 and CD73 (224). Using the Nur77-GFP reporter to gauge TCR signal strength, FR4^{hi}CD73^{hi} CD4$^+$ T cells were found to have GFP expression as high as Treg, suggesting they are also more self-reactive than conventional T cells (20). However, neither study examined whether FR4^{hi}CD73^{hi} CD4$^+$ T cells could be generated in the thymus as an alternative to clonal deletion. Among Foxp3$^-$ CD4SP thymocytes, we found a mildly elevated frequency of FR4^{hi}CD73^{hi} cells in NKO and BKO mice compared to WT (**Fig. 5-8A and 5-8B**). The number of FR4^{hi}CD73^{hi} Foxp3$^-$ CD4SP thymocytes was also elevated in NKO and BKO mice, with Bim deficiency having a greater impact (**Fig. 5-8C**). As in numerous other instances, combined deficiency of both proteins produced a much more dramatic phenotype such that the frequency and number of FR4^{hi}CD73^{hi} CD4SP were higher in DKO mice than either single knockout. Again, as with Treg selection, DKO (I) mice exhibited a dramatically increased frequency of anergic phenotype CD4SP thymocytes compared to normal DKO mice, while the absolute number was not much higher (**Fig. 5-8B and 5-8C**). In the periphery, we also found elevated an elevated frequency of FR4^{hi}CD73^{hi} Foxp3$^-$ CD4$^+$ T cells in BKO mice (**Fig. 5-8D**), which has been reported previously (20). While Nur77 deficiency was not sufficient to render a difference in the frequency or number of FR4^{hi}CD73^{hi} Foxp3$^-$ CD4$^+$ splenic T cells from WT mice, Nur77 does play a role in their generation as evidenced by a higher frequency and number of anergic phenotype cells in DKO compared to BKO mice (**Fig. 5-8D and 5-8E**). While these data do not prove that FR4^{hi}CD73^{hi} CD4$^+$ T cells can originate in the thymus, their increased frequency in BKO, NKO, and especially DKO thymi supports the

idea that this is an alternative mechanism of negative selection when clonal deletion is impaired. Bim and Nur77 may continue to affect their generation in the periphery as well.

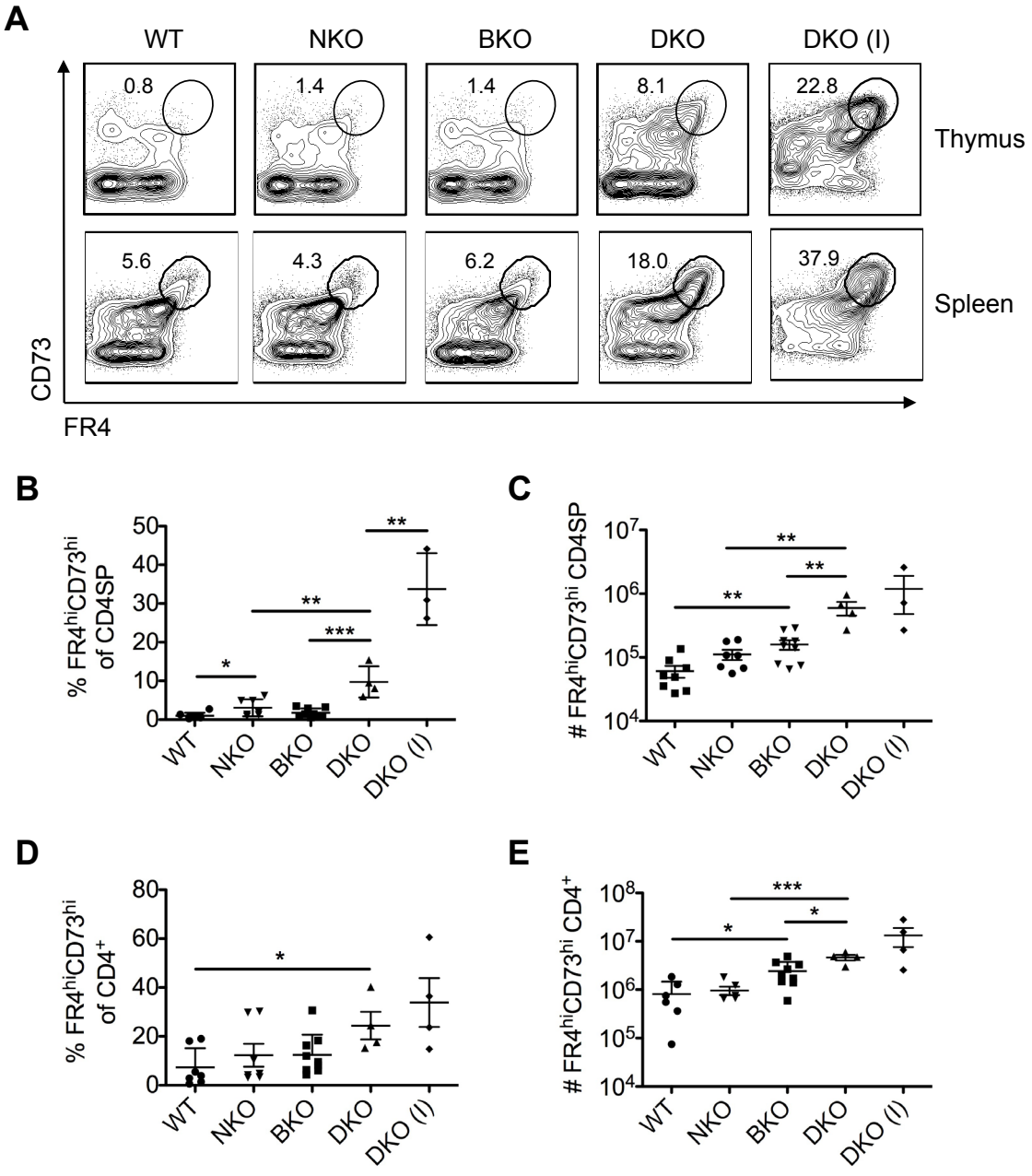

Figure 5-8. Increase in anergic phenotype CD4$^+$ T cells in the absence of Bim and Nur77. **(A)** Representative plots of FR4 and CD73 expression among Foxp3$^-$ CD4SP thymocytes and Foxp3$^-$ CD4$^+$ splenocytes from each strain. Compiled frequencies and absolute numbers of Foxp3$^-$ FR4^{hi}CD73^{hi} cells among CD4SP thymocytes **(B, C)** and CD4$^+$ splenocytes **(D, E)**. Data represents a minimum of 4 mice of each strain from 4 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Increase in DN thymocytes in BKO and DKO mice

In addition to altered development of SP thymocytes and T cells, we also observed a higher proportion of DN thymocytes (**Fig. 5-1A)** and splenic TCRβ^{+} DN cells (**Fig. 5-4B**) in BKO and DKO mice. In the thymus, the frequency of DN cells was significantly higher in BKO and DKO mice compared to WT and NKO mice (**Fig. 5-9A**), suggesting that the increase is associated with Bim deficiency. Of the four normal DKO mice, two had a higher frequency of DN than in any of the BKO mice. In terms of the absolute number of DN thymocytes, there was no difference between BKO and DKO mice, with both strains having significantly higher numbers than WT or NKO mice (**Fig. 5-9B**). The increase in DN thymocytes is consistent with what others have reported in polyclonal BKO mice (265), though the authors did not speculate on their origins or subsequent fate. We examined the distribution of TCRβ^{-} DN thymocytes among the DN1-DN4 stages to determine if BKO and DKO mice exhibited enhanced β-selection. WT mice typically have a ratio DN3 to DN4 thymocytes where the frequency of DN3 is higher than DN4 (**Fig. 5-9C**). Compared to WT mice, 6 of 11 NKO mice exhibited a ratio of DN3 to DN4 thymocytes that was close to 1:1 or was skewed towards the DN4 subset. BKO and DKO mice consistently showed a low ratio of DN3 to DN4 TCRβ^{-} thymocytes, indicative of enhanced β-selection. This is consistent with previous reports where Bim was shown to be important for survival of DN3 thymocytes, suggesting that it is important for progression through the β-selection checkpoint (266). DKO mice had a more highly skewed DN3:DN4 ratio than BKO mice; this may in part be due to an effect of Nur77 deficiency during β-selection, given that many of the NKO mice also showed an altered ratio. However, the increase in TCRβ^{-} DN1 thymocytes, which appeared unique to BKO mice, is also a factor. The DN1 subset is heterogeneous and may include NK cells, B cells, and DCs (267).

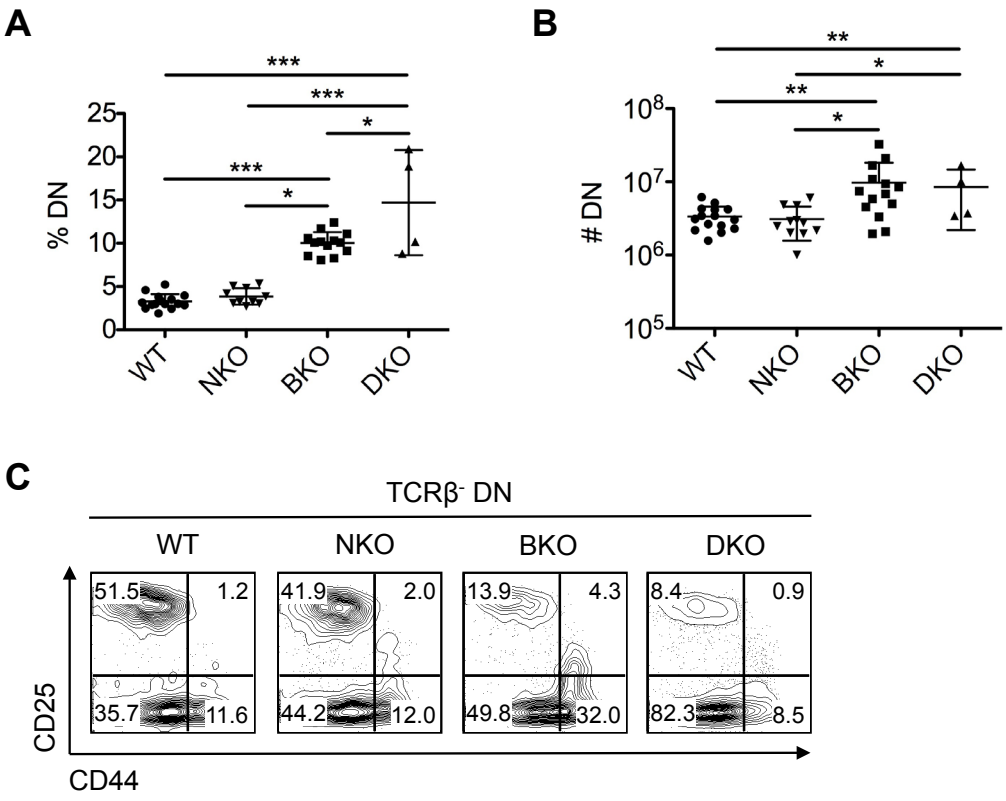

Figure 5-9. Increase in DN thymocytes in BKO and DKO mice. (A) The frequency and **(B)** absolute number of total DN thymocytes from each strain were quantified. **(C)** CD44 by CD25 profiles of total TCRβ$^-$ DN thymocytes. Data represents a minimum of 4 mice of each strain from 4 independent experiments. Data is depicted as mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Increase in post-selection TCRβ$^+$ DN4 thymocytes in BKO mice

Because the fate of thymocytes post-TCR selection is of particular interest to this thesis, we went on to examine TCRβ$^+$ DN thymocytes in particular. Specifically, we examined the DN4 subset since they were the most mature DN stage and thus likely to contain post-selection thymocytes. Whereas only a fraction of WT or NKO total DN4 thymocytes expressed TCRβ, the majority of DN were TCRβ$^+$ in BKO mice (**Fig. 5-10A**). The accumulation of TCRβ$^+$ DN4 thymocytes in BKO mice is consistent with previous reports (208). We additionally showed that while β-selection was enhanced in DKO mice as well, most DKO DN4 cells did not express TCRβ as in BKO mice. The dramatic increase in TCRβ$^+$ DN4 thymocytes in BKO mice could be due to accumulation of immature DN4 (for example, due to a block in transitioning to the DP stage) or an increase in a post-TCR-signaled population. To investigate their origin, we measured the amount of TCRβexpression on TCRβ$^+$ DN4 thymocytes. We found that in all strains of mice, the TCRβ$^+$ DN4 thymocytes expressed increased levels of TCRβthan DP thymocytes (**Fig. 5-10B**). Since TCR upregulation occurs following positive selection, this suggests that the TCRβ$^+$ DN4 are at a post-DP rather than pre-DP stage. In comparison to CD4SP thymocytes, however, they expressed a lower amount of TCRβ. The difference in TCRβ expression between TCRβ$^+$ DN4 and CD4SP appeared to be smaller in BKO and DKO mice. This may be due to a CD4^{lo}CD8$^-$ population that falls within both CD4SP and DN gates, which is always prominent in BKO mice, and is seen in DKO mice to a lesser extent (**Fig. 5-1A**).

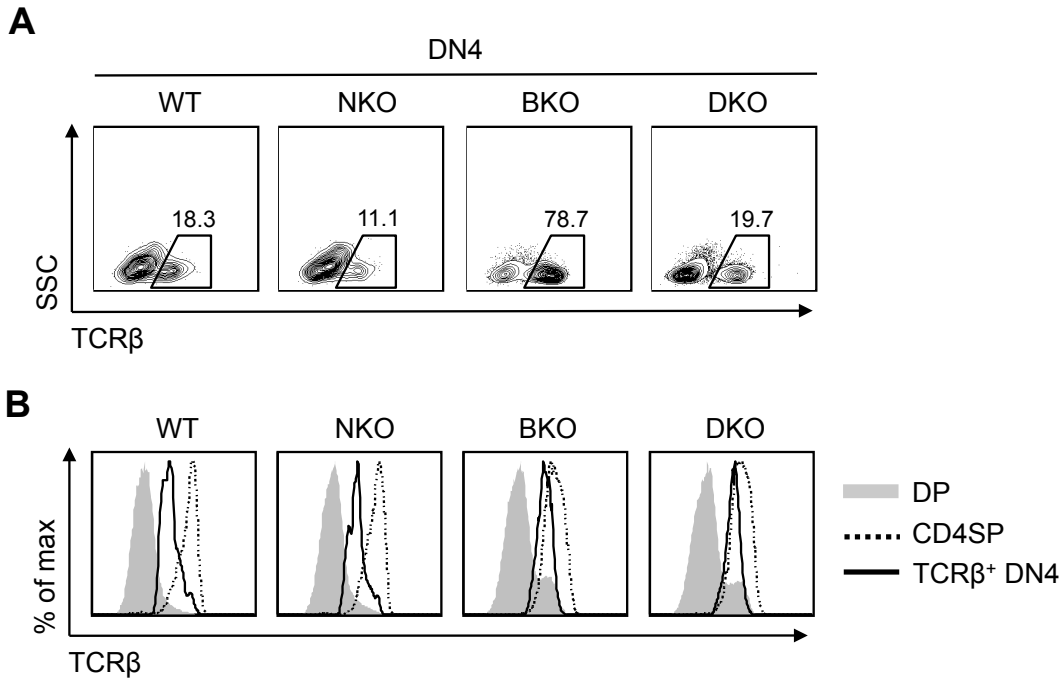

Figure 5-10. Increase in post-selection TCRβ$^+$ DN4 thymocytes in BKO mice. Total DN thymocytes were divided into developmental stages on the basis of CD44 and CD25 expression. **(A)** Representative frequency of TCRβ$^+$ cells among total DN4 thymocytes from each strain. **(B)** The amount of TCRβ on DP thymocytes, CD4SP thymocytes, and TCRβ$^+$ DN4 thymocytes is depicted as histogram overlays. Data represents a minimum of 4 mice of each strain from 4 independent experiments.

TCRβ$^+$ DN4 thymocytes express high affinity TCR signaling markers

In order to further investigate whether the TCRβ$^+$ DN4 thymocytes represent a post-selection stage, we assessed expression of Helios and PD-1. We found that TCRβ$^+$ DN4 thymocytes from all strains were Helios$^+$PD-1$^+$ (**Fig. 5-11A**), consistent with the cells recently having undergone TCR-mediated selection. In comparison to BKO mice, TCRβ$^+$ DN4 from DKO mice exhibited lower PD-1 expression, as evidenced by lower expression of PD-1 by cells within the Helios$^+$PD-1$^+$ population and an increase in Helios$^+$PD-1$^-$ cells. We also examined Helios and PD-1 expression among total DN4 thymocytes and found that Helios$^+$PD-1$^+$ cells only formed a majority of the population in BKO mice (**Fig. 5-11B**), consistent with BKO mice being the only strain to have a majority of DN that express TCRβ (**Fig. 5-10A**).

Based on our data, we speculate that both BKO and DKO mice have enhanced β-selection efficiency (**Fig. 5-9C**) due to the critical role of Bim in thymocyte survival during this process (266). The high frequency of TCRβ$^+$ and Helios$^+$PD-1$^+$ DN4 in BKO mice indicates that most of the DN4 compartment is occupied by post-selection thymocytes. The relative lack of TCRβ$^+$ and Helios$^+$PD-1$^+$ DN4 in DKO mice raises the possibility that additional Nur77 deficiency inhibited most thymocytes from adopting this fate and/or promoted alternative fates.

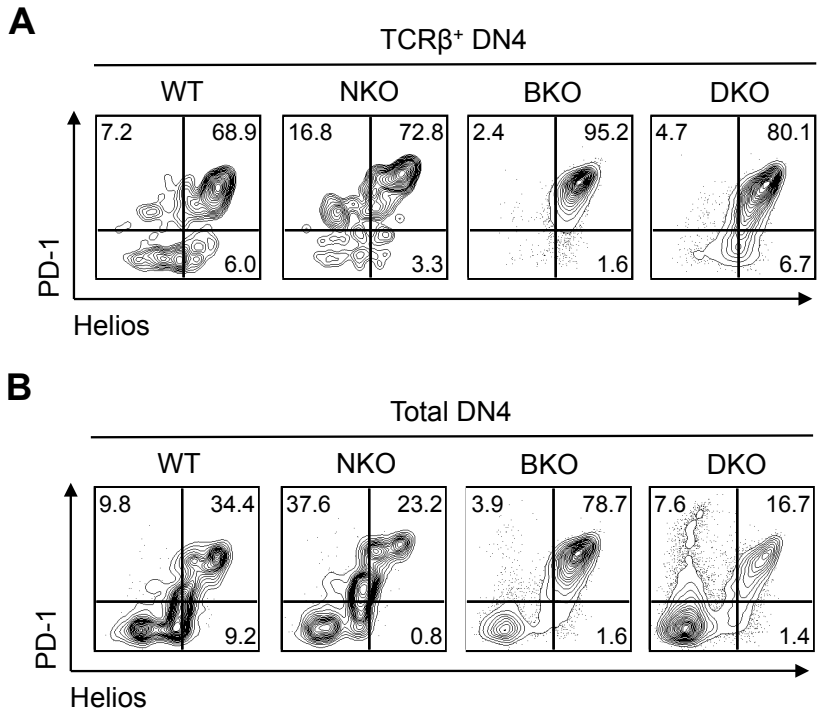

Figure 5-11. TCRβ$^+$ DN4 thymocytes express high affinity TCR signaling markers. Representative plots depicting Helios and PD-1 expression among TCRβ$^+$ DN4 thymocytes **(A)** and total DN4 thymocytes **(B)** from each strain. Data represents a minimum of 4 mice of each strain from 4 independent experiments.

Possible fates of TCRβ$^+$ DN thymocytes

Post-selection DN thymocytes have been reported to be CD8αα$^+$ IEL precursors. As such, they expressed CD122, the IL-2/IL-15 receptor β chain, and induced CD8ααin response to IL-15 *in vitro* (207). We found that among total DN thymocytes, the proportion of TCRβ$^+$CD122$^+$ to TCRβ$^+$CD122$^-$ cells was similar in all strains (**Fig. 5-12A**). Therefore, though BKO mice have a dramatically higher frequency of TCRβ$^+$ DN, DN thymocytes which do express TCRβ have a similar phenotype as in other mouse strains, and may be destined for the same fates.

Development into regulatory DN T cells is another potential fate for the TCRβ$^+$ DN thymocytes. While some groups have reported that regulatory DN T cells do not express Foxp3 (reviewed in (209)), others did find Foxp3 expression in DN T cells under various contexts (268-270). We found that few TCRβ$^+$ DN4 thymocytes expressed Foxp3 in WT or NKO mice (**Fig. 5-12B**). Therefore, unlike the CD4SP subset in which Nur77 deficiency results in increased Foxp3 expression (160), Nur77 may not have the same role in post-selection DN thymocytes. Though the frequency of Foxp3 expression was higher in BKO and DKO mice, Foxp3$^+$ cells still comprised a relatively small proportion of all TCRβ$^+$ DN4. Overall, the frequency of Foxp3$^+$ TCRβ$^+$ DN4 was similar between BKO and DKO mice. Liu *et al.* recently reported that the highest frequency of Foxp3 expression in DN thymocytes is found in the TCRβ$^-$CD25$^+$ fraction (270). When we gated on total Foxp3$^+$ DNs, we found the same results in WT and NKO mice, with the other substantial Foxp3-expressing fraction being TCRβ$^-$CD25$^-$ (**Fig. 5-12C**). However, the highest Foxp3-expressing populations in BKO and DKO mice remained in the TCRβ$^+$ fractions. As with the putative CD8αα$^+$ IEL precursors, the increase in TCRβ$^+$ Foxp3$^+$ DN in BKO and DKO mice may indicate increased lineage diversion when clonal deletion is impaired.

While it is not currently known whether any of the Foxp3$^+$ DN populations have regulatory function, it is possible that there are several subsets of regulatory DN with different origins. Since we are interested in fates following TCR-mediated selection, we are concerned only with TCRβ$^+$ DN, which we have shown to have a similar phenotype in all strains with respect to TCRβ, Helios, PD-1, CD122, and Foxp3. Therefore, we suggest that Bim and Nur77 deficiency is mostly altering the proportion of thymocytes that develop into these subsets.

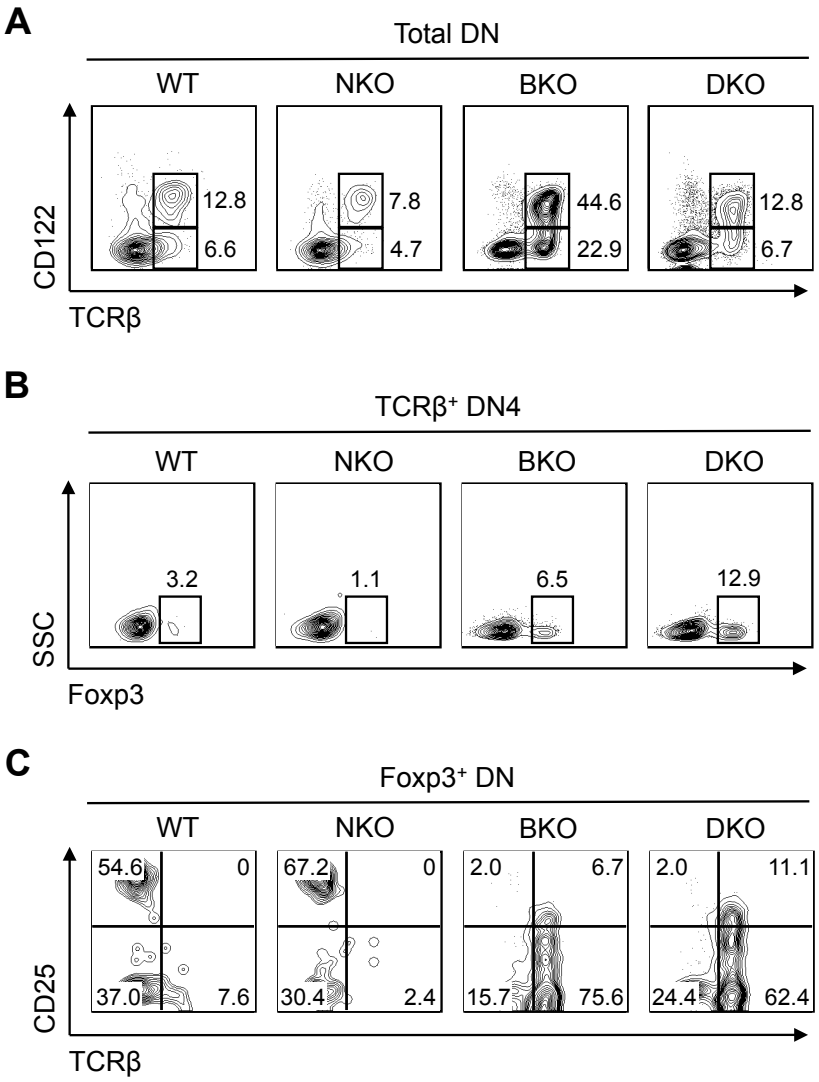

Figure 5-12. Possible fates of TCRβ$^+$ DN thymocytes. (A) TCRβ and CD122 expression among total DN thymocytes from each strain. **(B)** Foxp3 expression among TCRβ$^+$ DN4 thymocytes from each strain. **(C)** TCRβ and CD25 expression of total Foxp3$^+$ DN thymocytes from each strain. Data represents a minimum of 8 mice of each strain from 8 independent experiments, except in the case of DKO mice representing 2 mice from 2 independent experiments.

Increase in high affinity signaled DN T cells in the periphery of BKO and DKO mice

Lastly, we examined TCRβ$^+$ DN cells in the spleen to determine whether they were similar to TCRβ$^+$ DN thymocytes. Over all experiments, there was a significant increase in the frequency of TCRβ$^+$ DN splenocytes in BKO and DKO mice compared to WT and NKO mice (**Fig. 5-13A**). As mentioned previously, the increased proportion of DN correlated with a decreased proportion of CD4$^+$ T cells (**Fig. 5-4**). We also found a modest but significant decrease in the frequency of DN splenocytes in NKO compared to WT mice, as well as a non-significant decrease in DKO compared to BKO mice (**Fig. 5-13A**). This is consistent with a lower proportion of TCRβ$^+$ DN4 thymocytes in NKO and DKO mice compared to WT and BKO, respectively (**Fig. 5-10A**), and indicates a role for Nur77 in promoting this fate. In terms of absolute numbers of DN T cells in the spleen, it is Bim deficiency that is associated with an increase (**Fig. 5-13B**), similar to what we have seen with SP T cells. As in the thymus, BKO and DKO mice had an increased frequency of Foxp3$^+$ DN T cells in the spleen compared to WT and NKO mice (**Fig. 5-13C**). Both Foxp3$^+$ TCRβ$^+$ DN in the thymus (**Fig. 5-12B**) and spleen (**Fig. 5-13C**) had relatively low expression of Foxp3 compared to CD4$^+$ counterparts (**Fig. 5-7**). While Foxp3$^+$ cells comprised a minority of the splenic DN T cells, we nevertheless found that a considerable fraction of total DN T cells expressed Helios in all strains (**Fig. 5-13D**). In contrast, WT CD4$^+$ T cells in the spleen are mostly Helios$^-$ despite having a higher frequency of Foxp3$^+$ cells. Along with the fact that WT and NKO spleens barely have any Foxp3$^+$ DN, this suggests that Helios expression in most DN T cells is uncoupled from Foxp3 expression. We also found that BKO and DKO mice showed a considerably higher frequency of Helios$^+$PD-1$^+$ DN T cells compared to WT or NKO mice (**Fig. 5-13D)**. Though it is presently unclear what this means,

increased expression of the co-inhibitory molecule PD-1 may reflect an increase in anergic or regulatory DN T cells in the absence of Bim.

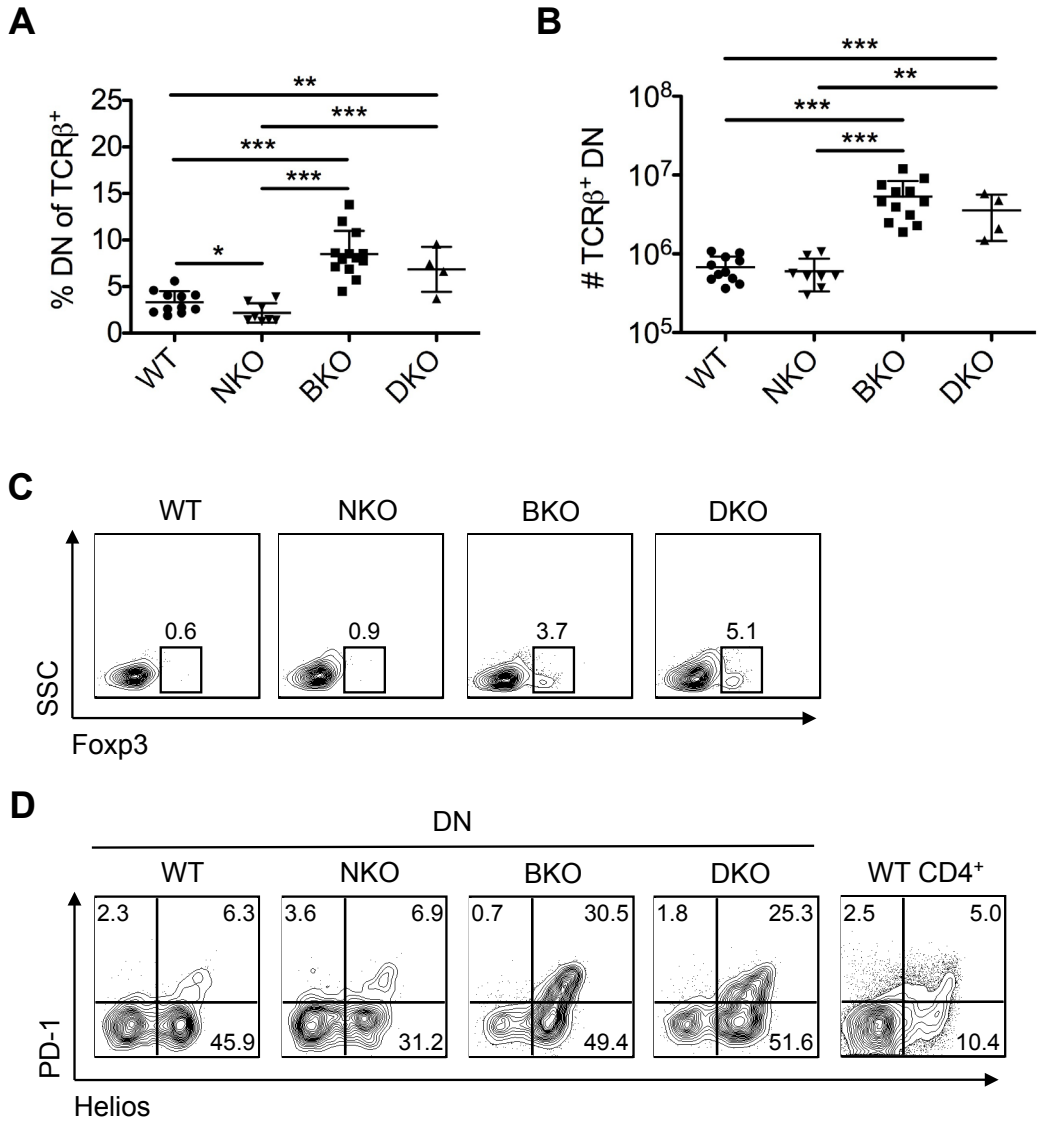

Figure 5-13. Increase in high affinity signaled DN T cells in the periphery of BKO and DKO mice. (A) Frequency of DN cells among TCRβ$^+$ splenocytes and **(B)** absolute number of TCRβ$^+$ DN splenocytes from each strain. **(C)** Foxp3 expression among TCRβ$^+$ DN splenocytes. Data represents a minimum of 4 mice of each strain from 4 independent experiments. **(D)** Helios and PD-1 expression among TCRβ$^+$ DN splenocytes from each strain (the four plots under the DN header) and among TCRβ$^+$ CD4$^+$ splenocytes from WT mice as a control. Data represents a minimum of 8 mice of each strain from 8 independent experiments, except in the case of DKO mice representing 2 mice from 2 independent experiments.

In summary, we explored T cell development in a polyclonal repertoire in this chapter and found evidence to support the conclusions we made in our TCR transgenic models. For example, the increase in high affinity TCR-signaled DP and particularly SP thymocytes in Bim-deficient mice is consistent with the role of Bim in UbA- and TRA-mediated clonal deletion. The increase in CD4SP and CD8SP thymocytes in BKO and DKO mice could also reflect increased positive selection, which we saw in TCR transgenic bone marrow chimeras. Analysis of non-TCR transgenic mice also allowed us to expand our investigation of non-deletional mechanisms of negative selection. Treg selection, CD4 anergy, and DN diversion all appeared to be promoted in mice in which clonal deletion was impaired. Since it is mostly Bim deficiency that is associated with impaired clonal deletion, yet significant increases in these alternative fates were found in DKO mice compared to BKO mice, we speculate that Nur77 deficiency may be promoting these fates through other mechanisms. Though selected through high affinity TCR-pMHC interactions, cells that adopt these alternative fates are supposed to be self-tolerant. Therefore, it is unclear why DKO mice tended to exhibit a phenotype reminiscent of inflammation despite having a higher ratio of tolerant to effector T cells. Whether DKO (I) mice show more direct markers of inflammation and whether this putative inflammation is caused by an increase in self-reactive T cells are key questions of interest that we are beginning to investigate.

CHAPTER 6: DISCUSSION

I. Summary on the roles of Bim and Nur77 in UbA-mediated negative selection

The work presented in this thesis examined thymocyte development, particularly outcomes that occur following high affinity TCR signaling, in multiple model systems. Though TCR transgenic mice have been instrumental to understanding antigen-specific negative selection, premature TCR expression and signaling in some mouse models limits the study of physiological selection (77, 79). Thus, previous findings in classical HY mice that concluded that Bim is critical for clonal deletion (141) are more representative of the role of Bim in DN and pre-TCR-signaled DP thymocytes. Indeed, a body of literature supports Bim as a key mediator of apoptosis during β-selection of DN thymocytes and death by neglect in DP thymocytes (137, 265, 266, 271). We investigated the role of Bim in UbA-mediated clonal deletion by utilizing the HY^{cd4} model designed to correct for premature TCR expression. Along with *in vitro* TCR stimulation experiments, we concluded that Bim is essential for TCR-induced caspase-mediated apoptosis and is important for TCR-induced CICD in DP thymocytes. However, Bim was ultimately not required for clonal deletion in the HY^{cd4} model. Since publishing this finding (97), another study using an alternative 'on-time' TCR transgenic model has supported the conclusion that Bim is not necessary for UbA-mediated clonal deletion (272). While Bim was dispensable for clonal deletion, we observed that HY^{cd4} $Bim^{-/-}$ M mice contained a higher proportion of DP thymocytes expressing the high affinity TCR-induced protein Helios, as well as higher numbers of $T3.70^{+}$ DP thymocytes. These data suggest that Bim deficiency did delay clonal deletion in the HY^{cd4} model and is consistent with a critical role for Bim in caspase-mediated and caspase-independent cell death. Nur77 deficiency, on the other hand, was insufficient to impair DP thymocyte

deletion *in vivo* and apoptosis *in vitro*. However, double deficiency in Bim and Nur77 resulted in a notably increased T3.70^{+} CD8SP population in two of four chimeras. Though this experiment was limited by a low number of chimeras, such an increase in T3.70^{+} CD8SP thymocytes was very rarely observed in HY^{cd4} Bim$^{-/-}$ M or HY^{cd4} Nur77$^{-/-}$ M mice, suggesting that deficiency in these two molecules increases the propensity for impaired deletion.

I-a. How are T3.70^{+} thymocytes eliminated in the absence of Bim?

One of the questions that remain concerns the mechanism that ultimately produces a lack of T3.70^{+} CD8SP thymocytes in HY^{cd4} Bim$^{-/-}$ M mice. During negative selection in the presence of Bim, caspase-3 activation marks T3.70^{+} DP thymocytes undergoing deletion *in vivo*. Activation of caspase-3 has been demonstrated in other models of negative selection as well (96, 234, 273). It has been shown that deletion of the caspase-3 gene largely abrogates thymocyte apoptosis uponanti-CD3 stimulation *in vitro*, with caspase-7 playing a relatively minor role (99). Moreover, others have shown that peptide stimulation of TCR transgenic thymocytes results in caspase-3 activation while the active forms of caspase-6 and caspase-7 were not detected (98). Though these studies were conducted in various model systems, the literature collectively indicates that caspase-3 is the predominant executioner caspase that functions during negative selection. Since we have shown that Bim is required for caspase-3 activation in T3.70^{+} thymocytes (97), we speculate that the lack of T3.70^{+} CD8SP thymocytes in HY^{cd4} Bim$^{-/-}$ M mice results from a caspase-independent mechanism of clonal deletion. Though it has been contested, caspase-independent clonal deletion is supported by studies involving deletion of caspase-3 or transgenic expression of a viral pan-caspase inhibitor on TCR transgenic

backgrounds (100-102). However, all of these studies are limited by the use of TCR transgenic models with premature TCR expression.

$T3.70^+$ DP thymocytes from HY^{cd4} $Bim^{-/-}$ M mice exhibited phosphatidylserine exposure comparable to HY^{cd4} M mice despite abrogation of caspase-3 activation, providing more direct evidence that clonal deletion is occurring. Caspase-independent phosphatidylserine exposure has been demonstrated in mature T cells and non-T cell lines (114, 274, 275), but to our knowledge, our observations in thymocytes undergoing negative selection constitute a novel finding. Though we do not think that residual caspase-3 activity (which was nearly zero) or other executioner caspases were responsible for phosphatidylserine exposure in HY^{cd4} $Bim^{-/-}$ M DP thymocytes, we also examined cell death upon plate-bound TCR stimulation *in vitro* in the presence of the pan-caspase inhibitor z-VAD-FMK to address this possibility. Consistent with *ex vivo* observations, the frequency of AV^+ DP thymocytes was clearly increased over unstimulated control even when stimulated in the presence of z-VAD-FMK. In another *in vitro* model involving peptide stimulation of TCR transgenic thymocytes in thymic slices, Dzhagalov *et al.* found little increase in caspase-independent phosphatidylserine exposure in DP thymocytes (273). One factor that may account for the discrepancy is the duration of stimulation. Dzhagalov *et al.* examined thymocytes only up to 4 h post-stimulation; in our hands, we did observe clear induction of phosphatidylserine exposure after 4 h in the presence of z-VAD-FMK, but maximal death over unstimulated controls occurred after 6 h. Another reason for the lack of AV^+ DP thymocytes may be due to phagocytosis in the thymic slice model.

Mitochondrial dysfunction is a widely cited feature common to both caspase-mediated and caspase-independent cell death (114, 237, 274), which we also observed during TCR-induced death in the presence of z-VAD-FMK. For both phosphatidylserine exposure and mitochondrial dysfunction, Bim deficiency resulted in a more severe impairment than abrogation of caspase activity. This is consistent with the Bcl-2 family acting as gatekeepers of mitochondrial integrity, thereby controlling caspase-mediated and caspase-independent cell death. In fact, Bim and other Bcl-2 proteins are known to interact with components of the mitochondrial permeability transition pore, thought to be involved in mediating CICD (274, 276). As with phosphatidylserine exposure, this has not been previously examined in the context of TCR-induced thymocyte death. We know that the frequency of *ex vivo* $TMRE^{lo}$ $T3.70^{+}$ DP thymocytes in HY^{cd4} $Bim^{-/-}$ M mice is at least comparable to HY^{cd4} M mice, but we chose not to include these data due to an assay concern that we are working to address definitively.

The signs of cell death that we observed suggest that the ultimate lack of $T3.70^{+}$ CD8SP thymocytes in HY^{cd4} $Bim^{-/-}$ M mice is in part the result of clonal deletion. An additional mechanism that could account for the lack of $T3.70^{+}$ CD8SP thymocytes is inhibition of positive selection. While it is possible that factors necessary for positive selection are only induced by low affinity TCR signaling, we do not favour this idea since $T3.70^{+}$ DP thymocytes should encounter both the positively selecting and the negatively selecting ligand in male mice, yet negative selection is the dominant outcome. $T3.70^{+}$ DP thymocytes removed from male mice continue to develop into CD8SP thymocytes *in vitro*, despite already showing signs of co-receptor downregulation, suggesting that negative selection requires an active program induced by high affinity TCR signaling (272). For example, it is possible that high affinity TCR signaling

induces both an active death program as well as a block in positive selection pathways. We showed that expression of Id3 and Runx3, both involved in CD8 lineage development, is similar between HY^{cd4} $Bim^{-/-}$ F and M DP thymocytes (97). It remains possible that these or other factors are negatively regulated by high affinity TCR signaling by post-transcriptional mechanisms.

I-b. What other proteins mediate UbA-mediated clonal deletion?

Failure to receive survival and differentiation cues associated with positive selection could result in DP thymocytes undergoing an inefficient form of cell death – that is to say, a pro-apoptotic mediator other than Bim may not be necessary for $T3.70^{+}$ thymocytes to be eliminated. However, Bcl-2 protein expression appears to correlate with TCR signaling strength since both HY^{cd4} M and HY^{cd4} $Bim^{-/-}$ M DP thymocytes express higher Bcl-2 than female counterparts. Overexpression of Bcl-2, as well as of Mcl-1, has been shown to impair clonal deletion in classical HY mice and promote diversion into post-selection DN fates in polyclonal mice (207, 277, 278). These data suggest that other pro-apoptotic proteins may be important for counteracting pro-survival Bcl-2 proteins when Bim is deficient. Among the BH3-only proteins expressed in mice and humans, only additional deficiency in Puma appreciably perturbed the ratio of DP to SP thymocytes in Bim-deficient polyclonal mice (144). Their data also showed that Puma and Bim cooperate in TRA-mediated deletion but did not examine antigen-specific deletion in a UbA-mediated model. The relatively minor role of Puma in thymocyte apoptosis compared to Bim could account for the delayed deletion of DP thymocytes we observe in HY^{cd4} $Bim^{-/-}$ M mice.

Nur77 initially stood out as a promising candidate for mediating Bim-independent clonal deletion since it is upregulated in numerous models of negative selection (81, 125-129), and along with family member Nor-1, Nur77 has been shown to be important for clonal deletion in classical TCR transgenic models (172, 178). We were particularly keen on investigating the role of Nur77 in negative selection in the HY^{cd4} model since Nur77 has been implicated in CICD in other cell types (169-171). Though Nur77 deficiency alone was insufficient to result in impaired deletion, consistent with older literature (177), results from HY^{cd4} DKO M chimeras revealed a contribution of Nur77 to clonal deletion when Bim was additionally absent. This was in part a thymocyte-intrinsic effect since the frequency of $T3.70^{+}$ CD8SP thymocytes of DKO origin was higher than those of WT origin in 3 of 4 mixed chimeras. While more repeats are needed to confirm impaired deletion in DKO chimeras, the unusual and striking increase in $T3.70^{+}$ CD8SP thymocytes suggests that Nur77 is involved in UbA-mediated clonal deletion, albeit in a minor role compared to Bim. These results also suggest that Nor-1 did not compensate for the absence of Nur77, since we know that Nor-1 transcripts are induced in HY^{cd4} M and HY^{cd4} $Bim^{-/-}$ M DP thymocytes over female counterparts. The conclusion that Nor-1 is redundant for Nur77 is based on studies in which Nur77 deficiency had no phenotype yet inhibition of Nur77 and Nor-1 impaired clonal deletion (172, 177, 178). An alternative interpretation is that Nur77 deficiency had no phenotype because Bim, the most prominent mediator of apoptosis in thymocytes, was still expressed. However, deletion of two relatively minor apoptotic proteins, Nur77 and Nor-1, could still result in a phenotype – whether functioning redundantly or not.

I-c. Does Nur77 mediate apoptosis during UbA-mediated clonal deletion?

Our data shows that neither Nur77 nor Nor-1 compensated for Bim deficiency in activating caspase-3 during negative selection in HY^{cd4} $Bim^{-/-}$ M mice. Furthermore, the ability of transgenic Nur77 to activate caspase-3 was reduced in the context of TCR signaling. While it is possible that Nur77 and Nor-1 contribute to caspase-independent clonal deletion, we raise the possibility that the main role of the NR4A family during UbA-mediated negative selection is not to mediate apoptosis. Regarding its role as a transcription factor, Nur77-regulated genes known to be involved in apoptosis are scarce. Fassett *et al.* reported that Bim induction in DP thymocytes is partly dependent on Nur77 in the context of MHC class II-restricted antigen stimulation *in vitro* as well as following plate-bound antibody stimulation (160). While Bim is the most plausible target gene for transcriptional regulation of apoptosis by Nur77 to date, neither stimulation condition represents MHC class I-restricted UbA-mediated selection *in vivo*. In fact, our preliminary data indicates that Bim expression is not impaired in HY^{cd4} $Nur77^{-/-}$ M DP thymocytes compared to DP from HY^{cd4} M mice, consistent with unimpaired deletion in the absence of Nur77. The only other known Nur77-dependent genes implicated in cell death are those that function via the external apoptosis pathway, which does not play a significant role in negative selection (87, 159, 229, 279).

In addition to being a transcriptional regulator, TCR signaling reportedly induces nuclear export of Nur77 to mitochondria, where it converts Bcl-2 into a pro-apoptotic form (163). However, we found no evidence of Bcl-2 conversion during UbA-mediated negative selection. In DO11.10 T cell hybridomas, forced nuclear export of Nur77 promotes apoptosis but no interaction with Bcl-2 was observed (165). Though this does not reflect Nur77 function in the context of TCR

signaling, it is possible that Nur77 has an extra-nuclear apoptotic function that is independent of Bcl-2. Even upon PMA and ionomycin stimulation of DO11.10 cells, the majority of Nur77 was localized in the nucleus, followed by the cytoplasmic fraction, with the mitochondrial fraction having the least amount of Nur77 (165). In another study using DO11.10 cells, it was found that TCR-induced Nur77 inhibited dexamethasone-induced apoptosis. Since Nur77 inhibited activation of the glucocorticoid response element, it was suggested that Nur77 and glucocorticoid receptor inhibit each other by binding to a shared co-activator (280). This study provides further evidence that TCR-induced Nur77 at least partly remains in the nucleus and does not necessarily promote apoptosis. TCR-induced export of Nur77 has been demonstrated in primary peripheral T cells, although this was argued to be an anti-apoptotic mechanism that does not occur in DP thymocytes (164). Though we are still working to overcome technical challenges associated with determining the localization of Nur77, its localization during negative selection could be argued for and against a role in apoptosis.

A key piece of data that leads us to speculate that Nur77 is minimally involved in UbA-mediated clonal deletion is the observation that a reduction in transgenic Nur77-mediated apoptosis was associated with contexts of TCR signaling. As described above, regulation of Nur77 by TCR signaling is well documented (163-165). Though studies to date have focused on regulation of Nur77 localization, which may be linked to its function, it is also possible that TCR signaling pathways directly regulate Nur77 function by modulating its ability to transactivate genes or interact with other proteins. An alternative interpretation is that transgenic Nur77-mediated apoptosis was inhibited due to upregulation of anti-apoptotic proteins by TCR signaling. While we showed that Bcl-2 expression, for example, correlates with TCR signaling strength, we do not

favour this interpretation because TCR signaling – high affinity at least –should induce both pro- and anti-apoptotic proteins. Yet caspase-3 activation was disproportionately low compared to Nur77 expression even in T3.70^{+} DP thymocytes from HY^{cd4} M Nur77-FL mice. Induction of anti-apoptotic proteins remains a possible factor in HY^{cd4} F Nur77-FL and OT-I Nur77-FL mice, as well as in polyclonal CD69^{+} DP thymocytes, which may include positively selected cells.

I-d. Conclusion

Though UbA-mediated clonal deletion of DP thymocytes is delayed by Bim deficiency, antigen-specific thymocytes are ultimately lacking. Since our data and that of others suggest that Nur77 and Puma have only minor contributions to UbA-mediated deletion, we hypothesize that a block in positive selection signals by high affinity TCR signaling is a major reason for the ultimate absence of T3.70^{+} CD8SP thymocytes. Rather than functioning as a major mediator of apoptosis, the role of Nur77 in this context may involve regulation of TCR signaling. A combination of delayed DP thymocyte apoptosis and altered TCR signaling may underlie the increase in T3.70^{+} CD8SP thymocytes in both F and M chimeras containing HY^{cd4} DKO-derived cells.

II. Summary of the roles of Bim and Nur77 in TRA-mediated negative selection

In the second chapter of this thesis, we examined the contributions of Bim and Nur77 to TRA-mediated negative selection, which differed from their roles in UbA-mediated negative selection. In contrast to its non-essential role in UbA-mediated clonal deletion, Bim deficiency results in severely impaired clonal deletion in the OT-I and OT-II Rip-mOva models (143, 144, 160). Furthermore, our data and that of others indicate that Nur77 also plays a greater role in TRA-mediated deletion compared to UbA-mediated deletion (97, 160). However, the difference in

importance of Bim vs. Nur77 to clonal deletion in the OT-I and OT-II Rip-mOva models suggests additional differences in the mechanism of MHC class I vs. MHC class II-restricted negative selection. Whereas double deficiency of Bim and Nur77 resulted in an increase in $T3.70^+$ CD8SP thymocytes compared to single knockout mice, the frequency of $V\alpha2^+$ CD8SP thymocytes in OT-I DKO → Rip-mOva chimeras was not higher than in OT-I BKO → Rip-mOva chimeras, owing to the total block in clonal deletion caused by Bim deficiency alone. However, OT-I DKO → Rip-mOva chimeras were the only situation in which diabetes developed, indicating that both Bim and Nur77 had important contributions to tolerance. In addition to the modest impairment in clonal deletion in the absence of Nur77, Nur77 deficiency may have promoted autoimmunity by increasing the function of T cells.

II-a. Differential contributions to clonal deletion against UbA vs. TRA by Bim and Nur77

Both Bim and Nur77 play larger roles in clonal deletion in the OT-I Rip-mOva model than in the HY^{cd4} model. Neither Bim nor Nur77 are required for clonal deletion in the HY^{cd4} model, but Bim deficiency delayed deletion of $T3.70^+$ DP thymocytes, and Nur77 deficiency contributed to the increase in $T3.70^+$ CD8SP thymocytes in HY^{cd4} DKO chimeras. In contrast, Bim deficiency completely blocked deletion of $V\alpha2^+$ CD8SP thymocytes, while Nur77 deficiency was sufficient to render a modest but significant impairment in deletion compared to OT-I Het/Het → Rip-mOva controls. One potential difference between the two model systems that could affect the mechanism of deletion is the affinities of the transgenic TCRs for cognate antigen and consequently the strength of TCR signaling. Though studies have been performed to determine the TCR/pMHC affinity of each TCR, the reported K_d values differ considerably from study to study (22, 23, 281). To our knowledge, no one has directly compared the affinity of the HY TCR

and OT-I TCR for cognate antigen in the same study. However, as in the HY^{cd4} model, Bim was also not required for deletion of OT-I thymocytes when Ova expression was ubiquitously driven by the actin promoter (143). In terms of TCR affinity for positively selecting or homeostatic ligands, data suggests that the OT-I TCR is of a higher affinity for self-antigens than the HY TCR (282). Our data suggested that Bim and Nur77 deficiency had a greater impact on increasing positive selection efficiency in the HY^{cd4} model than the OT-I model. Lower affinity of the HY TCR for positive selection ligands may mean that Bim or Nur77 deficiency has a greater effect on altering selection efficiency.

Another factor that may account for the different requirements of UbA- vs. TRA-mediated deletion is the stage at which the thymocytes encounter cognate antigen – at the DP stage in HY^{cd4} M mice and after commitment to the CD8 lineage in OT-I → Rip-mOva mice (64, 72). Studies have demonstrated that DP thymocytes are more sensitive to apoptosis than SP thymocytes (254-256). Furthermore, sensitivity to low affinity positively selecting ligands is decreased as DP thymocytes develop into mature T cells (283, 284). Higher sensitivity of DP thymocytes to TCR stimulation may mean that deletion can still occur when Bim or Nur77 is absent, whereas contributions by both molecules may be required for deletion of SP thymocytes.

Lastly, different stromal composition in the thymic cortex and medulla may induce different TCR signaling pathways during negative selection. While mTECs exclusively express TRAs, DCs can also cross-present the TRAs. DCs on a whole are more abundant in the medulla than the cortex, and certain subsets such as $CD8\alpha^+$ $SIRP\alpha^-$ resident DCs are preferentially localized in the medulla (76). Furthermore, clusters of a type of epithelial cells in the medulla called Hassall's

corpuscles are thought to activate dendritic cells, which in turn mediate selection of Treg cells (6). This is one of the reasons why Treg selection is thought to mainly occur in the medulla. While Hassall's corpuscles are small and scarce in mice compared to humans, a study has identified these structures as the expression site of the TRA pro-insulin in murine thymus (285). *In vitro* studies have also shown that only CD28 provides costimulation for deletion of DP thymocytes, whereas CD5 and CD43 can also provide costimulation for deletion of semi-mature $HSA^{hi}CD4^{+}CD8^{-}$ thymocytes (286). This may reflect receptivity to deletion by DCs in the medulla, which bear a wider array of costimulatory molecules than epithelial cells. Although it is difficult to reconcile the highly efficient negative selecting environment of the medulla with stricter molecular requirements for clonal deletion (at least in terms of Bim and Nur77), it is clear that the environments in which UbA- and TRA-mediated deletion takes place are markedly different.

II-b. Different roles for Bim and Nur77 in MHC class I and MHC class II-restricted TRA-mediated clonal deletion

In addition to different requirements for Bim and Nur77 in clonal deletion against UbA vs. TRA, our results on MHC class I-restricted TRA-mediated deletion highlight differences from MHC class II-restricted deletion. Data from the OT-II Rip-mOva model implies that Bim is restricted to the Nur77 pathway in antigen-specific thymocytes (160). By contrast, Bim deficiency has a much greater effect than Nur77 deficiency in the OT-I Rip-mOva model, suggesting that Nur77 would be but one of the regulators of Bim expression. Whether Nur77 even regulates Bim expression in the context of MHC class I-mediated negative selection is uncertain. The results presented in Chapter 4 show that baseline and high affinity-induced Bim expression is modestly

lower in Vα2^{+} CD8SP thymocytes from OT-I NKO → Rip-mOva mice compared to OT-I Het/Het → Rip-mOva mice. However, the fold induction of Bim between OT-I NKO →Rip-mOva and OT-I NKO →WT recipients was not different compared to Het/Het control pairs. As mentioned previously, preliminary data also indicates no impairment in Bim induction in DP thymocytes from HY^{cd4} NKO M compared to HY^{cd4} M mice. Given the redundancy of the DNA binding domains between NR4A family members (149), it is possible that Nor-1 may be inducing Bim in these contexts. However, Nur77 deficiency was sufficient to cause reduced Bim induction in the OT-II Rip-mOva model (160), suggesting some differences in the relationship between Nur77 and Bim in DP, CD8SP, and CD4SP thymocytes undergoing negative selection.

It has been previously shown that APCs and mTECs were needed to delete OT-II thymocytes, whereas mTECs could directly delete OT-I thymocytes (72). Professional APCs are thought to deliver a stronger deletion signal due to their superior array of costimulatory and adhesion molecules. Yet, Lck binds with higher affinity to the CD4 coreceptor, and the subsequent stronger TCR signal is thought to underlie commitment to the CD4 lineage (51). However, it is possible that the OT-II TCR has weaker affinity than the OT-I TCR for cognate antigen, resulting in a weaker TCR signal despite being CD4-committed. Other possibilities that have been raised include differences in antigen presentation efficiency (MHC class I/endogenous vs. MHC class II/exogenous pathways) and stability of the peptide-MHC complexes (72). Therefore, the outcome of this study may be particular to the TCRs involved. Nevertheless, differences in TCR signaling, antigen presentation, and gene expression all support the possibility of different molecular mechanisms used to delete CD4SP vs. CD8SP thymocytes.

In addition to differential involvement in clonal deletion, the apparent greater role of Nur77 in the OT-II Rip-mOva model may reflect other roles for Nur77 in CD4SP thymocytes (143, 144, 160). All members of the NR4A family have the ability to transactivate Foxp3 expression (148). Yet, our data and that of others show that Nur77 single deficiency results in an increased frequency of $Foxp3^+$ CD4SP thymocytes (160). This not only implies that Nor-1 fulfills the redundant function of driving Foxp3 expression in thymocytes, but also suggests that Nur77 deficiency promoted Treg selection – perhaps by lowering the requisite TCR signaling threshold. Since $Foxp3^+$ cells were not gated out of their analysis, the increase in OT-II CD4SP thymocytes in OT-II NKO Rip-mOva mice could be partly due to increased selection of Treg rather than Nur77 deficiency impairing deletion (160). In other words, the functions of Nur77 during negative selection include promoting Treg selection and mediating clonal deletion. It is possible that Nur77 is involved in both of those mechanisms in CD4SP thymocytes, whereas Nur77 is more involved in clonal deletion in CD8SP thymocytes.

II-c. A role for Nur77 in anergy induction and implications for autoimmunity

Neither Bim nor Nur77 single deficiency resulted in the development of diabetes in Rip-mOva recipients; deletion of both molecules was necessary to produce autoimmunity. Given that Bim deficiency already causes a near complete block in clonal deletion, it is unlikely that additional Nur77 deficiency promoted diabetes as a result of a further impairment in thymic deletion. Rather, we found evidence to suggest that Nur77 plays a role in inducing T cell anergy. All $V\alpha2^+$ $CD8^+$ T cells from Rip-mOva recipients show decreased function compared to WT recipients. However, $V\alpha2^+$ $CD8^+$ T cells from OT-I NKO → Rip-mOva chimeras showed increased CD69 induction and proliferation compared to other Rip-mOva recipients. Nur77 enforced T cell non-

responsiveness specifically after high affinity antigen encounter since no differences in function were observed between OT-I NKO → WT mice and other WT recipients. Thus, subsequent stimulation of OT-I NKO→ Rip-mOva T cells with Ova *in vitro* resulted in an enhanced response. Furthermore, $V\alpha2^{+}$ $CD8^{+}$ T cells from OT-I DKO → Rip-mOva chimeras appeared to have enhanced proliferation compared to T cells from OT-I BKO → Rip-mOva chimeras. We propose that the combination of blocked thymic deletion due to Bim deficiency and impaired anergy induction due to Nur77 deficiency is what allowed autoimmune disease to develop. Clonal deletion in the thymus is imperfect (179), requiring additional mechanisms to control autoreactive clones that escape deletion. It follows that impairment in more than one tolerance mechanism would be necessary for autoimmunity to occur.

Though Puma serves a modest but non-redundant role to Bim in TRA-mediated deletion of OT-I CD8SP thymocytes (144), Bim and Nur77 deficiency is the only combination known to result in autoimmune diabetes in this model to date. Adoptive transfer of polyclonal Puma and Bim double KO T cells into RAG KO recipients resulted in pancreatitis (144). However, since RAG KO recipients are lymphopenic, it is unclear to what extent this reflects increased T cell numbers due to lymphopenic proliferation combined with decreased apoptosis rather than an increase in autoreactive clones in the T cell repertoire. Regardless of increased clonal deletion escapees in OT-I Puma/Bim double KO Rip-mOva mice, the fact that they did not develop diabetes whereas OT-I Bim/Nur77 DKO mice did provides further argument that Nur77 deficiency impacted a non-deletional arm of tolerance. Another interesting contrast to our OT-I results is that OT-II Bim/Nur77 DKO Rip-mOva chimeras did not develop diabetes (160). One explanation could be that the Nur77 deficiency also resulted in increased numbers of Treg cells in this MHC class II-

restricted system. Furthermore, $CD8^+$ T cells play a fundamental role in the development of diabetes (287), and there would be few antigen-specific $CD8^+$ T cells in an OT-II system. Another possible explanation is that Nur77 is important for anergy induction in $CD8^+$ T cells but not $CD4^+$ T cells. Indeed, our data from polyclonal mice shows that Nur77 deficiency resulted in higher frequencies of anergic phenotype $CD4^+$ T cells.

II-d. Conclusion

The requirement for Bim or Nur77 during negative selection, and the relationship between the two molecules, is different between DP, CD8SP, and CD4SP thymocytes. The different molecular mechanisms likely reflect differences in both thymocytes and their environment. As in the HY^{cd4} chimeras, the combined deletion of Bim and Nur77 resulted in an increased frequency of antigen-specific CD8SP thymocytes compared to single deficiency in Bim or Nur77. In the case of positive selection, the effect of combined Bim and Nur77 deficiency was not as pronounced as in the HY^{cd4} model. For negative selection, combined Bim and Nur77 deficiency resulted in a similar or more severe impairment than in the HY^{cd4} model. The impairment in negative selection in OT-I DKO → Rip-mOva chimeras includes a complete block in clonal deletion, and perhaps defective anergy induction as well. It is currently not clear whether the impaired anergy induction stems from thymic negative selection or peripheral cognate antigen encounter. However, impairment in two mechanisms of tolerance is likely to account for the high incidence of autoimmune diabetes among OT-I DKO → Rip-mOva chimeras. These data provide further evidence of novel roles for Nur77 in T cells aside from mediating apoptosis.

III. Summary of the roles of Bim and Nur77 in a polyclonal T cell repertoire

In the third chapter of this thesis, we investigated the effects of Bim and/or Nur77 deficiency on T cell development in a polyclonal repertoire. One of the strongest phenotypes associated with Bim deficiency in BKO and DKO mice was an increase in the proportion of CD4SP and CD8SP thymocytes. By using the high affinity TCR signaling markers Helios and PD-1, we were able to conclude that at least part of the increase was due to impaired clonal deletion. Given our results with BKO and DKO chimeras in HY^{cd4} F and OT-I → WT models, it is likely that part of the increased frequency of SP thymocytes in polyclonal mice is due to enhanced positive selection as well. In addition, Bim deficiency resulted in an accumulation of $Helios^{+}PD\text{-}1^{+}$ DP thymocytes, supporting the conclusion of delayed UbA-mediated deletion made in the HY^{cd4} model. In the periphery, homeostatic mechanisms largely evened out the proportion of $CD4^{+}$ and $CD8^{+}$ T cells to a distribution similar to WT mice. However, BKO and DKO mice exhibited notable increases in absolute T cell numbers compared to WT mice, reflecting the major role of Bim in T cell apoptosis. Despite both BKO and DKO mice having higher T cell numbers, only combined deficiency of Bim and Nur77 resulted in frequent occurrence of a phenotype suggestive of inflammation. This is reminiscent of diabetes development in OT-I DKO → Rip-mOva chimeras, though we have not yet examined polyclonal DKO mice for autoimmune pathology. Curiously, DKO mice – even the ones with the putative inflammation phenotype – showed significant increases in subsets thought to be anergic or suppressive such as $Foxp3^{+}$ $CD4^{+}$ Treg, $FR4^{hi}CD73^{hi}$ $CD4^{+}$ T cells, and DN T cells. Though indicative of diversion to alternative fates in the face of impaired clonal deletion, it remains unclear how autoimmunity may still occur.

III-a. Autoimmunity in the absence of Bim and Nur77

Currently, our supposition of an increased propensity for DKO mice to develop inflammation is based on a severe reduction in DP thymocytes. Preliminary analysis of sera from the various polyclonal strains suggests that IL-6 and TNFα are elevated in BKO and DKO mice. However, more work is required to conclude whether any cytokines are elevated in DKO mice with the putative inflammatory phenotype compared to 'normal' DKO mice. The HY^{cd4} WT + HY^{cd4} DKO → M mixed bone marrow chimeras revealed that the increase in antigen-specific thymocytes was not only a result of impaired clonal deletion, but also due to thymocyte-extrinsic factors caused by Bim and Nur77 deficiency. This is evidenced by the observation that $T3.70^{+}$ CD8SP thymocytes of HY^{cd4} WT origin were also increased compared to intact HY^{cd4} M mice. Factors other than impaired clonal deletion may contribute to the inflammation in polyclonal DKO mice and autoimmunity in OT-I DKO → Rip-mOva chimeras as well. One extrinsic factor may be an enhanced inflammatory macrophage phenotype, as this has been linked to Nur77 deficiency, resulting in exacerbation of atherosclerosis in mouse models (152-154). Increased production of IL-6 and/or TNFα by Nur77-deficient macrophages was reported in some studies but not in all. In the future, chimeras will be generated using a bone marrow mix derived mostly from a WT donor with a minor contribution by a DKO donor in order to determine whether autoimmunity still arises when the majority of hematopoietic cells are WT. Non-hematopoietic cells may also be a significant producer of inflammatory cytokines in polyclonal DKO mice, while this is unlikely in OT-I DKO → Rip-mOva chimeras since the recipients are still capable of Bim and Nur77 expression.

Another factor that may contribute to heightened inflammation in polyclonal DKO mice is an increase in Tfh cells. Accumulation of Tfh cells has been linked to excessive B cell activation and autoimmunity (260-262). Preliminary data suggests that DKO mice have an increased frequency of $CXCR5^{+}PD\text{-}1^{+}$ $CD4^{+}$ T cells in the spleen compared to other strains. We are working on confirming this observation by examining expression of the Tfh-lineage specific transcription factor Bcl-6 (288).

While combined deficiency in Bim and Nur77 resulted in a high incidence of diabetes in the OT-I Rip-mOva model and putative inflammation in polyclonal mice, we did not observe any signs of autoimmunity in HY^{cd4} WT + HY^{cd4} DKO → M chimeras. HY TCR transgenic $CD8^{+}$ T cells have long been known to exhibit poor proliferation, IFNγ production, and cytolytic activity in response to antigen (289-291). Nevertheless, antigen-specific T cells appear to expand in the periphery of HY^{cd4} M mice since the frequency of $T3.70^{+}$ $CD8^{+}$ splenocytes is higher than the frequency $T3.70^{+}$ CD8SP thymocytes in male mice or in the periphery of HY^{cd4} F mice (97). Though some of this expansion may be due to less competition because of clonal deletion of $T3.70^{+}$ thymocytes (i.e. not antigen-driven proliferation), HY^{cd4} mice contain a substantial fraction (~30%) of non-HY TCR-transgenic thymocytes, which expand to make up the majority of peripheral T cells. Furthermore, $V\alpha2^{+}$ $CD8^{+}$ T cells from OT-I DKO → Rip-mOva chimeras also exhibited poor proliferation and IFNγ production. Both $T3.70^{+}$ $CD8^{+}$ from HY^{cd4} M mice and $V\alpha2^{+}$ $CD8^{+}$ T cells from Rip-mOva recipients showed downregulation of CD8 compared to non-cognate antigen expressing controls, consistent with poor function. One reason why there may not have been inflammation in the HY^{cd4} WT + HY^{cd4} DKO → M chimeras is because, unlike OT-I DKO → Rip-mOva chimeras and polyclonal DKO mice, the HY^{cd4} mixed bone

marrow chimeras contained both TCR transgenic WT T cells as well as B6 T cells. As discussed earlier, we speculate that Nur77 is important for anergy induction in $CD8^+$ T cells. Thus, the admixture of $T3.70^+$ WT and B6 T cells considerably decreases the amount of $T3.70^+$ DKO T cells, which may otherwise be hyper-responsive and capable of mediating autoimmunity. Secondly, the presence of B6 T cells means that these mixed bone marrow chimeras contain more $Foxp3^+$ $CD4^+$ Treg than the full OT-I bone marrow chimeras. However, polyclonal DKO mice also contain a high ratio of Treg (as well as anergic phenotype T cells) to effector T cells. In the absence of any functional assays, we cannot conclude that the increased populations of Treg or $FR4^{hi}CD73^{hi}$ $CD4^+$ T cells in polyclonal DKO mice are fully suppressive or anergic. For example, Treg have been shown to acquire IFNγ and IL-17 production in an inflammatory milieu, though others argue that their suppressive function remains intact (292).

III-b. Potential role for Nur77 in modulating TCR signaling and implications for thymocyte development

As mentioned in the previous section, data from the OT-I Rip-mOva model suggests that Nur77 plays a role in the induction of anergy in $CD8^+$ T cells. We do not know whether this occurs during high affinity antigen encounter in the thymus and/or periphery. Given the modest role of Nur77 in TRA-mediated deletion of CD8SP thymocytes, it is tempting to speculate that induction of Nur77 during thymic selection may serve additional functions like promoting anergy. Egr2 is a transcription factor thought to be important in anergy induction (218, 219). While we did not find any differences in Egr2 expression between $V\alpha2^+$ CD8SP thymocytes or $CD8^+$ T cells from OT-I Het/Het and OT-I NKO → Rip-mOva mice, Nur77 may regulate expression of other anergy factors such as the related Egr3.Furthermore, the role of Nur77 in

anergy is complicated by potentially opposing effects of Nur77 deficiency in $CD8^+$ vs. $CD4^+$ T cells. Whereas $V\alpha2^+$ $CD8^+$ T cells from OT-I NKO → Rip-mOva mice have increased function compared to OT-I Het/Het → Rip-mOva, polyclonal NKO mice have an increased population of anergic phenotype CD4SP thymocytes and $CD4^+$ T cells. Though one observation concerns function and the other development of anergic T cells, as well as involving different model systems, different requirements for anergy induction in $CD4^+$ vs. $CD8^+$ T cells has been suggested (293, 294).

The most notable difference between OT-I Het/Het and OT-I NKO → Rip-mOva chimeras was a tendency for $V\alpha2^+$ CD8SP thymocytes to be divided into distinct TCR-signaled (Helios, PD-1, Bim, CD44) and unsignaled populations in OT-I Het/Het → Rip-mOva mice. It is unclear if or how this observation would reflect anergy induction. A more direct explanation would be a role for Nur77 in modulating the synchronicity of TCR signaling. Differences in high affinity-induced TCR signaling involving Helios and CD69 were also observed in DP thymocytes from HY^{cd4} NKO M mice compared to HY^{cd4} M mice. Though the altered phenotypes associated with Nur77 deficiency were different between the HY^{cd4} and OT-I models, together these data support a role for Nur77 in modulating TCR signaling.

In addition to anergy, which results from high affinity antigen encounter, the putative altered TCR signaling due to Nur77 deficiency may also underlie enhanced positive selection in HY^{cd4} DKO F and OT-I DKO → WT chimeras. Increased positive selection efficiency is likely to account for a considerable part of the increase in CD4SP and CD8SP thymocytes in polyclonal DKO mice as well. One finding that was observed in multiple settings is an increase in $CD24^{lo}$

SP thymocytes in the absence of Bim and/or Nur77. In the OT-I → WT chimeras, there was a dramatic increase in the proportion of $V\alpha2^{+}$ $CD24^{ultra\text{-}lo}$ CD8SP thymocytes in recipients of DKO bone marrow compared to other recipients. This is reminiscent of the marked increase in $CD24^{ultra\text{-}lo}$ CD4SP and CD8SP thymocytes in polyclonal DKO mice. Though some of this increase may be attributed to recirculating peripheral T cells, the majority of $CD24^{lo}$ SP thymocytes did not express CD44. Our data suggests that CD44 is upregulated during the course of thymocyte maturation, though to lower levels than on high affinity signaled thymocytes or activated T cells. We did observe an increase in $CD44^{int}$ $CD24^{ultra\text{-}lo}$ CD4SP thymocytes in polyclonal DKO mice, suggesting that increased retention in the thymus is a large factor. A third explanation for the increase in mature SP thymocytes may involve an altered TCR signaling threshold that promotes thymocyte selection and maturation. Next to DKO mice, HY^{cd4} BKO F, OT-I BKO → WT, and polyclonal BKO mice exhibited the greatest increases in positive selection and thymocyte maturation, demonstrating a major role for Bim deficiency in these processes. However, the strongest phenotype always occurred in DKO mice, indicating a role for Nur77 as well. While Nur77 deficiency may promote thymocyte survival like Bim deficiency, based on the limited role of Nur77 in clonal deletion, we favour a role for Nur77 in modulating TCR signaling. This could be achieved through direct interactions between Nur77 and proteins involved in TCR proximal signaling or as a result of Nur77-induced transcriptional changes.

In both positive and negative selection models, we found evidence of altered TCR signaling associated with Nur77 deficiency. Furthermore, in polyclonal DKO mice, we observed dramatic increases in the frequencies of Treg and anergic phenotype CD4SP and $CD4^{+}$ T cells compared to other genotypes. One of the greatest topics of interest in the field is how agonist selection is

distinguished from clonal deletion, since both fates result from encounter with higher affinity antigen than positive selection. Although Treg are anergic and suppressive, constituting a critical arm of self-tolerance like clonal deletion, one fate results in a survival and differentiation program and the other in death. In accordance with other studies (20, 160), we found that Bim or Nur77 deficiency promoted increased Treg selection, as well as generation of anergic phenotype CD4SP thymocytes. Bim plays a large role in clonal deletion of CD4SP thymocytes (144), so loss of this important apoptotic mediator may prevent clonal deletion and divert autoreactive clones to regulatory and anergic fates. Nur77 deficiency may act in the same way, but again, our data did not show a large role for Nur77 in deletion. The NR4A family has been shown to drive Foxp3 expression (148), yet Nur77 single deficiency results in increased Treg selection. This apparent contradiction could be reconciled if Nur77 deficiency lowers the TCR signaling threshold to allow more thymocytes to undergo agonist selection while Nor-1 drives Foxp3 expression. Bim deficiency was already sufficient to cause increased T cell numbers in the thymus and spleen, with no further increase observed in DKO mice, implying that Nur77 deficiency had little additional affect in promoting T cell survival. However, polyclonal DKO mice exhibited a dramatically increased frequency of Treg cells compared to BKO mice, supporting the theory that Nur77 deficiency promoted their selection by a mechanism unrelated to apoptosis.

III-c. Clonal diversion to DN T cells

In addition to clonal deletion, anergy induction, receptor editing, and Treg selection, recent data demonstrate that clonal diversion into DN thymocytes is another fate of autoreactive thymocytes (207). Post-selection $\alpha\beta TCR^{+}$ $CD5^{hi}$ DN thymocytes are thought to be precursors for $CD8\alpha\alpha^{+}$

IELs, which have been proposed to maintain self-tolerance in the gut (295). HY^{cd4} mice, which by design have a greatly reduced $T3.70^+$ DN population compared to classical HY mice, also exhibit reduced development of $CD8\alpha\alpha^+$ IELs (77). In addition to $CD8\alpha\alpha^+$ IEL precursors, DN T cells have been shown to possess regulatory roles in other contexts (209). In the thymus of HY^{cd4} $Bim^{-/-}$ M mice, we did not observe much increase in $T3.70^+$ DN thymocytes compared to HY^{cd4} M mice. Though the $T3.70^+$ DN population is considerably larger in the periphery of HY^{cd4} $Bim^{-/-}$ M compared to HY^{cd4} M mice (97), this is unlikely to indicate increased clonal diversion during thymic selection because there is little difference in the frequency of $T3.70^+$ DN thymocytes between the two strains. Since clonal deletion is intact in the HY^{cd4} model when Bim is absent, most antigen-specific thymocytes are likely deleted rather than become developmentally diverted.

Overall, the frequency of $V\alpha2^+$ DN thymocytes was not consistently increased in any particular OT-I chimera. They are unlikely to result from high affinity TCR-mediated selection since they have also been found to occur in WT recipients. Furthermore, if they were representative of clonal diversion in lieu of clonal deletion, we would expect OT-I BKO → Rip-mOva and OT-I DKO → Rip-mOva chimeras to preferentially contain an increased frequency of $V\alpha2^+$ DN thymocytes, yet we commonly observe this in OT-I NKO → Rip-mOva chimeras as well. At present, we are unsure what an increase in $V\alpha2^+$ DN thymocytes may signify.

In contrast to the TCR transgenic models, we consistently observed an elevated frequency of $TCR\beta^+$ DN thymocytes from polyclonal BKO and DKO mice compared to WT or NKO mice. Furthermore, the $TCR\beta^+$ DN thymocytes that were $Helios^+$ had a high level of Helios expression

comparable to SP thymocytes, indicative of post-high affinity TCR signaling. In the spleen, many TCRβ$^+$ DN cells maintained a level of Helios expression comparable to splenic TCRβ$^+$ CD4$^+$ cells, though Helios expression was lower than on DN thymocytes. This could mean that the splenic DN population was derived from the thymic DN population. Alternatively, TCR stimulation in the periphery may have induced Helios expression. Bone marrow transplantation into thymectomized or sham-thymectomized mice reveals that DN T cells arise from both a thymic-dependent and independent pathway (296). Extrathymic DN T cells can be derived from downregulation of CD4 or CD8 co-receptor (296, 297). There was a modest but significant decrease in the frequency of TCRβ$^+$ CD4$^+$ splenocytes in polyclonal BKO mice compared to WT. This was also true in polyclonal DKO mice, except the decrease in the frequency of the CD4$^+$ subset was not as large as in BKO mice. We do not know whether TCRβ$^+$ DN splenocytes preferentially originate from CD4$^+$ T cells in the absence of Bim; this correlation could also reflect competition between the two T cell subsets for a similar niche. For example, in polyclonal BKO and DKO mice, only the CD4$^+$ and DN T cell subsets contained an appreciable Foxp3$^+$ population, and these Foxp3$^+$ populations may compete for the same niche. However, the role of Foxp3 in regulatory DN T cell function remains unclear. One study reported that Foxp3$^+$ CD4$^+$ T cells lost Foxp3 expression when they converted into regulatory DN T cells (297). We observed that the level of Foxp3 expressed by splenic TCRβ$^+$ Foxp3$^+$ DN cells was lower than that in splenic TCRβ$^+$ Foxp3$^+$ CD4$^+$ cells. We do not know whether this reflects a population that is downregulating Foxp3, nor whether a stable low level of Foxp3 expression is sufficient to confer suppressive functions. Most WT Foxp3$^+$ DN thymocytes are TCRβ$^-$ (270), suggesting that they are not of a post-TCR-mediated selection stage. In contrast, most Foxp3$^+$ DN thymocytes in BKO and DKO mice are TCRβ$^+$, supporting the occurrence of increased clonal diversion when

clonal deletion is impaired. An important distinction between BKO and DKO mice is that a much larger proportion of DN thymocytes in polyclonal BKO mice are post-selection; most DN thymocytes from DKO mice were TCRβ$^-$ Helios$^-$ PD-1$^-$. This suggests that the absence of Nur77 inhibits certain fates from being adopted and/or promotes the adoption of other fates. These data add to the evidence arguing that Nur77 plays a role in thymocyte development apart from clonal deletion. Preliminary data argues against NKT cells as a large contributor to the TCRβ$^+$ DN thymocytes or splenocytes, and the frequency of NK1.1$^+$ TCRβ$^+$ DN cells was not different between any of the strains. Rather, the post-selection DN thymocytes may include CD8αα IEL precursors or other regulatory DN cells. We intend on performing functional assays in the future to characterize these possibilities.

III-d. Conclusion

The results of this chapter extend the conclusions made from TCR transgenic models to selection in a polyclonal TCR repertoire (**Fig. 6-1**). Bim is an important contributor to clonal deletion among all thymocyte subsets, while the contribution of Nur77 is little, if any. In terms of clonal deletion efficiency, as assessed by accumulation of high affinity-signaled thymocytes, polyclonal DKO mice exhibited no appreciable difference compared to polyclonal BKO mice. Instead, the impact of Nur77 deficiency was apparent in positive selection, Treg selection, and generation of anergic CD4$^+$ T cells. Development of these fates may be promoted by Nur77 deficiency at the expense of clonal diversion to DN thymocytes, since polyclonal DKO mice had less high affinity-signaled DN compared to polyclonal BKO mice. These findings again highlight previously unappreciated roles for Nur77 in T cell tolerance.

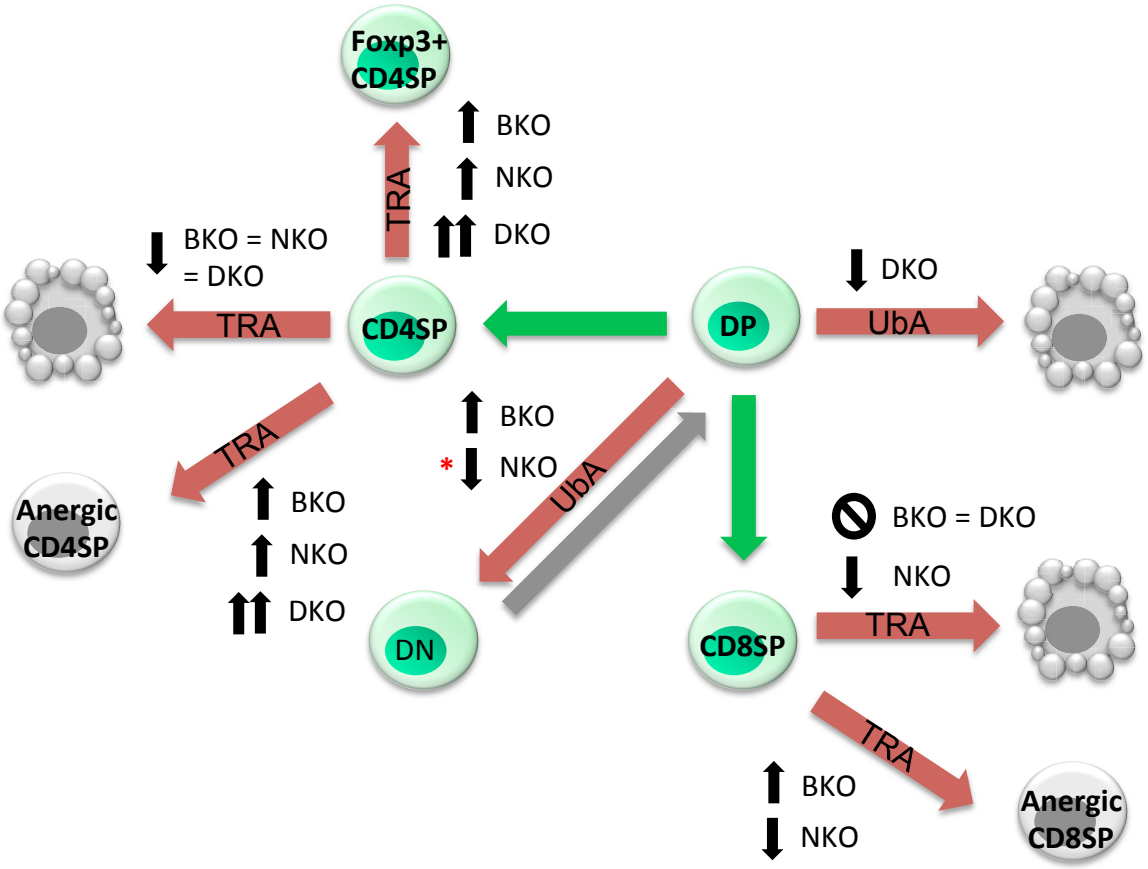

Figure 6-1. Effects of Bim and/or Nur77 deficiency on different fates following high affinity TCR-mediated selection. UbA-mediated clonal deletion of DP thymocytes requires neither Bim nor Nur77, but both may contribute in different ways. Following positive selection and development into CD4SP or CD8SP thymocytes, TRA-mediated clonal deletion can occur; the impact of Bim or Nur77 deficiency differs between the two subsets. When clonal deletion is inhibited, other tolerance mechanisms become more prominent. For Treg selection and generation of anergic CD4SP thymocytes, Bim and Nur77 deficiencies have a synergistic effect. For clonal diversion to DN thymocytes (* = inferred from DKO, not directly observed in NKO) and anergy induction in $CD8^+$ T cells, Bim and Nur77 may play opposing roles. Grey arrow, TCR-independent development; green arrow, low affinity TCR signal; red arrow, high affinity TCR signal; black up arrow, increase observed with gene deficiency; black down arrow, decrease observed with gene deficiency; no symbol, abrogation.

CLOSING REMARKS

The data presented in this thesis challenges traditional roles ascribed to Bim and Nur77 in T cell development and biology. Though Bim is an important mediator of clonal deletion in multiple settings, it is not always required for the elimination of autoreactive thymocytes. While Nur77 has historically been considered an apoptotic protein, it contributes little to clonal deletion in some settings, and recent studies expand its role in T cells beyond a matter of life and death. Through our findings on Bim and Nur77, we have increased our understanding of several mechanisms used to maintain self-tolerance – namely clonal deletion, clonal diversion, and anergy. It is the breaking of more than one tolerance mechanism that leads to autoimmunity, highlighting the complex nature of these diseases.

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