Characterizing tolerance in CD8 T cells in the absence of Bim-mediated clonal deletion to tissue restricted antigens

by

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Abstract

T cells are part of the adaptive immune system that eliminate pathogen-infected cells, but do not harm healthy cells. Precursor T cells, termed thymocytes, develop in the thymus where they randomly generate and express unique antigen specific T cell receptors (TCR) during the $CD4^+CD8^+$ double positive (DP) stage of development. Due to the random nature of generation, the specificity of the TCR needs to be screened by a selection process controlled by TCR affinity for self-peptide presented in major histocompatability complex (MHC) proteins. DP thymocytes that express TCRs with low or moderate affinity with self-peptide-MHC are positively selected. These cells then migrate from the cortex of the thymus to the medulla to mature into CD4 single positive (SP) or CD8SP thymocytes. DP thymocytes that express potentially harmful TCRs that have high affinity for self-peptide-MHC are negatively selected. The exact mechanisms and pathways that mediate this binary fate decision remain unclear. One protein that may be involved in this fate decision is the pro-apoptotic Bcl-2 family protein Bim. Bim plays an important role in negative selection by inducing cell death in thymocytes that recognize self-antigen with high affinity. However, in the case of ubiquitous self-antigens (UbA), self-antigens expressed by all cells in the body, it has been shown that Bim is not required for the elimination of self-reactive thymocytes, suggesting the existence of non apoptotic negative selection mechanisms. However, the role of Bim in negative selection to tissue-restricted antigens (TRA), self-antigen expressed only in certain tissues (insulin for example), is unclear, as negative selection to TRA occurs in a

developmentally, temporally and physically distinct manner than negative selection to UbA.

I generated bone marrow (BM) chimeras by transplanting Bim-deficient OT-I TCR transgenic BM, a MHC class I restricted TCR specific for chicken ovalbumin (OVA), into mice expressing membrane bound OVA as a TRA under control of the rat insulin promoter (RIPmOVA). In these mice, I demonstrated that Bim is required for clonal deletion to TRA. While Bim-deficient OT-I thymocytes are not deleted in RIPmOVA chimeras, they are rendered anergic and are unable to proliferate OVA-pulsed splenocytes or cause diabetes. This anergic state persists in the periphery with OT-I Bim-deficient T cells being unable to proliferate or produce cytokines in response to stimulation with OVA-pulsed splenocytes. While proliferation can be rescued by stimulation with plate-bound αCD3/αCD28 antibodies, cytokine production cannot. Persistent antigen encounter is required to maintain this anergic state, as both proliferation and cytokine production is rescued in chimeras where OVA is expressed only in the thymus. Taken together, these data demonstrate that Bim is required for clonal deletion to TRA and highlights the fact that the immune system has "fail-safe" mechanisms in place to control auto-reactive T cells and limit their potential for autoimmunity. These data also provide some insight into methods of breaking immunological tolerance as might be desirable in adoptive T cell cancer immunotherapies.

Preface

This thesis is an original work by Alexander Yung Wen Suen. It contains content co-authored by collaboration and republished with permission:

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Table of Contents

Table of Figures

Table of Tables

List of Abbreviations

AICD	Activation induced cell death
AIRE	Autoimmune regulator
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy
ARE	AU-rich elements
BCR	B cell receptor
BH3	Bcl-2 homology domain 3
BM	Bone Marrow
BTLA	B and T lymphocyte attenuator
CD	Cluster of differentiation
CD4SP	$CD4^+$ single positive
CD8SP	$CD8^+$ single positive
CFSE	Carboxyfluorescein diacetate, succinimidyl ester
CMJ	Cortico-medullary junction
cTEC	Cortical thymic epithelial cell
CTL	Cytotoxic lymphocytes
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTV	Celltrace Violet
DC	Dendritic cell
DN	Double negative
DP	Double positive
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalitis
Egr2	Early growth response gene 2
ERK	Extracellular signal-regulated kinase
FTOC	Fetal thymic organ culture
GITR	Glucocorticoid-induced TNFR-related protein
HSC	Hematopoietic stem cells
HVEM	Herpes virus entry mediator
IFNγ	Interferon γ
IL	Interleukin
JNK	c-Jun NH2-terminal kinase

Lag-3	Lymphocyte activation gene 3
LIP	Lymphopenia induced proliferation
LM-OVA	*L. monocytogenes* secreting ovalbumin
LM-GAG	*L. monocytogenes* secreting GAG
LN	Lymph node
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MLR	Mixed lymphocyte reactions
Mls	Minor lymphocyte stimulatory
MMTV	Mouse mammary tumor virus
MOG	Myelin oligodendrocyte glycoprotein
mTEC	Medullary thymic epithelial cell
NFAT1	Nuclear factor of activated T cells 1
NK	Natural killer
OT-I	Ovalbumin transgenic class I
OVA	Chicken ovalbumin
PAMP	Pathogen associated molecular pattern
PCC	Pigeon cytochrome c
PD-1	Programmed death 1
pLN	Pancreatic lymph node
pMHC	Peptide presented on self MHC
qRT-PCR	Quantitative real-time polymerase chain reaction
SA	Superantigen
SEB	Staphylococcal enterotoxin
TCR	T cell receptor
TEC	Thymic epithelial cell
T_h1	T helper type 1
T_h2	T helper type 2
Tim-3	T cell Ig- and mucin-domain-containing molecule–3
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily
TNFRSF14	Tumor necrosis factor receptor superfamily, member 14
TRA	Tissue restricted antigen
T_{reg}	T regulatory

TSSP	thymus-specific serine protease
UbA	Ubiquitous antigen
UTR	Untranslated region

Chapter 1: Introduction

The body must contend with pathogens such as bacteria and viruses and sometimes itself in the form of cancer. In order to repel these health threats, a robust and efficient immune system is essential. The immune system can be broadly divided into two arms: innate immunity and adaptive immunity.

Innate immune system

The innate immune response can be generally described as aspects of the immune system that are hard wired into the host's germ line DNA. The role of the innate immune system is to be the first line of defense and control a pathogen while at the same time activating the adaptive immune system. This includes physical barriers such as epithelial and mucosal barriers whose main function is to prevent pathogens from entering the body. The innate immune system is generally activated by pathogen associated molecular patterns **(PAMPs)**.

Soluble proteins and molecules such as complement, defensins and ficolins are constitutively present in biological fluids (1). These molecules are capable of a variety of functions including direct antimicrobial or antiviral activity, identifying targets for destruction or modulation of innate and/or adaptive cellular responses (1-4). In addition to these constitutively present molecules, there are others that are secreted by activated cells such as cytokines, chemokines, lipid mediators of inflammation, reactive free radical species and inflammatory enzymes (1).

Phagocytic cells, including neutrophils, macrophages, monocytes and dendritic cells **(DC)**, are adept at phagocytosing microbes and particles marked by

complement or immunoglobulin. These cells can then act as antigen presenting cells **(APC)** by breaking down the phagocytized microbes and particles and presenting antigen in the context of major histocompatibility complex **(MHC)** molecules on their surface to adaptive immune cells (1). DCs are particularly proficient as APCs and depending on the subset of DCs that encounters antigen and the cytokine environment, DCs can activate or regulate T cell activation in the adaptive arm of the immune system (5). These fates depend on the expression of co-stimulatory molecules and secretion of cytokines by DCs (5)

Natural killer **(NK)** cells target host cells with decreased expression of MHC class I **(MHC-I)** and eliminate them primarily through cytotoxic degranulation (1, 6, 7). NK cells can modulate adaptive and innate immune responses through the expression of cell surface ligands and receptors as well as through the secretion of cytokines and chemokines. NK cells are also able to recruit effector cells to sites of inflammation through the secretion of chemokines (7).

The recognition molecules used by the innate system are widely expressed over a variety of cell types, allowing the cellular components of this system to rapidly react to an invading pathogen (1). This initial response is important for the early immune response and through complex interplay with the adaptive immune system, the innate immune system can help shape the entire immune response (1, 7).

Adaptive Immune System

The adaptive immune system differs from the innate in that it is antigen specific and generates a long-lived memory population capable of being reactivated should cognate antigen be encountered again (1). Adaptive responses primarily

involve B cells and T cells that express an antigen specific, somatically rearranged, clonal antigen receptor, B cell receptor **(BCR)** or T cell Receptor **(TCR)**, respectively (1). T cells recognize antigen in the context of self-MHC molecules through their TCR receptor (1). T cells expressing the $CD4^+$ co-receptor ($CD4^+$ T cells) recognize antigen presented in the context of MHC class II **(MHC-II)** molecules whereas T cells expressing the CD8 co-receptor ($CD8^+$ T cells) recognize antigen presented in the context of MHC-I molecules (8-10). This difference in preferences for MHC molecules means the different subsets will encounter antigens from different sources. $CD8^+$ T cells can take on cytotoxic functions after activation. These cytotoxic lymphocytes **(CTL)** are able to directly target and kill infected cells (11). $CD4^+$ T cells generally take on a helper role and depending on the activating environment, they can help direct the immune response. IL-12, IL-2 or IFNγ, skew the activation of $CD4^+$ T cells towards becoming T helper type 1 **(T_h1)** cells, which drive a cellular immune response through secretion of IL-2 and IFNγ where by macrophages and CTLs are activated (12-15). IL-4, on the other hand, skews the activation of $CD4^+$ T cells into becoming T helper type 2 **(T_h2)** cells (16). The T_h2 response is characterized by the activation of B cells and the generation of a humoral response to pathogens (12, 13).

MHC class I molecules are generally ubiquitously expressed on most nucleated cells (17). Generally, MHC-I antigens are generated from cytosolic proteins by degradation through the proteasome, after which antigens are loaded on to MHC-I molecules in the endoplasmic reticulum before trafficking to the cell surface (18). As a consequence of this process, in the context of an immune response, antigens tend to be derived from cytosolic foreign proteins. However, there exists a mechanism within

DCs, termed cross-presenting whereby endocytosis of antigens is followed by their transfer into the cytosol and subsequent processing for MHC-I presentation (18). Constitutive expression of MHC-II molecules is limited to professional APCs, which includes most DCs, B cells and thymic epithelial cells **(TEC)**, but MHC-II expression is inducible in most cell types. MHC-II molecules bind antigens generated from the proteolytic activity of endosomes and lysosomes. This allows presentation of antigens that enter the cell through receptor-mediated endocytosis, macropinocytosis, phagocytosis, and autophagy (18).

The TCR is a dimeric antigen receptor composed of a TCRα chain and TCRβ chain. These chains are each made up of several domains; the variable (V), joining (J) and constant (C) domains in the TCRα chain; and the V, J, diversity (D) and C domains in the TCRβ chain. Within the *Tcra* and *Tcrb* loci, there exist multiple potential V, D and J domains and one or two C domains respectively. The RAG recombinases mediate recombination at these two loci, resulting of the joining of one of each V, J, C and in the case of the TCRβ chain, one D domain to generate a TCRα chain and TCRβ chain (19). As a result of this process, it has been estimated, in humans, that the total potential diversity of TCR specificities that could be generated is between to 10^{16} to 10^{20} (20). This coupled with the plasticity of T cell antigen recognition (21) allows for sufficient coverage within the T cell population of a wide range of foreign antigens. However, as a result of this process there is also significant chance that TCRs specific for self-peptides are also produced. In the case where tolerance to peptides derived from self-proteins is broken or not induced, it is possible that these peptides will become self-antigens and drive a T cell response. In order to combat this possibility,

T cells undergo education processes in the thymus, known collectively as central tolerance. Lederberg first proposed this concept in relation to immature antibody producing lymphocytes, suggesting that antigen encounter would suppress the activity of these immature lymphocytes (22).

In regards to T cell development, these processes are known as positive selection and negative selection. These processes ensure that T cells can respond to antigen in the context of self-MHC molecules but T cells that show high affinity to self-peptide in the context of self-MHC are eliminated, respectively. Recent estimates of the total TCR diversity in human blood have focused on estimating the diversity of the CDR3 region of the TCR β chain, the most variable region in the TCR (23). Robins *et al.* calculated that there were approximately 3 to 4 million unique TCR β CDR3 sequences in the blood of human adult males. In addition to this variety, there exists variability within other regions of the TCR β chain as well as variability in the TCR α chain that would pair with the TCR β chain to form a mature α/β TCR, thus increasing the actual diversity within the blood (23). This reduction from the potential diversity of the TCR repertoire highlights the effectiveness of positive and negative selection.

Thymocyte Development

The thymus consists of two compartments, the outer cortex and inner medulla. The two compartments differ primarily in the subset of TEC that reside within the compartments: cortical TECs **(cTECs)** and medullary TECs **(mTECs)** (24). Despite arising from the endodermal epithelium, both subsets have distinct features and functions in T cell development(24).

In addition to the classical antigen processing mechanism present in DCs and mTECs, cTECs also exhibit two unique antigen processing mechanisms. In the context of antigen processing for MHC-I, cTECs express the unique catalytic proteasome subunit β5t. Incorporation of this subunit generates what is known as the thymoproteasome with a distinct substrate preference compared to proteasomes from mTECs and DCs (24, 25). It is thought that the thymoproteasome generates peptides that may bind more weakly to MHC-I (26) or more weakly interact with TCR in the context of MHC-I (27). In regards to MHC-II antigen processing, cTECs express the unique lysosomal proteases cathepsin L and thymus-specific serine protease **(TSSP)**. Mice deficient of these proteases exhibit impaired development of $CD4^+$ T cells (28, 29). Together, these unique antigen-processing mechanisms are thought to be important for positive selection.

mTECs differ from cTECs and thymic DCs in that they are able to promiscuously express tissue restricted antigens **(TRA)**, which would normally not be expressed outside of specific tissues (24, 25). TRA expression is restricted to the medulla with expression of many TRA controlled by the *AIRE* gene (30). The importance of Aire-driven TRA mediate negative selection is highlighted by the fact that mutations in *AIRE* lead to a defect in T cell clonal deletion and onset of the autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome **(APECED)** (31). Similarly, the transcription factor Fezf2 has recently been shown to induce the expression of Aire-independent TRA in mTECs(32). The expression of over 60% of mTEC-specific TRAs are under control of Fezf2 and Aire (32). In addition to mTECs, the medulla is home to the majority of thymic DCs. These DCs do not

express TRAs, however they are able to acquire TRA from mTECs and cross-present these antigens to thymocytes (33). These cellular differences result in two compartments with segregated function in T cell development.

Despite continuously outputting T cells for a large portion of a person's life, the thymus does not contain a resident stem cell population (34). T cell progenitors arise in from hematopoietic stem cells **(HSC)** in the bone marrow. These progenitors traffic through the blood and are recruited into the thymus at the cortico-medullary junction **(CMJ)**(35).

After entry into the thymus these developing T cells, termed thymocytes, express neither the CD4 or CD8 co-receptor and are referred to as double negative (DN) thymocytes **(Fig 1-1).** DN thymocytes can be further sub-divided into four sequential developmental stages based on CD44 and CD25 expression. After entering the thymus at the CMJ, DN1 ($CD44^+CD25^-$) thymocytes progress to towards the capsule, entering the DN2 ($CD44^+CD25^+$) stage (36) at which point somatic rearrangement of the *Tcrb* locus begins. Arriving at the subcapsular zone thymocytes enter the DN3 ($CD44^-CD25^+$) stage(36) when rearrangement of the *Tcrb* locus is complete (37). Successful dimerization of a functional and rearranged TCRβ chain with an invariant pre-TCRα chain allows thymocytes to progress to the DN4 ($CD44^-CD25^-$) stage (36, 37). DN4 thymocytes rapidly undergo a burst of proliferation, commence rearrangement of the *Tcra* locus, upregulate both CD4 and CD8 co-receptors becoming $CD4^+CD8^+$ double positive **(DP)** thymocytes and begin to travel back through the cortex (36-38).

Upon expression of a mature α/β TCR, DP thymocytes may undergo one of three fates depending on the affinity of their TCR for self-peptide presented on self-MHC **(pMHC)** on cTECs: death by neglect, negative selection or positive selection (39). If the TCR cannot functionally interact with self-pMHC, the thymocytes die by neglect. These cells fail to undergo positive selection and do not receive the accompanying survival cues (40). Thymocytes expressing a TCR with high affinity for self-pMHC undergo negative selection resulting in the elimination of that particular TCR specificity from the T cell repertoire, however cTECs do not appear to be particularly proficient at inducing clonal deletion and require assistance from rare cortical DCs (33). The primary mechanism of negative selection is clonal deletion whereby thymocytes are thought to primarily undergo apoptosis (39). Apoptosis can occur via two pathways. The cell extrinsic pathway is activated by death receptors such as Fas/FasL, whereas the cell intrinsic pathway, which is thought to be responsible for the majority of clonal deletion, is mediated by Bcl2 family members. Anergy, whereby thymocytes are intrinsically functionally impaired, and receptor editing have been described as additional mechanisms of negative selection, though clonal deletion is thought to be the more prominent mechanism. While thymic anergy induction has been described in several models (41, 42), the molecular mechanisms and functional characteristics are not fully understood.

Low or moderate affinity interactions between the TCR and self-pMHC result in positive selection, leading to thymocyte survival and subsequent differentiation into $CD4^+$ single positive **(CD4SP)** or $CD8^+$ single-positive **(CD8SP)** thymocytes (39). These positively selected thymocytes then undergo CCR7-dependent migration to the

thymic medulla (43). During the 3-4 day residence in the medulla (44), SP thymocytes are subjected to a further round of negative selection through interactions with mTEC and medullary DCs (45, 46). Cells that survive this final round of negative selection become mature SP thymocytes and egress into the periphery.

Interestingly, some T cell subsets are positively selected by high affinity antigen encounter in the thymus (47). One such example are T regulatory **(T_{reg})** cells which are believed to require high affinity interactions to develop in the medulla (48). It remains unknown what drives this distinction between negative selection and T_{reg} cell development, however, a TCR signal strength reporter mouse, thymocytes undergoing T_{reg} differentiation exhibited expression levels of the fluorescent reporter between cells undergoing positive and negative selection (49). It has been suggested that an intermediate TCR avidity, a measure of the combined strength of interaction between TCRs on a single T cell and pMHCs on APCs, which is a function of the combination of TCR affinity and abundance of selecting pMHCs, between that required for positive of negative selection drives T_{reg} differentiation (50, 51). Secondary signals have also been implicated. CD28 co-stimulation (52) and IL-2 signaling (53, 54) are essential for the induction of Foxp3 in $CD4^+$ SP thymocytes, which in turn specifies these thymocytes to become T_{reg} cells(55). While, Foxp3 induces proapoptotic proteins Puma and Bim, common gamma chain (γc)-dependent cytokine signaling can rescue $Foxp3^+$ thymocytes from death (56). Additionally, TGF-β may also play a role in overcoming cell death (57). Tumor necrosis factor **(TNF)**

receptor superfamily members **(TNFRSF)** GITR, OX40 and TNFR2 have been shown to be essential for the development of thymic natural T_{reg} cells (58).

It is clear that two different classes of self-antigens induce negative selection: ubiquitous self-antigens **(UbA)** and TRA. After much debate, recent data suggests that negative selection of DP thymocytes to UbA primarily occurs in the cortex without medullary involvement (33), while positive selection and CCR7 dependent migration to the medulla are required for negative selection to TRA (45). Once in the medulla, $CD4^{+}CD8^{lo}$ or $CD4^{lo}CD8^{lo}$ thymocytes can interact with mTECs that directly present TRA or DCs that can cross-present TRA derived from mTEC (46). Therefore, negative selection to UbA and TRA differ with respect to the developmental stage of the thymocyte, the anatomical location within the thymus as well the APCs inducing the process and the antigens they present.

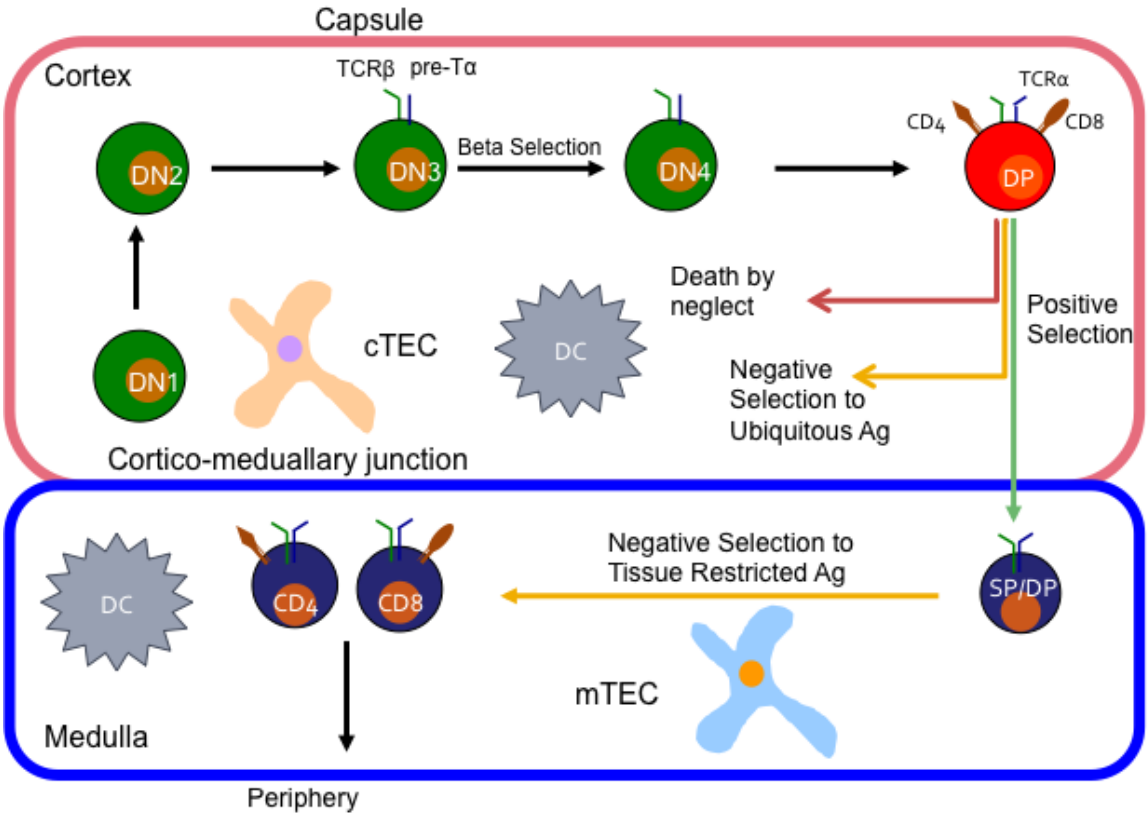


Figure 1-1: Thymocyte development and central tolerance
T cell precursors enter the thymus at the CMJ and become $CD4^{-}CD8^{-}$ DN thymocytes. As DN thymocytes progress to the DN3 stage, they rearrange the *Tcrb* locus by somatic rearrangement to generate a TCR β chain. Dimerization of a functional TCR β chain with an invariant pre-Tα chain allows thymocytes to progress through β selection and rapidly expand and develop into $CD4^{+}CD8^{+}$ DP thymocytes. Depending on the affinity of their TCR for ubiquitous self-pMHC, DP thymocytes can undergo positive selection (low/moderate affinity) upon cTECs, negative selection (high affinity) upon cTECs or DCs, or death by neglect (no affinity). DP thymocytes that are positively selected commit to the CD4 or CD8 lineage depending on whether their TCR is specific for MHC-II or MHC-I respectively and traffic to the medulla. Here they may another round of negative selection against TRA on mTECs and DCs. Mature CD4 and CD8 SP thymocytes can then egress from the thymus.

Positive vs Negative selection

While it is unclear how the same TCR can transduce a signal for positive or negative selection, several different molecular pathways and cellular requirements have been implicated. Co-stimulation seems to be important for negative selection but not positive selection. *In vitro*, it was demonstrated that anti-CD28 antibodies could enhance apoptosis in DP thymocytes (59, 60). A similar effect was shown with monoclonal antibodies **(mAb)** against CD28, CD43 and CD5 (60). Blockade of CD80 and CD86, both co-stimulatory ligands of CD28, as well as TNF, rescued development in a negatively selected $CD4^+$ SP population in fetal thymic organ culture **(FTOC)**(61). Additionally, inhibition of negative selection by perinatal blockade of CD80 and CD86 (62) further supports the importance of CD28 activation to negative selection. Consistent with these results, CD28- and CD43-deficient neonatal mice injected with superantigen **(SA)** staphylococcal enterotoxin **(SEB)** showed impaired clonal deletion (60). However, in regards to TCR transgenic mice, CD28 deficiency did not impact negative selection (63) nor did CD5/CD28 (64) or CD80/CD86 (65) double-deficiency in the context of SA-mediated deletion.

Using a SA model, it was shown that the absence of CD40 ligand (CD40L) resulted in impaired negative selection (66). Interestingly, CD40L does not need to be expressed on all thymocytes to mediate negative selection, suggesting that CD40L may affect the expression of MHC molecules and co-stimulatory proteins on APCs (65), which in turn would affect the ability of these APCs to induce negative selection.

Through the use of two-photon microscopy to observe motility and calcium flux within MHC-I restricted TCR transgenic thymocytes on thymic lobe slices, it was

shown that in the presence of positively selecting ligands, thymocytes exhibit short transient signaling events followed by longer periods of low calcium levels and relatively high motility (67). In contrast, thymocytes encountering negatively selecting ligands underwent rapid migratory arrest and a sustained increase in intracellular calcium two hallmarks of TCR signaling (67, 68).

Differential mitogen activated protein kinase **(MAPK)** signaling appears to play a role a in the binary fate decision between negative and positive selection (69, 70) **(Fig 1-2)**. It is thought that the kinetics of activation of MAPK pathways differentiates positive and negative selection (69). Both c-Jun NH2-terminal kinase **(JNK)** and p38 are activated with similar kinetics in both positive and negative selection (70). In contrast, positive selection is associated with weak but sustained induction of extracellular signal-regulated kinase **(ERK)** activity while negative selection is associated with a transient burst of ERK activity (70). As a result of this differential activation of the ERK pathway, maximal ERK activation occurs after JNK and p38 in positive selection, whereas in negative selection maximal ERK activation occurs before JNK and p38 (69)**.** However, contrary to this observation, while gene deletion experiments suggest ERK1/2 and 3 are critical for thymocyte positive selection (71-73), ERK1/2 activation is not required for negative selection (74). Ultimately, this differential activation of MAPK signaling pathways leads to the activation of different transcriptional programs (75).

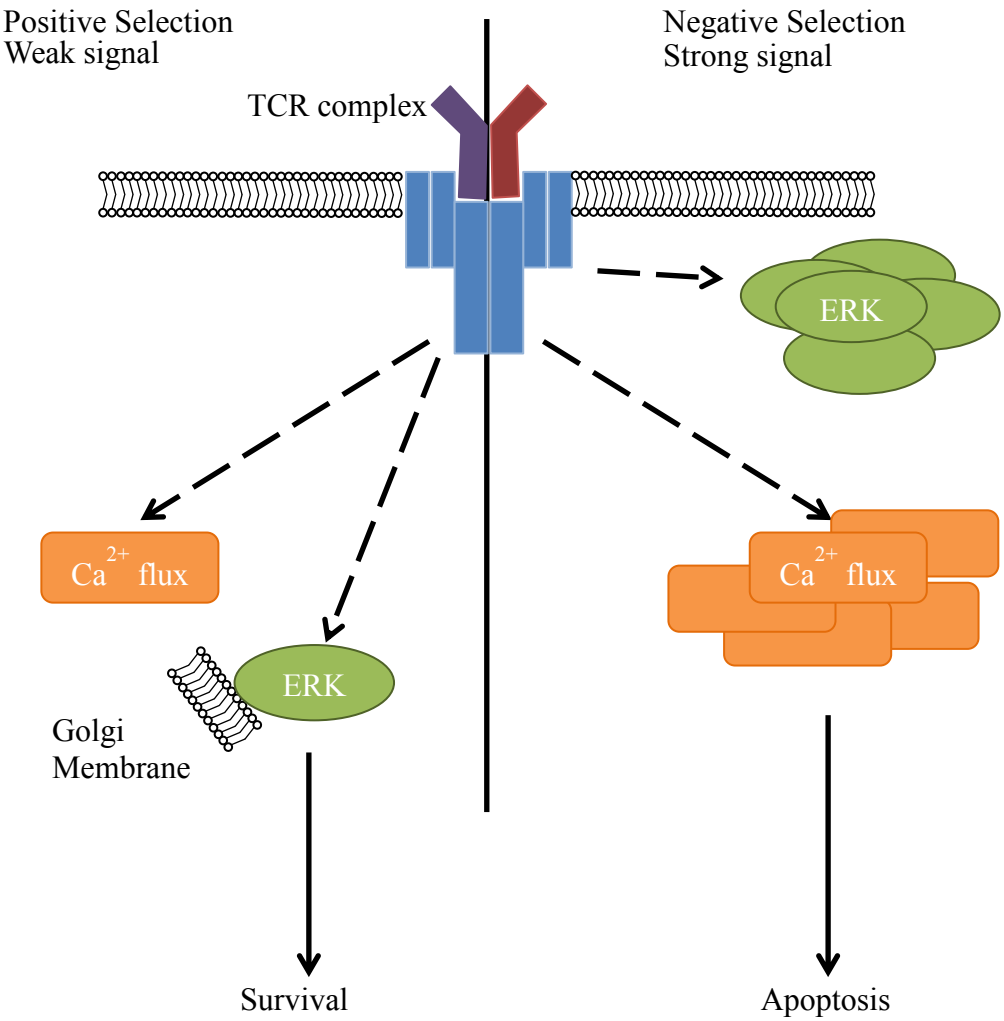


Figure 1-2: Simplified view of TCR signaling pathways during positive and negative selection. Low affinity interactions between the TCR and pMHC drive a weak signal through the TCR complex. This results in a low and sustained ERK activation localized to the Golgi membrane as well as slow and sustained calcium mobilization. High affinity interactions between the TCR and pMHC drive a strong signal through the TCR complex and results in a transient burst of membrane localized ERK activation as well as rapid but transient calcium mobilization.

Positive (76) and negative selection (77) both induce upregulation of the anti-apoptotic protein Bcl-2. While Bcl-2 overexpression could rescue thymocytes from various apoptotic signals (78, 79), it could not rescue thymocytes from negative selection (78). In addition to Bcl-2, negative selection has been shown to upregulate the pro-apoptotic molecule Nur77, a member of the orphan nuclear receptor Nr4A family (77). Expression of a dominant-negative form of Nur77 blocks negative selection (80), however, in both classical (80-82) and physiological (83) models of negative selection to UbA, Nur77 deficiency alone does not impair clonal deletion. Interestingly, in a $CD4^+$ model of negative selection to TRA, Nur77 deficiency impaired clonal deletion (84). While Nur77 has been associated with apoptotic functions, its mechanism of action has not been elucidated (40).

Helios, an Ikaros transcription factor family member initially thought to be dispensable to T cell differentiation and function(85), has been shown to be upregulated during negative selection in a model of negative selection against UbA (86) as well as in a MHC class II-restricted TCR $CD4^+$ model against TRA (87). In the $CD4^+$ model against TRA, Helios was expressed at low levels in DP thymocytes and was further downregulated in positively selected cells, suggesting the Helios could be used as a marker of high affinity antigen encounter (87). While it has been suggested that Helios marks thymically derived T_{reg} cells, more recent data has shown that Helios is also expressed in peripheral T_{reg} cells as well as in activated, differentiating and dividing T cells (88-92). Additionally Helios has been shown to be essential for the stable inhibitory activity of $CD4^+$ and $CD8^+$ T_{reg} cells (93).

Negative selection also induces expression of the pro-apoptotic, Bcl-2 homology domain 3 **(BH3)** only Bcl-2 family member Bim (77, 94), which is essential for the intrinsic pathway of apoptosis (95), thought to be the primary mechanism of clonal deletion. It is not clear if Bim interacts directly with and activates Bax/Bak at the mitochondrial membrane or if Bim inhibits anti-apoptotic Bcl-2 family members from interacting with Bax/Bak.

It was generally held that Bim-mediated apoptosis was required for negative selection (96) as the loss of Bim protected thymocytes and immature T cells from deregulated calcium flux-mediated apoptosis (97). This was supported by the fact that Bim-deficient mice develop late onset autoimmune disease and that NOD mice, which spontaneously develop diabetes, have a defect in the induction of Bim (94, 97, 98). In contrast to this paradigm, recent studies demonstrated that while Bim is required for thymocyte apoptosis, it is not required for physiological clonal deletion of HY TCR transgenic thymocytes to the ubiquitous s-mcy male peptide (99, 100). These models utilized the HY TCR, however unlike classical TCR transgenics, the expression of the TCRα chain was delayed until the DN to DP transition, as it would in a non-transgenic thymocyte (99, 100). Bim was also not required in a model of SA-mediated clonal deletion (101), suggesting a role for Bim-independent mechanisms of clonal deletion. These conflicting reports may arise from the different classes of antigens examined in these models. As negative selection to UbA and TRA occur in distinct locations with in the thymus and at different stages of thymocytes development, these factors may result in differential molecular requirements for

negative selection to UbA versus TRA, though no such thymocyte intrinsic differences downstream of the TCR have been reported.

Peripheral Tolerance

Due to the high level of cross-reactivity of the TCR recognition (21), if negative selection in the thymus is too stringent, there is a risk of depleting the T cell repertoire of sufficient TCR specificities to maintain adequate immune surveillance (102). Thus it is more effective to allow thymocytes with some limited degree of self-reactivity to escape negative selection (102). Additionally, some degree of weak self-reactivity is required by all T cells to maintain T cell homeostasis. Indeed, central tolerance is not perfect and self-reactive T cells are present in the periphery of healthy individuals (103, 104). Consistent with deletion of T cells bearing TCRs with high affinity for self antigens, these peripheral self-reactive T cells are more likely to have low affinity for self-antigens (105). To control these self-reactive T cells, there are peripheral tolerance mechanisms in place. These mechanisms can be divided into two groups: T cell intrinsic and T cell extrinsic. T cell intrinsic mechanisms arise from the T cell itself, whereas T cell extrinsic mechanisms involve external cell types acting on the tolerized T cell.

T cell intrinsic mechanism of peripheral tolerance includes: ignorance, anergy, phenotypic skewing and deletion **(Fig 1-3).** If self-antigen is sequestered in an immune privileged site or not easily accessible to circulatory or lymph systems (106, 107), it is possible a self-reactive T cell will never encounter its cognate antigen. For example, transplant of MHC class I K^b specific BM into neonatal Rag deficient hosts expressing K^b on skin keratinocytes resulted in mice tolerant to K^b tumor cells, while

adult recipients were not tolerant (106). This was attributed to the ability of peripheral T cells to traffic to and engage keratinocytes expressing K^b in neonatal mice, but not adult mice.

Like antigen encounter in the thymus, peripheral antigen encounter can also induce anergy in self-reactive T cells, whereby the T cell are intrinsically unable to respond to antigen. Classically, anergy has been studied *in vitro* in the context of $CD4^+$ T cell clones that have been stimulated through the TCR in the absence of CD28 co-stimulation and rendered proliferatively non-responsive (41). The CD28/B7 co-stimulation pathway appears to be essential in preventing the induction of anergy, though it is not clear what is the exact mechanism by which CD28 signaling prevents anergy. This could be through direct inhibition of the production or function of anergy molecules or indirect effect by driving cell cycle progression and enhancing the release of growth factors such as IL-2. In many cases, exogenous IL-2 can rescue T cells from this *in vitro* form of anergy, and it is difficult to assess any direct effect of CD28 signaling (41). Cyclosporin A, a calcineurin inhibitor, was shown to block anergy induction in *in vitro* models. It is thought that that calcineurin is acting through nuclear factor of activated T cells 1 **(NFAT1)**, to induce expression of anergy genes (41, 108, 109).

There have been several models that have characterized this phenomenon using both $CD4^+$ and $CD8^+$ TCR transgenic T cells (110-114). Initial experiments involved the transfer of naïve male antigen specific HY TCR transgenic T cells from female donors into athymic male mice. $CD8^+$ HY T cells expanded rapidly in these hosts before contracting and leaving behind a small anergic population (111, 112).

The study of peripheral anergy in $CD4^+$ T cells involved the transfer of pigeon cytochrome c **(PCC)** specific T cells into irradiated hosts expressing the cognate antigen (114) or the transfer of chicken ovalbumin **(OVA)** specific T cells into intact hosts who were then immunized to induce anergy (113). It is not clear what mechanisms are driving anergy in these models, however its been suggested that impaired proximal TCR signaling through LAT (41) may play a key role. These models generated anergic populations with very similar characteristics including: impaired proliferation to antigen and APC; initial activation of the anergic T cells; impaired effector function and requirement for persistent antigen encounter to maintain anergy.

While a self-reactive T cell may be activated in the periphery to self-antigen, the functional response of the T cell could be altered to maintain tolerance. This could include modulating cytokine production or altering T cell trafficking (102). In models of experimental autoimmune encephalitis **(EAE)** and autoimmune diabetes, diseases both mediated by T_h1 responses, autoimmunity can be combatted by T_h2 cytokines and chemokines (115, 116). This phenotypic skewing could prevent activated self-reactive T cells from being in the right place or in proximity to the appropriate cells to mediate an immune response. Finally, peripheral encounter with self-antigen can lead to the deletion of self-reactive T cells. Repetitive engagement of the TCR can lead to activation-induced cell death **(AICD)**. It has been suggested that Fas and FasL may be important for peripheral deletion and Pten may be involved (102). However, rarely is peripheral deletion complete. In many cases there remains

a small population of anergic cells, highlighting the notion that these mechanisms work in tandem and not separately to control peripheral tolerance (102).

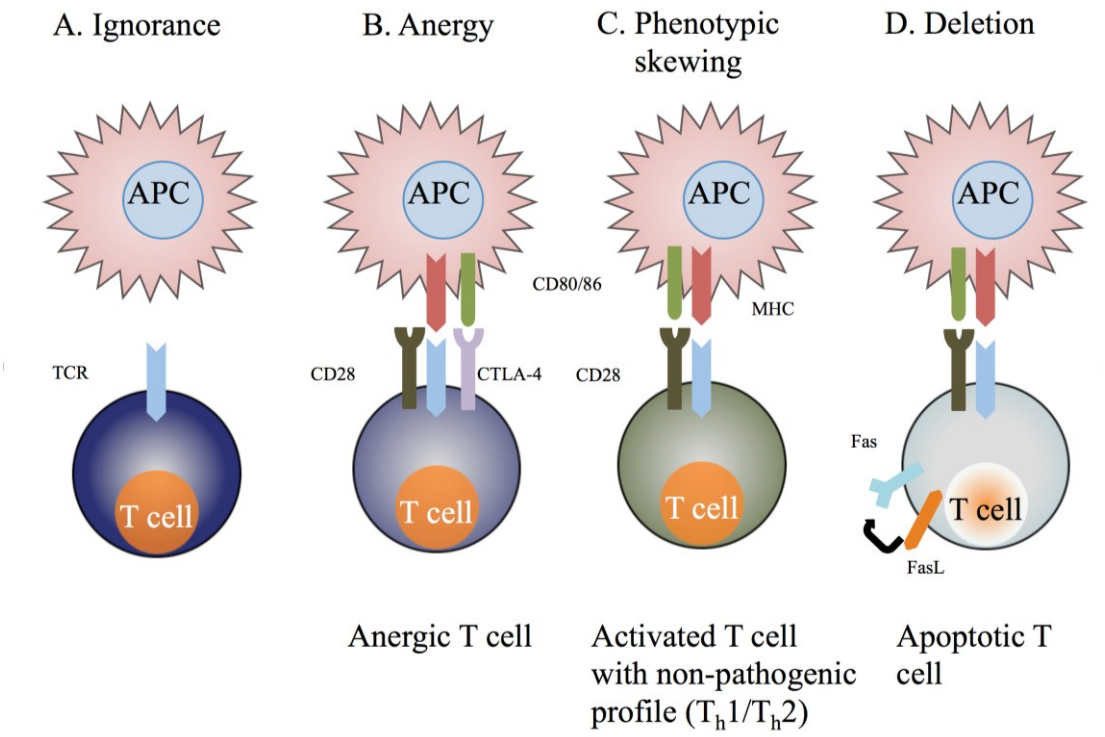


Figure 1-3: Cell intrinsic peripheral tolerance mechanisms
A. Ignorance: T cell is unable to access antigen presented in the context of self-MHC. B. Anergy: T cells are intrinsically impaired in their ability to response to antigen encounter but persist and survive in this state. C. Phenotypic skewing: T cells are activated by antigen however T cell function is modulated by cytokines. D. AICD: T cells are activated but rapidly undergo apoptosis.

There are two primary mechanisms of T cell extrinsic peripheral tolerance: tolerogenic DCs and T_{reg} cells. There are a wide variety of DCs capable of interacting in different contexts with immune cells. Tolerogenic DCs can control self-reactive T cells by modulating T cell activation. Tissue-resident immature DCs can readily present antigens, however due to low expression of co-stimulatory molecules or the absence of an inflammatory cytokine milieu, they are poorly immunogenic (5, 117). Tolerogenic DCs are a heterogeneous population, inducible by a variety of models from a range of DC subsets, with an assortment of mechanisms of action (117). It is thought that PAMP recognition or danger signals might be required to induce DC maturation (118, 119). Certain subsets of DCs in the intestinal lamina propria and mesenteric lymph nodes **(LN)** can uptake dying cells and present cellular antigens, ultimately inducing tolerance in $CD8^+$ T cells (120, 121).

In addition to being generated in the thymus by agonist selection, T_{reg} cells can be induced in the periphery, in part by tolerogenic DCs (117). Just as there are a large variety of tolerogenic DCs, there is a similarly wide array of T_{reg} cells beyond the prototypical $CD4^+CD25^+Foxp3^+$ T_{reg}, including $Foxp3^+$ CD8 and $Foxp3^-$ populations (117, 122). T_{reg} cells can modulate immune responses by directly targeting effector T cells or by suppressing the ability of APCs to activate effector T cells (123). T_{reg} can suppress responder T cells in several ways: through the secretion of cytokines such as IL-10, TGF-β and IL-35, to drive cell cycle arrest in responders; competing for IL-2; directly killing effector T cells; and expression of cell surface molecules that drive cell cycle arrest in effector T cells (123). Expression of molecules such as cytotoxic T lymphocyte-associated antigen 4 **(CTLA-4)**, lymphocyte activation gene 3 **(Lag-3),**

CD39 and neuropilin on T_{reg} cells allow them to modulated to ability of DCs to interact with effector T cells. CTLA-4 will compete for CD80/CD86 on DCs and impair CD28 co-stimulation of effector T cells. Lag-3 can bind to MHC-II and impair DC maturation and antigen presentation. CD39 can break down extracellular ATP, which has an inflammatory effect on DCs. Neuropilin promotes extended interactions between T_{reg} cells and DCs, impairing the ability of DCs to present antigens to effector T cells (123). These peripheral tolerance mechanisms often overlap and complement one another, highlighting the complexity and redundancies included in mammalian immune system.

TCR Transgenic Mice and T cell development

The development of TCR transgenic mice was a major boon in the study of T cell development and biology. Examining T cell development through a polyclonal population is difficult due to the very reason the T cell repertoire is so effective at immune surveillance: the astronomical potential for TCR specificities. Through introduction of pre-rearranged *Tcra* and *Tcrb* genes to the germ line, T cells within TCR transgenic mice express a TCR of defined specificity, allowing for the study of T cell biology in the context of a single specificity to a defined antigen. For thymocyte development, this also allowed the examination of a clonal population in isolation to a positively or negatively selecting ligand both *in vitro* and *in vivo.* However, in classical TCR transgenic mice, expression of the mature α/β TCR begins early in development. This non-physiological expression of mature α/β TCR can affect the efficiency of β selection (124) and the progression of thymocytes through the normal developmental stages (125, 126). This non-physiological expression is particularly

pertinent to the study of negative selection. If the negatively selecting antigen is expressed as a UbA, early expression of the α/β TCR can mediate early deletion between the DN and DP stage of development (39). However delaying the expression of the TCRα chain until the DP stage or limiting expression of the negatively selecting antigen to the medulla can restore physiological deletion between the DP and SP stages (46, 126).

For the study of thymocyte development to TRA, the OT-I transgenic TCR and RIPmOVA systems are well characterized (46). The OT-I TCR is comprised of the Vα2 and Vβ5 TCR chains and is specific for the SIINFEKL peptide of chicken ovalbumin **(OVA)** in the context of H-2K^b (127). The RIPmOVA mouse expresses a membrane-bound form of OVA consisting of the first 118 residues of the human transferrin receptor, including the cytoplasmic tail/anchor domain, linked to residues 139-385 of OVA under control of the rat insulin promoter **(RIP)**(128). The RIPmOVA mouse is an excellent model for the study of negative selection to TRA for several reasons: this promoter has been shown to be active in mTECs (129); OVA is membrane bound, restricting OVA encounter to mTECs or cross-presenting APCs in the thymus and thus limiting any confounding effects soluble antigen may have; thymic expression of OVA and not peripheral OVA from the blood or migrating APCs mediates deletion of OVA specific T cells (130); finally, development of OT-I thymocytes in chimeras generated from OT-I bone marrow **(BM)** transplanted into lethally irradiate RIPmOVA recipients (OT-I→RIPmOVA) has been well characterized and deletion is known to occur after positive selection in the DP stage (46).

Rationale

Given the importance of Bim to thymocyte apoptosis (96) and the onset of autoimmunity in its absence (94, 97, 98), various studies have examined the role of Bim in clonal deletion but have found Bim to be not essential for negative selection. (99-101). However, these studies examined only negative selection to UbA or superantigens and, in some cases, used classical TCR transgenics that result in early expression of the TCR. Given the differences in anatomical location and thymocyte developmental stages, differential molecular requirements for negative selection to TRA and UbA may reconcile these conflicting reports. The importance of negative selection to TRA is overlooked in these studies and is highlighted when the expression of many TRAs in the thymus is compromised by mutations in *AIRE,* leading to the onset autoimmune disorders such as APECED (31). To characterize the role of Bim in negative selection to TRA, we will examine the effects of Bim deficiency in OT-I Bim$^{-/-}$→RIPmOVA BM chimeras.

In addition to clonal deletion, the primary mechanism of negative selection, there exists other mechanisms, thymic and peripheral, capable of controlling self-reactive T cells. In the event of impaired of clonal deletion, these mechanisms might be able to compensate and control the self-reactive T cells.

Hypothesis

Bim is required for clonal deletion of thymocytes to TRA. In the absence of clonal deletion, high-affinity antigen encounter in the thymus will still induce non-deletional mechanisms negative selection. These mechanisms will maintain immunological tolerance in the periphery.

Research Objectives

1. Determine if Bim is required for negative selection to TRA
2. Examine how Bim deficiency in a model of negative selection to TRA affects T cell function

Chapter 2: Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute and The Jackson Laboratory. C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA) mice (128), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice (131), C57BL/6-Tg(CAG-OVA)916Jen/J (Act-mOVA) mice (132) and B6.Cg-FoxN1^{nu}/J (nude B6) mice were purchased from The Jackson Laboratory. OT-I Bim$^{-/-}$ mice were kindly provided by Dr. Maureen McGargill (St. Jude Children's Research Hospital, Memphis, TN). All mice, except Act-mOVA, were bred and maintained in our colony at the University of Alberta, treated in accordance with protocols approved by the University of Alberta Animal Care and Use Committee.

Bone Marrow Chimeras

Donor mice were injected i.p. with 100 µg of purified anti-Thy1.2 Ab (clone 30H12) on days -2 and -1 prior to BM harvest to deplete T cells. BM from the femur, tibia and humerus was harvested in EasySep Media (PBS, 2% FCS, 2mM EDTA) and was passed through 70µm nylon cell strainers (FisherBrand). Recipient mice were irradiated twice at 500 Gy 4 hours apart to lessen the impact of irradiation on the gastrointestinal tract. Between 5 x 10^6 to 1 x 10^7 BM cells were injected into the tail vein of recipient mice. Mice were provided with antibiotic water (40 mg neomycin, 15 mg polymyxin per 1L) for 4 weeks post injection in order to protect chimeras from infection. While this would likely negative affect the microbiota of the BM

chimeras, all mice were treated with the same regiment. Mice were allowed to reconstitute for at least 8 weeks before analysis.

Thymus Transplant Chimera

Thymi were removed from 1- to 3- day old neonatal RIPmOVA or B6 mice and were then cultured for 7 to 10 days on filters (MF-Millipore Membrane Filter, mixed cellulose esters, Hydrophilic, 0.45 µm, 13 mm, white, gridded, HAWG01300) placed on 70µm nylon mesh cell strainers (Fisher, #22363548), placed within the wells of a 6 well plate containing 1.35 mM 2'-deoxyguanosine (2-dG; Sigma-Aldrich) in complete DMEM-10 (DMEM containing 4.5 g/l glucose, 0.584 g/l L-glutamine and 3.7 g/l NaHCO3) for the depletion of hematopoietic cells from the thymus. Media was changed as needed during the culture. Thymi were then washed for 2 hours in DMEM-10 before transplantation under the kidney capsule of nude B6 mice. Reconstitution of the T cell compartment was confirmed by the presence of $CD4^+$ and $CD8^+$ T cells in peripheral blood, 8 weeks after transplant. Mice were allowed to age at least 4 months before generating BM chimeras as previously described.

Antibodies and flow cytometry

Antibodies (Ab) used are listed in Table 1. Cells were stained with Ab cocktails in FACS buffer (PBS, 1% FCS, 0.02% sodium azide) for 30 minutes on ice. Cells were washed twice with FACS buffer between primary and secondary staining cocktails. Cells were treated with the BD Fix/Perm kit (BD Biosciences) for intracellular staining for active caspase 3 or intracellular cytokines. Cells were treated with the Foxp3 staining kit (eBiosciences) for intracellular staining for Foxp3, Egr2, Helios,

and c-Myc. Cell events were collected on a FACSCanto II (BD Biosciences) or BD Fortessa SORP (BD Biosciences) and analysed by FlowJo software (Tree Star).

Table 1: Antibodies

Antigen	Fluorochrome	Clone	Company	Cat. No.
BTLA	Biotin	8F4	eBiosciences	13-5956
CD3e	Functional	145 2C11		
CD4	PerCp-eFluor710	RM4-5	eBiosciences	46-0042
CD4	BV650	RM4-5	BD Biosciences	563747
CD8a	Various	53-6.7	eBiosciences	XX-0081
CD8a	BV711	53-6.7	BD Biosciences	563046
CD24	eFluor 450	M1/69	eBiosciences	48-0242
CD25	Various	eBio7D4	eBiosciences	53-0252
CD28	PE-Cyanine7	37.51	eBiosciences	25-0281
CD28	Functional	37.51	eBiosciences	16-0281
CD44	Various	IM7	eBiosciences	XX-0441
CD45.1	APC-eFluor780	104	eBiosciences	47-0454
CD45.2	PE-Cyanine7	A20	eBiosciences	25-0453
CD62L	Various	MEL-14	eBiosciences	XX-0621
CD69	Various	H1-2F3	eBiosciences	XX-0691
CD103	Biotin	2E7	eBiosciences	13-1031
CD127	Various	A7R34	eBiosciences	XX-1271
c-Myc		D84C12	Cell Signaling	5605S
Cleaved Caspase-3(D175)			Cell Signaling	9661L
CTLA-4	Biotin	UC10-489	eBiosciences	13-1522
Egr2	PE	erongr2	eBiosciences	12-6691
Foxp3	Alexa Fluor 647	FJK-16s	eBiosciences	51-5773
GITR	Biotin	DTA-1	eBiosciences	13-5874
Helios	APC	22F6	eBiosciences	17-9883
IFNγ	Various	XMG1.2	eBiosciences	XX-7311
Nur77	PE	12.14	eBiosciences	12-5965
PD-1	PerCP-eFluor710	J43	eBiosciences	46-9985
Streptavidin	Various		eBiosciences	XX-4317
Streptavidin	BV711		BD Biosciences	563262
Tim-3	Bio	8B.2C12	eBiosciences	13-5871
TNFα	APC	MP6-XT22	eBiosciences	17-7321
Vα2	Various	B20.1	eBiosciences	XX-5812
Vβ5	PE	MR9-4	BD Biosciences	553190

Proliferation assay

Stimulator splenocytes were harvested from WT B6 or B6 Ly5.1/5.2 mice, washed and resuspended at 20 x 10^6 cells/mL in pure FCS and pulsed with 100nM SIINFEKL peptide at 37°C for 1 hour with gentle shaking every 15 – 20 minutes. Stimulators were washed with three times with 2% FCS – PBS and resuspended in RP10 (RPMI, 10% FCS, 5 mM HEPES, 50U(mg)/mL penicillin/streptomycin, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 50 mg/mL gentamicin sulfate) at 2 x 10^7 cells/mL. Non tissue culture treated plates were prepared with 10 µg/mL of α-CD3 and 5 µg/mL of α-CD28 overnight at 4°C. Effectors were harvested from indicated mice, washed and resuspended in sterile PBS at 1 x 10^7 (for CFSE staining) or 5 x 10^6 (For CTV staining) cells/mL. For CFSE staining, 1 µL of a 1.25 mM solution of CFSE in DMSO was added per 1 x 10^7 cells, which were then incubated for 10 minutes at 37°C with regular mixing. Staining was quenched with 4 volumes of RP10 and cells were washed once in RP10 and the cells were resuspended in RP10 to 1 x 10^7 cells/mL. For CTV staining, 1 µL of 5mM CTV solution in DMSO was added per 5 x 10^6 cells for a final concentration of 5µM. Cells were stained for 20 minutes at 37°C with regular mixing. Staining was quenched with at least 4 volumes of RP10. Cells were washed once in RP10 and the cells were resuspended in RP10 to 1 x 10^7 cells/mL. Effectors were mixed with stimulators at a ratio of 4:1 and allowed to incubate at 37°C for the indicated time points in the presence or absence of IL-2 (5 U/mL) or effectors were added to α-CD3 and α-CD28 treated plates at 2.5 x 10^6 cells/mL. Division and proliferation indices were calculated by Flowjo software (Tree Star). The division

index represents the average number of divisions all cells within the population undergo. The proliferation index represents that average number of divisions that only proliferating cells undergo.

Cytokine Assay

Effectors and stimulators were treated as above, but without CFSE labeling, and mixed at a ratio of 4:1 in RP10. On day 3 post-stimulation, cells were washed and resuspended in RP10 plus 1ng/mL IL-2. On day 5, effectors were restimulated with freshly pulsed stimulators at a 4:1 ratio or restimulated with 100 nM OVA peptide. 3 µg of Brefeldin A per 1 mL of culture volume was added to each culture 4 hours prior to harvest and analysis. Cytokines where detected by internal staining and flow cytometry.

Blood glucose levels

Blood glucose levels were determined using a OneTouch® UltraMini® system with OneTouch® Ultra® Test Strips. Blood samples (~5 µL) were obtained by tail vein bleeding. Readings were taken once per week and mice were considered diabetic when blood glucose levels exceeded 300mg/dL for 2 consecutive weeks. Blood glucose levels were measured for 8 to 14 weeks after BM graft.

Diabetes induction experiments

5 x 10^6 magnetic bead purified (StemCell Technologies) $CD8^+$ T cells from OT-I $Bim^{-/-}$ →RIPmOVA or OT-I $Bim^{-/-}$→B6 chimeras were injected into sublethally irradiated

(500 Gy) RIPmOVA or B6 recipients. Blood glucose levels were measured as described every 2 days. Mice were considered diabetic when blood glucose levels exceeded 300 mg/dL for 2 consecutive readings. Mantel-Cox *p* value was calculated by using Prizm Software (GraphPad Software).

Lymphopenia induced proliferation

Cells were isolated from the lymph nodes or thymi of OT-I Bim$^{-/-}$→RIPmOVA or OT-I Bim$^{-/-}$→B6 chimeras. 2 x 10^6 CFSE or CTV labeled lymph node T cells or thymocytes were injected by tail vein injection into sublethally (500 Gy) irradiated or unirradiated congenic B6 hosts. Spleens and lymph nodes were harvest on day 7 post injection and analyzed by flow cytometry for CFSE or CTV dilution.

STAT5 phospho-flow

Cells were isolated for the spleens and thymi from OT-I Bim$^{-/-}$→RIPmOVA or OT-I Bim$^{-/-}$→B6 chimeras. Cells were washed and resuspended in serum free RPMI at 1 x 10^7 cells/mL. 1 x 10^6 cells were placed per well in a 96 well plate and allowed to rest for 20 minutes at 37°C. Cells were then stimulated with 25 ng/mL of IL-7 for 20 minutes. BD Phosflow Lyse/Fix (Cat. No.: 558049) and BD Phosflow Perm Buffer III (Cat. No.: 558050) were prepared as instructed. Cells were surfaced stained for 15 minutes on ice, then fixed for 10 minutes at 37°C in 200 µL of 1x BD Phosflow Lyse/Fix buffer. Cells were washed 2 times with 100 µL FACS buffer (10 minutes @ 250 x *g*) and the FACS buffer was aspirated. The pellet was loosened by gentle vortexing and ice cold BD Phosflow Perm Buffer III was slowly added drop by drop to

permeabilize cells. Cells were then incubated on ice for 30 minutes. Cells were washed 2 times with 100 µL of FACS buffer and then stained with Alexa Fluor 647 Mouse anti STAT5 (pY694) antibody (BD biosciences Cat. No.:612599).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were harvested from the spleens and lymph nodes of OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras. Samples were enriched for CD8^{+} T cells using magnetic beads (StemCell Technologies). RNA was isolated from 1 x 10^{6} unstimulated CD8^{+} enriched cells from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras on day 0. 2.5 x 10^{6} CD8^{+} enriched cells from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras were stimulated with plate-bound α-CD3/α-CD28 mAb as previously described for 3 days. Cells were transferred to an uncoated well for 2 days before being restimulated with 100nM OVA peptide on day 5. RNA was then harvested using RNeasy® Plus Mini Kits (QIAGEN, Cat. No. 74134) from restimulated cells from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras. cDNA was generated from RNA samples using SuperScript® III first-strand synthesis supermix for qRT-PCR (Invitrogen, Cat. No.: 11752-050). qRT-PCR was performed using a Mastercyler® ep *realplex* 2S (Eppendorf) with PerfeCTa® SYBR® Green FastMix® (Quanta, Cat. No.: 95072-250) and primers listed in Table 2-2. Results were normalized to β-actin expression in each sample. Samples from the same experiment, stimulated in wells coated by the same antibody mixture were examined as pairs.

Table 2: qRT-PCR primers	
IFNγ spliced 5'	ACT GGC AAA AGG ATG GTG AC
IFNγ spliced 3'	GCT GAT GGC CTG ATT GTC TT
TNFα 5'	TGG CCT CCC TCT CAT CAG TT
TNFα 3'	TCC TCC ACT TGG TGG TTT GC

Statistical Analysis

Mean, SD and 2-sided paired Student's t-test *p* values were calculated using Prizm Software (GraphPad Software).

Chapter 3:
Bim is required for clonal deletion to tissue restricted antigens

Introduction

The pro-apoptotic BH3 only Bcl-2 family member Bim is induced by negative selection (77, 94) and is essential for thymocyte apoptosis (95). Onset of autoimmune disorders in Bim-deficient mice supported the initial assertion that Bim-mediated apoptosis was critical for negative selection (94, 96-98). However, recent studies examining SA-mediated (101) and UbA-mediated (99, 100) clonal deletion suggest that while Bim is required for thymocyte apoptosis, it is not required for clonal deletion. However, these models did not examine clonal deletion to TRA. Unlike negative selection to UbA, which occurs in the cortex without medullary involvement (33), negative selection to TRA requires positive selection and migration of thymocytes to the medulla (45). The differences in anatomical location and the development stage of thymocytes during negative selection to these two classes of antigen may result in differential molecular requirements for negative selection. As these UbA models are not easily manipulated to examine clonal deletion to TRA, we sought to utilize the OT-I TCR transgenic system. OT-I thymocytes express a TCR specific for the SIINFEKL peptide of OVA in the context of H-2K^b and can be detected using an antibody against the Vα2 TCRα chain of the OT-I TCR. Furthermore, mice that express membrane OVA ubiquitously or in a tissue restricted fashion are available.

We generated BM chimeras using mice expressing membrane bound chicken ovalbumin (mOVA) under control of the chicken β-actin promoter (Act-mOVA) (132)

or rat insulin promoter (RIP-mOVA)(128). Act-mOVA mice express membrane bound OVA on the surface of all cells in the body, allowing the study of the OT-I system in a model of negative selection to UbA. RIP-mOVA chimeras express membrane bound OVA in the pancreas and mTECs in the thymus generating a model of negative selection to TRA for the OT-I system. Control chimeras were generated with B6 mice to examine the role of Bim in a non-deleting model for the OT-I TCR (Fig 3-1).

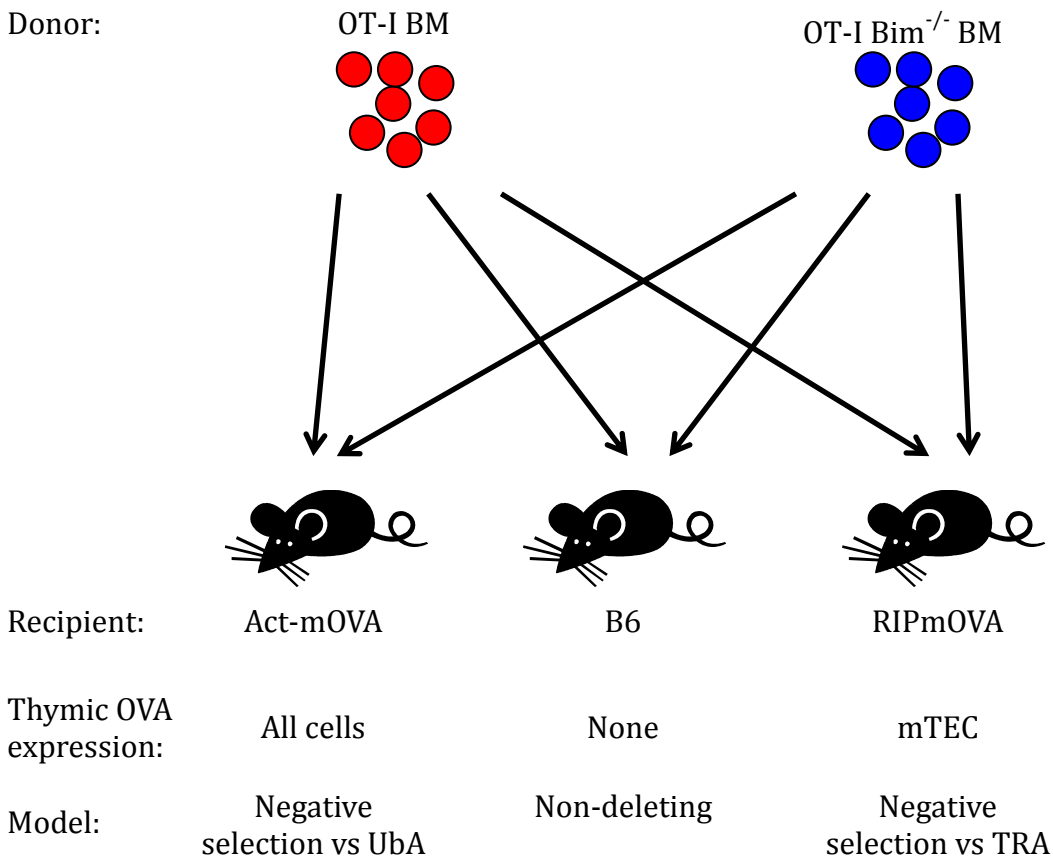


Figure 3-1: OT-I bone marrow chimera models to study the role of Bim in negative selection
Donors were depleted of T cells by i.p. injection with 100 µg of purified anti-Thy1.2 (clone 30H12) on days -2 and days -1. BM was harvested and between 5 x 10^6 to 1 x 10^7 BM cells were injected into recipients lethally irradiated with two doses of 500 Gy

Results

Bim is not required for clonal deletion to UbA

To ensure that clonal deletion of OT-I thymocytes to UbA was Bim independent, we generated BM chimeras using Act-mOVA recipients and either OT-I or OT-I $Bim^{-/-}$ BM donors.

We found that Bim deficiency did not affect the frequency of $V\alpha2^+$ thymocytes recovered from the thymi of OT-I $Bim^{-/-}$→B6 chimeras or OT-I→B6 chimeras compared to an intact OT-I mouse (**Fig. 3-2A)**. In both OT-I $Bim^{-/-}$→B6 chimeras and OT-I→B6 chimeras, DP, $CD4^+CD8^{int}$, and CD8SP thymocyte percentages of $V\alpha2^+$ thymocytes were similar **(Fig. 3-2B)**. These gates were drawn using thymocytes from an intact B6 control mouse. Compared to both B6 chimeras and intact OT-I mice, the Act-mOVA chimeras displayed a paucity of antigen specific $V\alpha2^+$ thymocytes **(Fig. 3-2A)**.

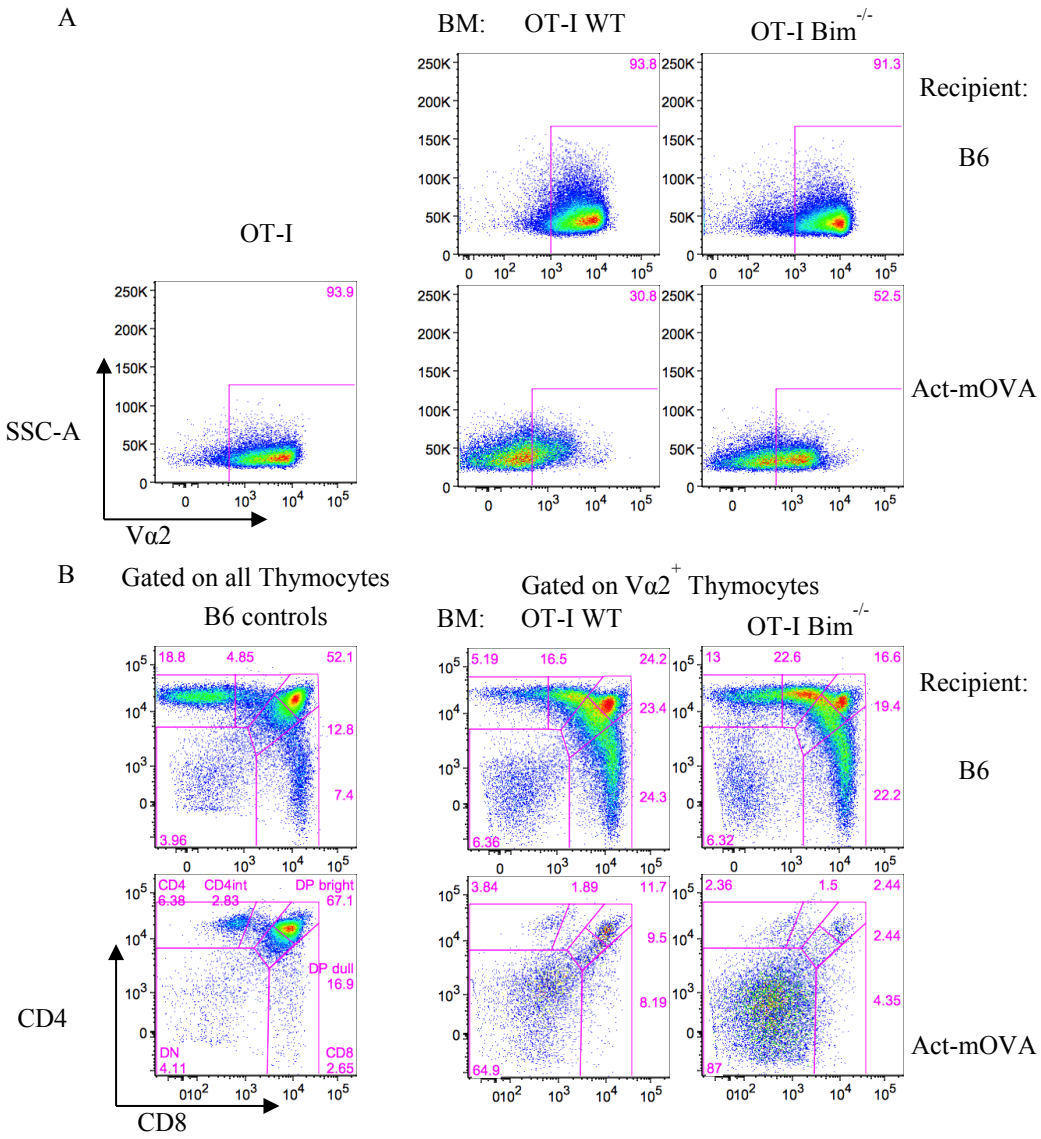


Figure 3-2: Bim is not required for clonal deletion to ubiquitous antigen.
A. Vα2 by side scatter profile of thymocytes from OT-I→B6 (n = 12), OT-I→Act-mOVA (n = 3), OT-I Bim-/-→B6 (n = 46) and OT-I Bim-/-→Act-mOVA (n = 3). **B.** CD4 by CD8 profile of thymocytes from control intact B6 mice and Vα2+ thymocytes from indicated chimeras. Data is representative of the indicated number of experiments.

Additionally, in both Act-mOVA chimeras, the majority of $V\alpha2^+$ thymocytes were $CD4^-CD8^-$ double negative (DN) with reductions in DP, $CD4^+CD8^{int}$, and CD8SP populations, indicating deletion of OT-I thymocytes (**Fig. 3-2:B)**. The increased percentage of DN thymocytes was more pronounced in the OT-I $Bim^{-/-}$→Act-mOVA chimeras. Furthermore, both Act-mOVA chimeras demonstrated a reduction in the proportion of mature $CD24^{lo}$, $V\alpha2^+$, CD8SP thymocytes, compared to the B6 chimeras **(Fig. 3-3A).** Bim deficiency did not affect the maturation of thymocytes from OT-I and OT-I $Bim^{-/-}$→B6 chimeras, as both showed similar proportions and numbers of mature $CD24^{lo}V\alpha2^+$ thymocytes. **(Fig 3-3A and B).** There was a similar reduction in $V\alpha2^+$ DP and $V\alpha2^+CD24^{lo}$ CD8SP cell numbers in OT-I→Act-mOVA compared to OT-I→B6 and in OT-I $Bim^{-/-}$→Act-mOVA compared to OT-I $Bim^{-/-}$→B6 chimeras (**Fig. 3-3B)**.

The similarities in cell numbers and frequencies of thymocytes between OT-I→B6 and OT-I $Bim^{-/-}$→B6 chimeras indicate that Bim deficiency alone does not impact the development of thymocytes. The reduction in frequency of $V\alpha2^+$ thymocytes and the reduction in cell numbers of $V\alpha2^+CD8^+CD24^{lo}$ and $V\alpha2^+DP^{bright}$ thymocytes in OT-I→Act-mOVA and OT-I $Bim^{-/-}$→Act-mOVA chimeras clearly demonstrate deletion of OVA-specific thymocytes.

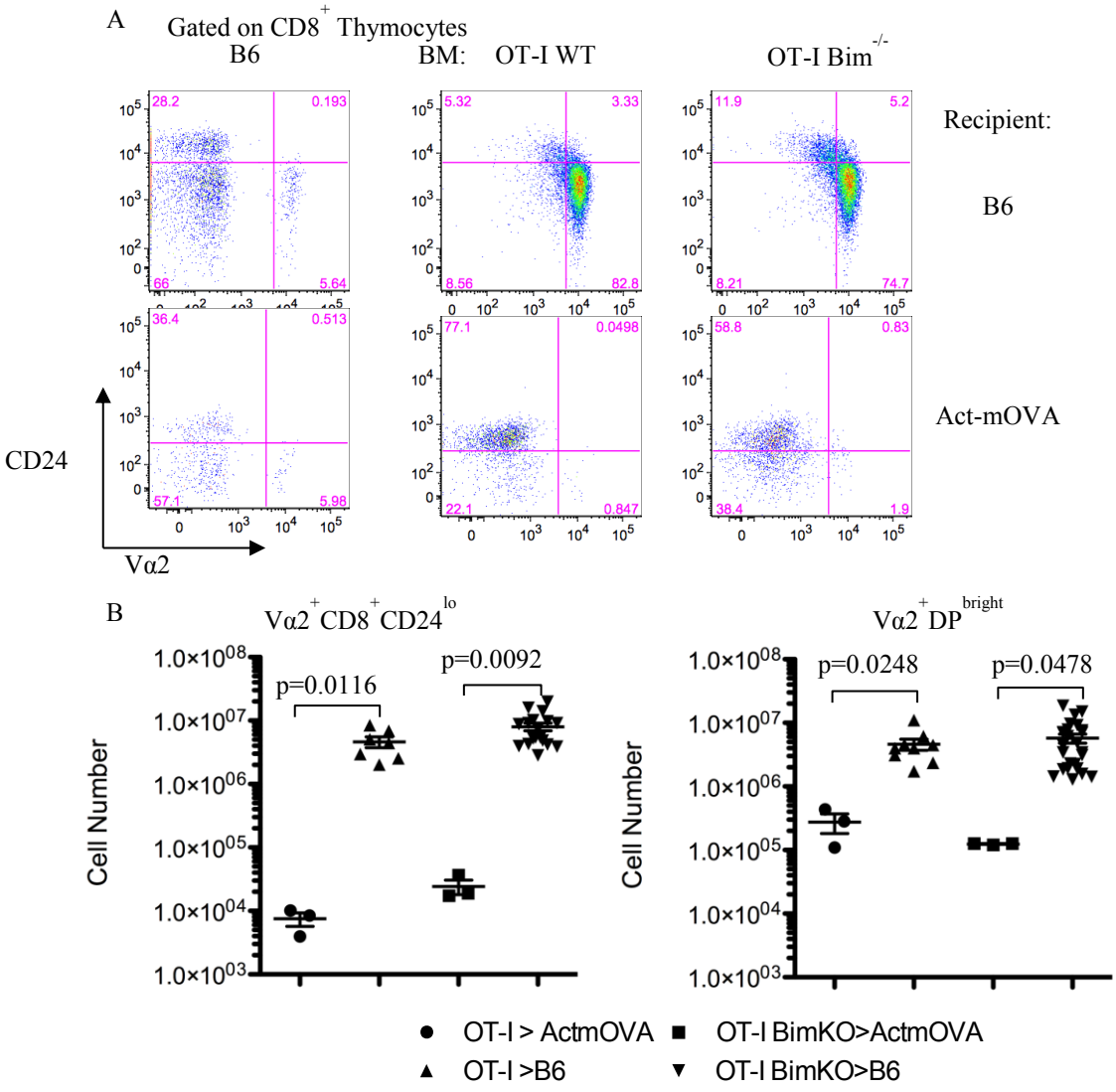


Figure 3-3: Clonal deletion to UbA can occur in a Bim-independent manner
A. CD24 by Vα2 profile of CD8+ thymocytes from OT-I→B6 (n = 12), OT-I→Act-mOVA (n = 3), OT-I Bim-/-→B6 (n = 46) and OT-I Bim-/-→Act-mOVA (n = 3). **B.** Number of mature Vα2+CD24lo CD8SP and Vα2+ DP thymocytes from OT-I→B6 (n = 7), OT-I→Act-mOVA (n = 3), OT-I Bim-/-→B6 (n = 18) and OT-I Bim-/-→Act-mOVA (n = 3).

Deletion occurs early at the DN stage in OT-I→Act-mOVA chimeras

As Bim is involved in the intrinsic arm of apoptosis, which ultimately results in the cleavage and activation of caspase 3, we examined caspase 3 cleavage in Vα2$^+$ thymocytes to determine if deletion was occurring in a Bim-independent manner. Anti-cleaved caspase 3 staining showed significant caspase 3 activity in Vα2$^+$ thymocytes from OT-I→Act-mOVA chimeras and OT-I→B6 chimeras. In contrast, anti-cleaved caspase 3 staining was dramatically reduced in Vα2$^+$ thymocytes from both OT-I Bim$^{-/-}$→Act-mOVA and OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 3-4A)**. Cells positive for cleaved caspase 3 in OT-I→Act-mOVA chimeras were mainly DN whereas those from OT-I→B6 chimeras were primarily DP^{dull} and CD4$^+$CD8^{int} **(Fig. 3-4B)**.

The detection of cleaved caspase 3 in Vα2$^+$ thymocytes from OT-I→B6 and OT-I→Act-mOVA chimeras, but not the OT-I Bim$^{-/-}$ counterparts, indicates that Bim is actively taking part in selection processes in the thymus. The absence of cleaved caspase 3 staining in Vα2$^+$ thymocytes from OT-I Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$→Act-mOVA demonstrates that caspase 3 cleavage is Bim dependent. Regardless of Bim expression, and subsequent caspase 3 activation, OT-I thymocytes were deleted in Act-mOVA chimeras.

The increased frequency of DN thymocytes and reduced frequency of DP thymocytes in combination with the enrichment of DN thymocytes in the cleaved caspase 3$^+$ compartment chimeras suggests that clonal deletion is occurring at the DN stage in OT-I→Act-mOVA. Even with the absence of a substantial cleaved caspase 3$^+$ population, OT-I Bim$^{-/-}$→Act-mOVA thymocytes show a similar distribution to OT-

I→Act-mOVA chimeras. This suggests that Bim-independent mechanisms are able to mediate clonal deletion. In contrast to the OT-I→Act-mOVA chimera, $V\alpha2^{+}$ cleaved caspase 3^{+} thymocytes from OT-I→B6 chimeras are primarily in the DP^{dull} and $CD4^{+}CD8^{int}$ stage, suggesting that deletion is occurring after positive selection.

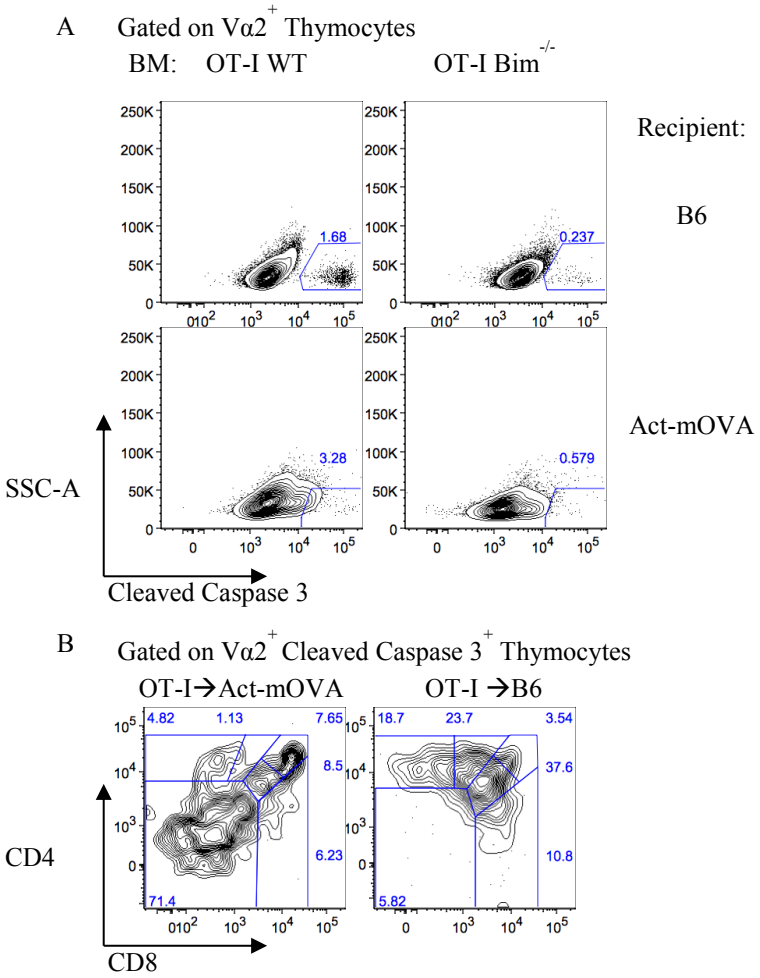


Figure 3-4: Caspase 3 activation suggests early deletion in OT-I→Act-mOVA chimeras
A. Active cleaved caspase 3 profile of Vα2+ thymocytes from OT-I→B6 (n = 7), OT-I Bim$^{-/-}$→B6 (n = 8), OT-I→Act-mOVA (n = 3) and OT-I Bim$^{-/-}$→Act-mOVA (n = 3) chimeras. **B.** CD4 by CD8 profile of Vα2+ cleaved caspase 3+ thymocytes. Data is representative of the indicated number of samples.

OT-I T cells are not detected in the periphery of Act-mOVA chimeras

Splenocytes were examined for the presence of OT-I T cells to ensure that deletion in the thymus was complete and that no, or few OT-I T cells egressed into the periphery. OT-I→B6 and OT-I Bim$^{-/-}$→B6 chimeras had a similar frequency and number of Vα2^{+}CD8^{+} T cells as well as a similar number of total Vα2^{+} T cells **(Fig. 3-5A and B)**. In contrast, there was a marked reduction in numbers of total Vα2^{+} T cells as well as a reduction in numbers and frequency of Vα2^{+}CD8^{+} T cells in the spleen of OT-I→Act-mOVA and OT-I Bim$^{-/-}$→Act-mOVA chimeras compared to their B6 counterparts **(Fig. 3-5A and B)**.

The absence of OT-I T cells from the spleens of OT-I→Act-mOVA and OT-I Bim$^{-/-}$→Act-mOVA chimeras indicates that OT-I thymocytes are efficiently deleted in the thymus and few escape into the periphery of these chimeras.

These results recapitulate those observed in previous studies (99-101), demonstrating that the OT-I model is comparable to other models. They also provide further support that Bim is not required for clonal deletion to UbA and that Bim-independent mechanisms can compensate in the absence of Bim.

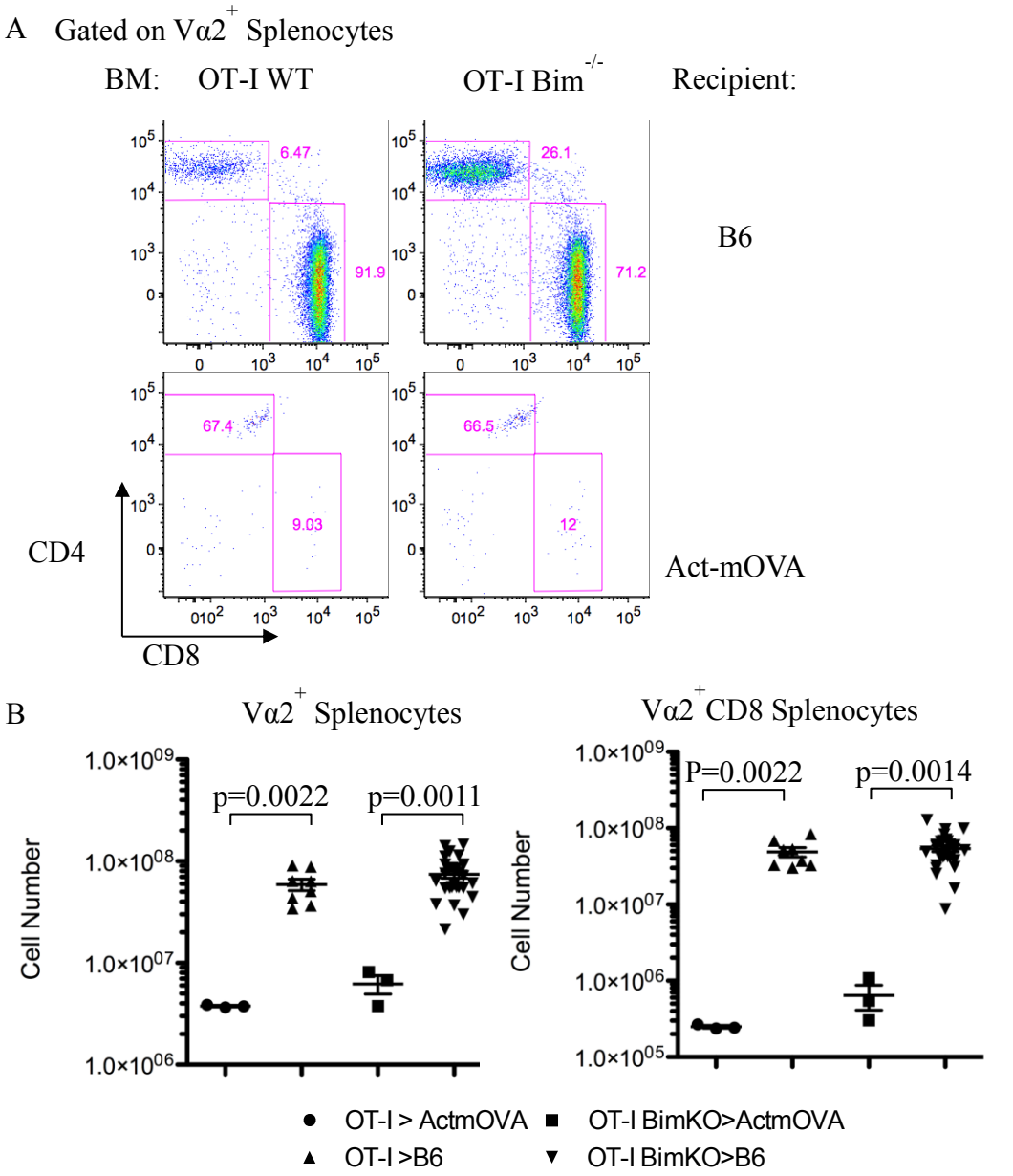


Figure 3-5: Antigen specific T cells are absent from the periphery of Act-mOVA chimeras
A. CD4/CD8 profile of Vα2+ T cells from OT-I→B6 (n = 12), OT-I→Act-mOVA (n = 3), OT-I Bim$^{-/-}$→B6 (n = 46) and OT-I Bim$^{-/-}$→Act-mOVA (n = 3) spleens. **B**. Number of Vα2+ and Vα2+CD8+ T cells from OT-I→B6 (n = 8), OT-I→Act-mOVA (n = 3), OT-I Bim$^{-/-}$→B6 (n = 27) and OT-I Bim$^{-/-}$→Act-mOVA (n = 3) spleens.

Bim is required for clonal deletion to TRA

Despite the lack of requirement for Bim in clonal deletion in the OT-I model and other models, the fact remains that Bim deficiency leads to autoimmune disorders (97). Whereas the previous models examined clonal deletion to UbA or SA they did not address clonal deletion to TRA, which differs with respect to the developmental stage of the thymocyte and anatomical location within the thymus. We hypothesized that these distinctions may result in a difference in the requirement for Bim in clonal deletion to TRA.

To generate a model of clonal deletion to TRA, we grafted OT-I or OT-I $Bim^{-/-}$ BM to RIP-mOVA recipients. The rat insulin promoter drives expression of mOVA in the pancreas, the kidney and in the medulla of the thymus (128). In this model, OT-I thymocytes should first undergo positive selection in the cortex before encountering their cognate antigen in the medulla. Thymocytes were harvested from the chimeras and examined for the presence of clonal deletion.

Unlike Act-mOVA chimeras, OT-I→RIPmOVA and OT-I $Bim^{-/-}$→RIPmOVA chimeras do not exhibit a reduction in $V\alpha2^+$ thymocytes, with similar frequencies to those seen in the B6 chimera **(Fig. 3-6A)**. The CD4/CD8 thymic profiles of $V\alpha2^+$ thymocytes from OT-I→B6 and OT-I $Bim^{-/-}$→B6 chimeras were not drastically altered by Bim deficiency (**Fig. 3-6B).** Whereas, the OT-I→RIP-mOVA chimeras displayed a significant reduction in the proportion of $V\alpha2^+$ CD8SP thymocytes with an increase in the DN compartment **(Fig. 3-6B)** consistent with previous data (133).

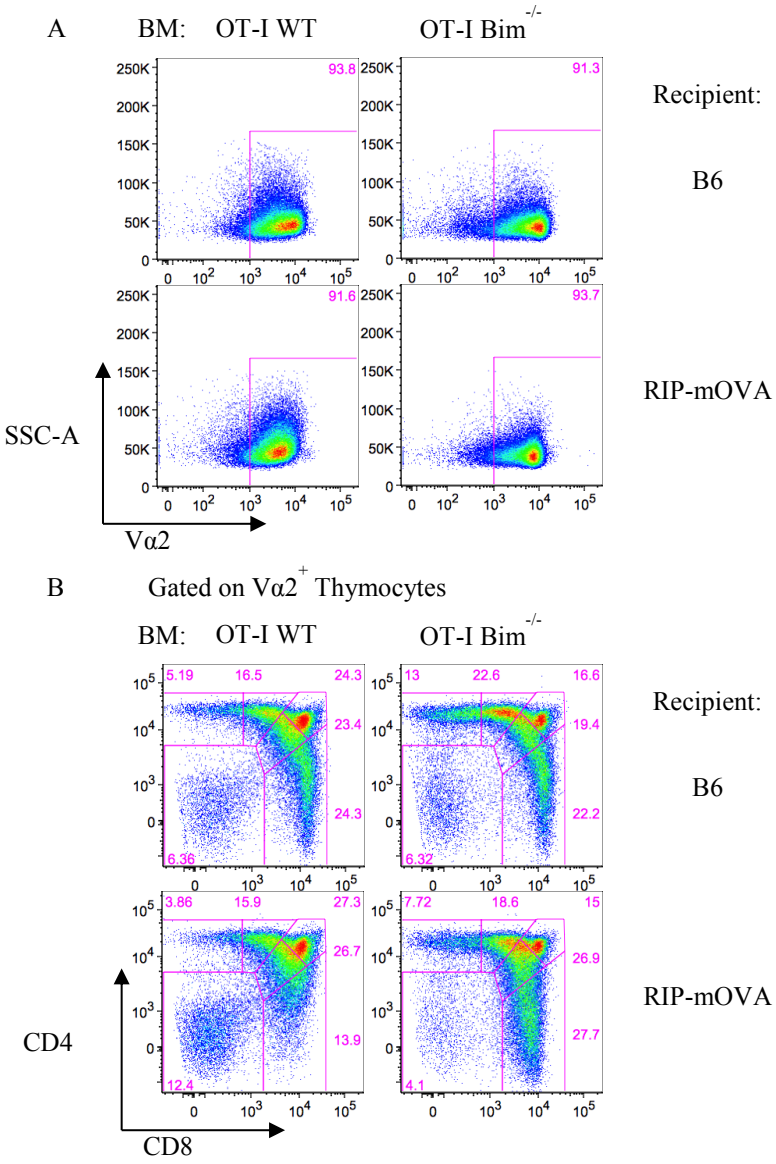


Figure 3-6: Bim is required for negative selection to TRA
A. Vα2 by side scatter profile of thymocytes from OT-I→B6 (n = 12), OT-I→RIPmOVA (n = 18), OT-I Bim$^{-/-}$→B6 (n = 46) and OT-I Bim$^{-/-}$→RIPmOVA (n = 46) chimeras. **B.** CD4 by CD8 profile of Vα2^{+} thymocytes from chimeras. Data is representative of the indicated number of samples.

In contrast, the OT-I Bim$^{-/-}$→RIP-mOVA chimeras showed no reduction in the frequency of Vα2^{+}CD8SP thymocytes compared to the OT-I Bim$^{-/-}$→B6 chimeras, suggesting that clonal deletion is not occurring (**Fig. 3-6B)**. The presence of a robust DP^{bright} population in OT-I Bim$^{-/-}$→RIPmOVA and OT-I→RIPmOVA chimeras suggest that in RIPmOVA chimeras, deletion is not occurring at the DN stage as observed with ActmOVA chimeras **(Fig. 3-2B)**.

Normally, thymocytes would not encounter TRA until after undergoing positive selection at the DP stage. Deletion of only the CD8SP population in OT-I→RIPmOVA chimeras indicates that deletion is occurring in a physiological manner after thymocytes have trafficked medulla where they will encounter OVA **(Fig. 3-6B).**

Interestingly, CD8 expression was slightly downregulated and Nur77 was slightly upregulated in the OT-I Bim$^{-/-}$→RIP-mOVA chimeras and may be indicative of high affinity antigen encounter **(Fig. 3-7A and B)**. The observation of both of these in Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras suggests that they have encountered OVA and that Bim deficiency is not affecting the presentation of OVA in the medulla **(Fig. 3-6 and Fig. 3-7)**.

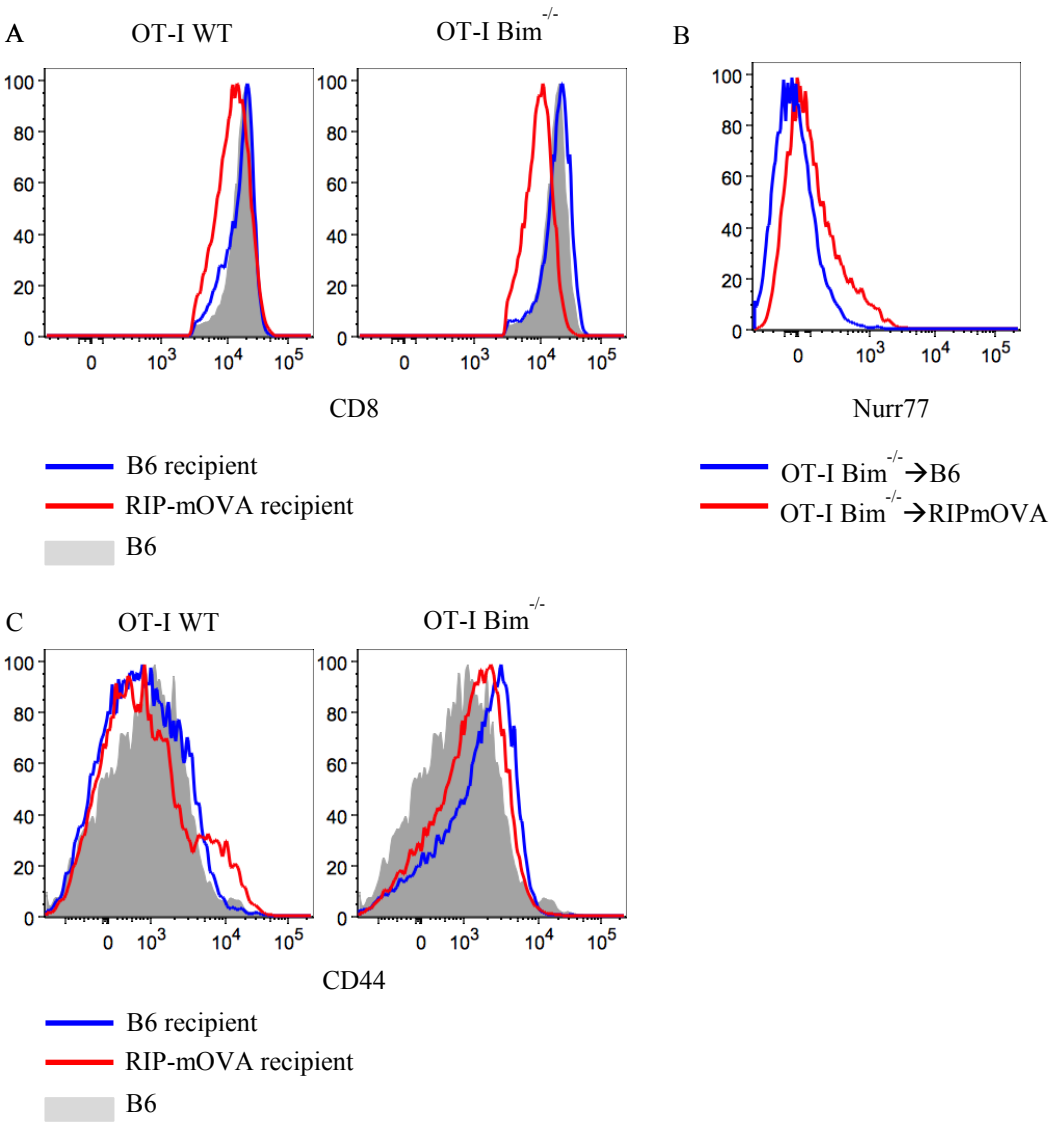


Figure 3-7: OT-I CD8SP thymocytes encounter antigen in the thymus
A. CD8 in Vα2⁺ CD8SP thymocytes. OT-I→B6 (n = 12), OT-I→RIPmOVA (n = 18), OT-I Bim$^{-/-}$→B6 (n = 46) and OT-I Bim$^{-/-}$→RIPmOVA (n = 46) chimeras **B**. Nur77 expression in Vα2⁺ CD8SP thymocytes. OT-I Bim$^{-/-}$→B6 (n = 5) and OT-I Bim$^{-/-}$→RIPmOVA (n = 5) chimeras **C.** CD44 expression in Vα2⁺CD8SP thymocytes. OT-I→B6 (n = 4), OT-I→RIPmOVA (n = 4), OT-I Bim$^{-/-}$→B6 (n = 8) and OT-I Bim$^{-/-}$→RIPmOVA (n = 8) chimeras

Vα2^{+}CD8SP thymocytes from both OT-I Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$→RIPmOVA chimeras appeared to slightly upregulate CD44 expression compared to OT-I WT chimeras **(Fig. 3-7C)**. However, CD44 expression in all chimeras was relatively similar to CD44 expression in an intact B6 mouse **(Fig. 3-7C).** This relatively low expression level suggests that Vα2^{+}CD8SP thymocytes are not overtly activated in the thymi of OT-Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6.

We also examined CD24 expression on Vα2^{+} CD8SP thymocytes to assess the maturity of the thymocytes. When compared to either OT-I or OT-I Bim$^{-/-}$→B6, OT-I→RIP-mOVA chimeras showed a considerable reduction in the proportion of mature, CD24^{lo}, Vα2^{+} CD8SP thymocytes while the proportion of mature OT-I thymocytes in OT-I Bim$^{-/-}$→RIP-mOVA chimeras was equivalent to that seen in OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 3-8A)**. Cell numbers reinforced these observations with OT-I and OT-I Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$→RIP-mOVA displaying similar absolute numbers of mature OT-I CD8SP thymocytes while the number of these cells was reduced approximately fifty fold in OT-I→RIP-mOVA chimeras compared to OT-I→B6 chimeras **(Fig. 3-8B)**. With the exception of the Vα2^{+} CD8SP compartment, the number of Vα2^{+} thymocytes in the other compartments was similar between OT-I→B6 and OT-I→RIPmOVA chimeras, indicating clonal deletion was occurring at the CD8SP stage **(Table I).**

The rescue of the mature Vα2^{+}CD24^{lo} CD8SP population by frequency and total numbers in OT-I Bim$^{-/-}$→RIPmOVA chimeras clearly demonstrates that Bim is essential for clonal deletion of TRA-specific thymocytes **(Fig. 3-6 and Fig. 3-8)**.

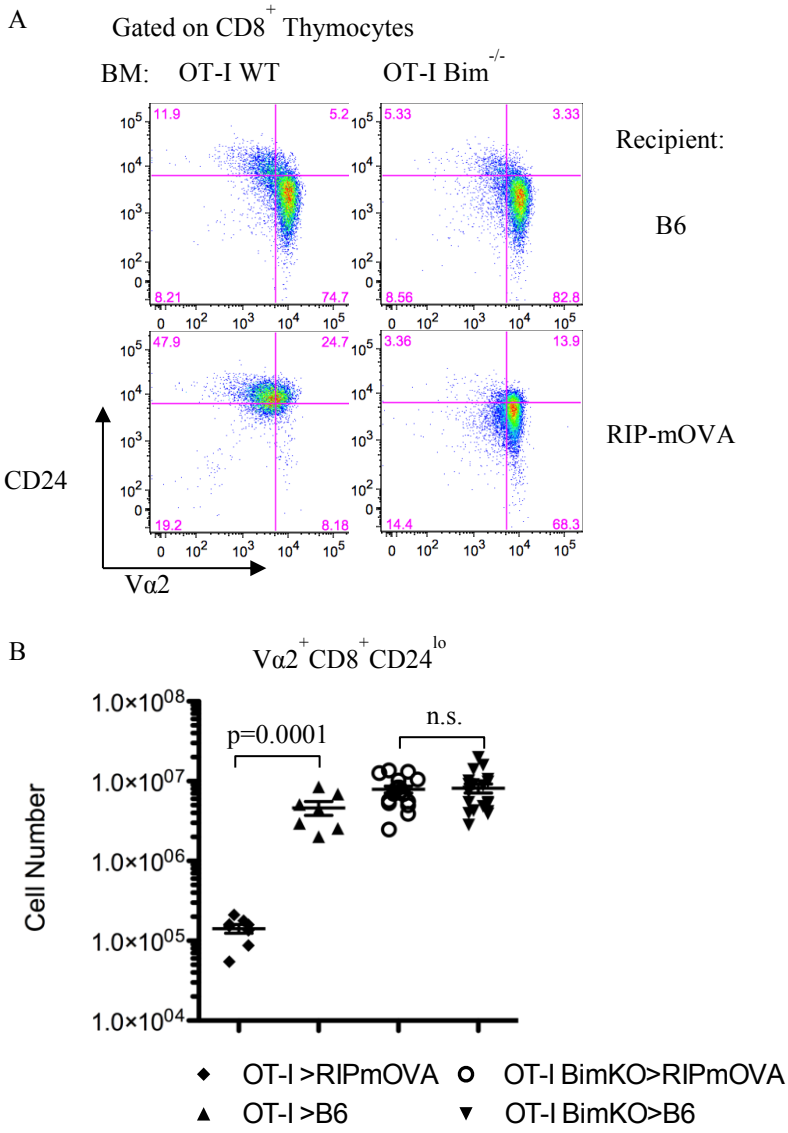


Figure 3-8: Bim deficiency rescues mature Vα2⁺CD8SP thymocytes from deletion
A. Vα2 by CD24 profile of CD8SP thymocytes from OT-I→B6 (n = 12), OT-I→RIPmOVA (n = 18), OT-I Bim-/-→B6 (n = 46) and OT-I Bim-/-→RIPmOVA (n = 46) chimeras**. B.** Cell numbers of mature Vα2⁺CD8⁺CD24^{lo} thymocytes from OT-I→B6 (n = 7), OT-I→RIPmOVA (n = 8), OT-I Bim$^{-/-}$→B6 (n = 18) and OT-I Bim$^{-/-}$→RIPmOVA (n = 15) chimeras. Data is representative of the indicated number of samples.

Table 3: Bim is required for clonal deletion to TRA

Chimeras	OT-I→B6	OT-I→RIP-mOVA	OT-I Bim$^{-/-}$→B6	OT-I Bim$^{-/-}$→RIP-mOVA
Vα2^{+}DP^{Bright}	4.62 ± 2.78	4.82 ± 2.56	5.74 ± 4.61	3.42 ± 2.69
Vα2^{+}CD4^{+}CD8^{int}	6.56 ± 4.57	3.06 ± 1.67	8.33 ± 4.42	5.44 ± 3.56
Vα2^{+}DP^{Dull}	4.78 ± 3.72	5.63 ± 2.33	10.4 ± 5.72	10.7 ± 5.83
Vα2^{+}CD8SP	6.40 ± 2.77	2.57 ± 1.10	8.72 ± 4.69	9.87 ± 4.18

OT-I→B6 (n=9), OT-I→RIPmOVA (n=8), OT-I Bim$^{-/-}$→B6 (n-25), OT-I Bim$^{-/-}$→RIPmOVA (n=22)

Clonal deletion occurs after positive selection in OT-I→RIPmOVA chimeras

Similar to the Act-mOVA chimeras, cleaved caspase 3 was detected only in Vα2^{+} thymocytes from OT-I Bim-sufficient BM recipients and not OT-I Bim-deficient BM recipients **(Fig. 3-9A)**. Unlike in OT-I→Act-mOVA chimeras, Vα2^{+} cleaved caspase 3^{+} thymocytes in OT-I→RIPmOVA chimeras were primarily DP^{dull} and CD4^{+}CD8^{int} with similar frequencies to what is seen in OT-I→B6 chimeras **(Fig. 3-9B).** This data is consistent with clonal deletion occurring after positive selection in the cortex of OT-I→RIPmOVA chimeras and suggests that clonal deletion is occurring after OVA encounter in the medulla. Collectively, this suggests that the OT-I→RIPmOVA and OT-I Bim$^{-/-}$→RIPmOVA chimeras successfully generate a model of negative selection to TRA, while OT-I→B6 and OT-I Bim$^{-/-}$→B6 chimeras represent a model of non-deletion in OT-I mice.

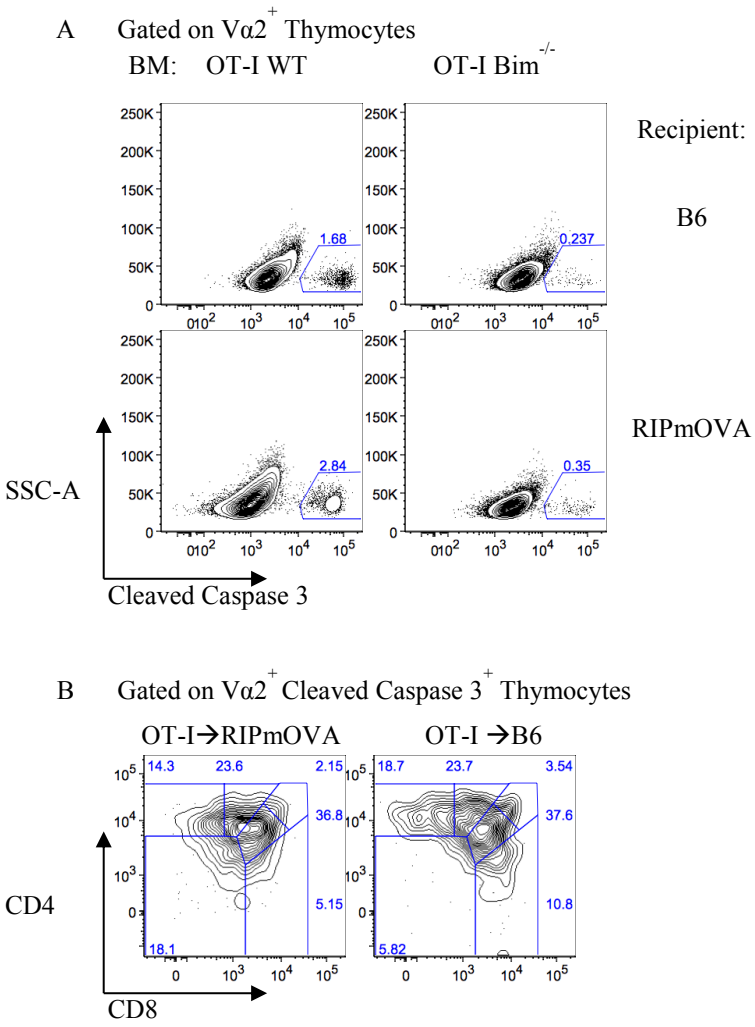


Figure 3-9: Clonal deletion in OT-I→RIPmOVA chimeras occurs after positive selection
A. Active cleaved caspase 3 profile of Vα2+ thymocytes from OT-I→B6 (n = 7), OT-I Bim-/-→B6 (n = 8), OT-I→RIPmOVA (n = 8) and OT-I Bim-/-→RIPmOVA (n =7) chimeras. **B.** CD4 by CD8 profile of Vα2+ cleaved caspase 3+ thymocytes. Data is representative of the indicated number of samples.

TRA-specific T cells persist in the periphery in the absence of Bim

OT-I Bim$^{-/-}$→RIPmOVA Vα2^{+}CD8SP thymocytes clearly escape clonal deletion in the thymus. However, they can still encounter OVA peripherally in the pancreatic lymph nodes **(pLN).** This could lead to peripheral deletion of these cells or activation (102).

To determine the fate of the OT-I Bim$^{-/-}$ CD8SP thymocytes that escaped clonal deletion in the thymus, we examined splenocytes from the four chimeras for deletion or activation.

The number of Vα2^{+} splenocytes was similar in the OT-I→B6 chimeras and the OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 3-10A)**. In the OT-I→RIP-mOVA chimeras, the number of Vα2^{+} splenocytes was significantly reduced compared to the OT-I→B6 control chimera, whereas in the OT-I Bim$^{-/-}$→RIP-mOVA chimeras, the number of Vα2^{+} cells was similar to what was seen in the OT-I Bim$^{-/-}$→B6 chimeras **(Fig 3-10A)**. Further examination of the V α 2^{+} splenocytes for CD4 and CD8 expression revealed a decrease in the proportion of CD8^{+} T cells in the OT-I Bim$^{-/-}$→B6 chimeras compared to OT-I→B6 chimeras with a reciprocal increase in the proportion of CD4^{+} T cell population in the OT-I Bim$^{-/-}$→B6 mice **(Fig. 3-10B)**. This is likely due to the rescue of non-OT-I T cells in the CD4 compartment from deletion in OT-I Bim$^{-/-}$→B6 chimeras that would normally be deleted in OT-I→B6 chimeras.

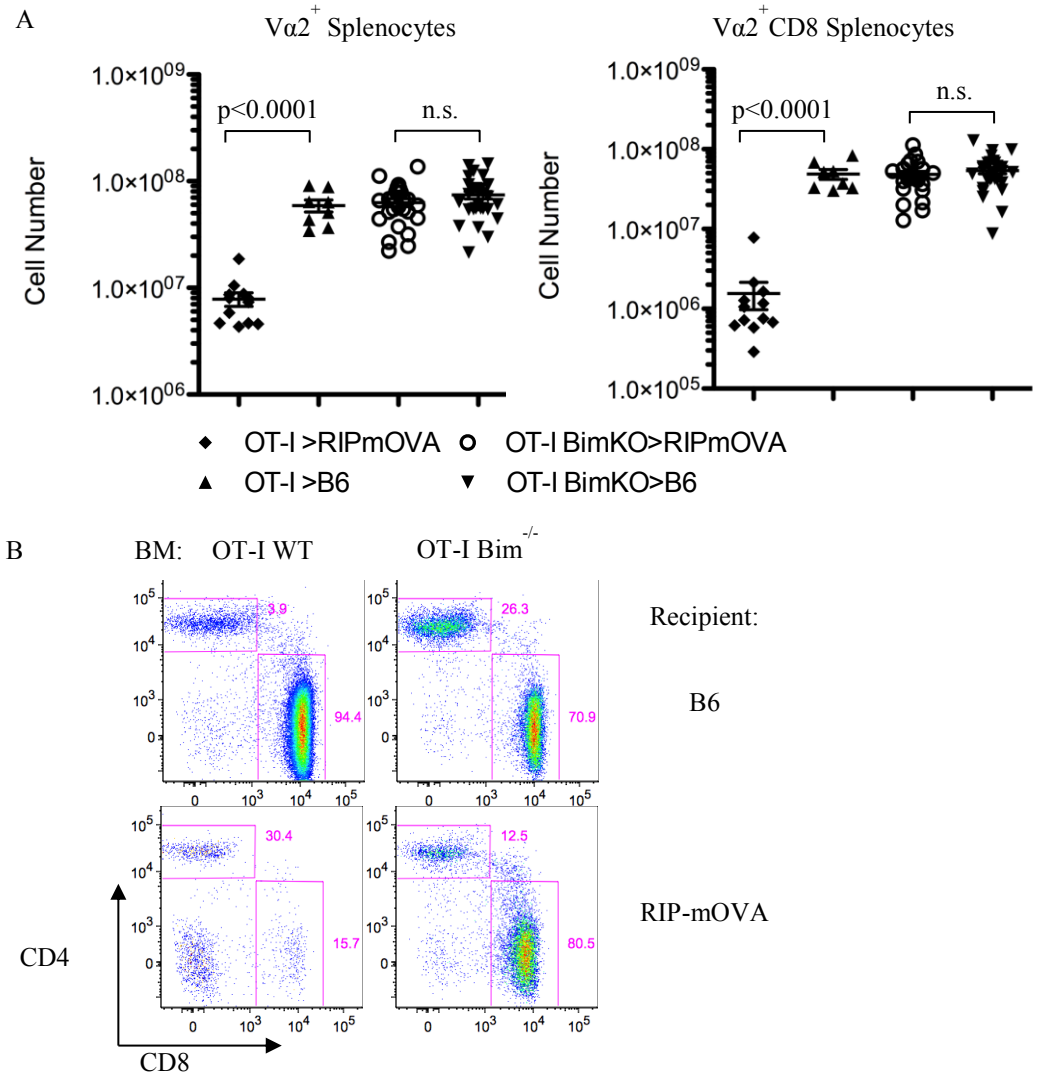


Figure 3-10: TRA specific T cells persist in the periphery in the absence of Bim
A. Cell numbers of the indicated populations from OT-I→B6 (n = 8), OT-I→RIPmOVA (n = 12), OT-I Bim$^{-/-}$→B6 (n = 28) and OT-I Bim$^{-/-}$→RIPmOVA (n = 25) chimeras. **B.** CD4/CD8 profile of Vα2^{+} T cells from spleens of OT-I→B6 (n = 8), OT-I→RIPmOVA (n = 12), OT-I Bim$^{-/-}$→B6 (n = 28) and OT-I Bim$^{-/-}$→RIPmOVA (n = 25) chimeras. Data is representative of the indicated number of samples.

In contrast, OT-I→RIP-mOVA chimeras had a dramatically reduced proportion of $CD8^+$ T cells within the $V\alpha2^+$ population compared to the OT-I→B6 and OT-I $Bim^{-/-}$→B6 chimeras that was not observed in OT-I $Bim^{-/-}$→RIPmOVA chimeras **(Fig. 3-10B)**. Similar to the OT-I→B6 and OT-I $Bim^{-/-}$→B6 chimeras, the $V\alpha2^+$ splenocytes from OT-I $Bim^{-/-}$→RIP-mOVA chimeras were largely $CD8^+$. OT-I→B6 and OT-I $Bim^{-/-}$→B6 chimeras had similar numbers of $V\alpha2^+$ $CD8^+$ T cells, demonstrating that Bim does not significantly alter mature peripheral T cell numbers **(Fig. 3-10A)**. As expected, $V\alpha2^+$ $CD8^+$ T cell numbers were significantly reduced in the OT-I→RIPmOVA chimeras demonstrating effective deletion of these auto-reactive T cells through central and peripheral tolerance mechanisms **(Fig. 3-10A)**. However, in the OT-I $Bim^{-/-}$→RIPmOVA chimeras there was an abundance of OT-I $Bim^{-/-}$ T cells in the periphery, similar in number to OT-I→B6 and OT-I $Bim^{-/-}$→B6 chimeras, suggesting that not only was thymic clonal deletion abrogated in the absence of Bim, but peripheral deletion was impaired as well **(Fig. 3-10A)**. Alternatively, the abundance of OT-I $Bim^{-/-}$ T cells might suggest that peripheral deletion is not induced in this model system.

As in the thymus, we observed downregulation of the CD8 co-receptor as well as a slight upregulation of Nur77 in OT-I $Bim^{-/-}$→RIPmOVA splenocytes **(Fig. 3-10A and B, 3-11A and B)**. This suggests peripheral T cells encounter antigen, however it is not clear if this due to antigen encounter in the pLN or simply a residual effect of antigen encounter in the thymus.

The few OT-I T cells that did enter the periphery in OT-I→RIP-mOVA chimeras expressed high levels of CD44 **(Fig. 3-11A)**. It is unclear whether CD44

induction occurred in response to antigen or lymphopenia. Interestingly, $V\alpha2^+$ $CD8^+$ T cells from the OT-I $Bim^{-/-}$→RIP-mOVA chimeras had a $CD44^{lo}$ phenotype, expressing similar levels of CD44 as OT-I and OT-I $Bim^{-/-}$→B6 chimeras, suggesting they are not overtly activated **(Fig. 3-11A)**.

It has been previously shown that adoptive transfer of 5 x 10^6 OT-I T cells into a RIP-mOVA mouse will result in rapid onset of diabetes (134). However, blood glucose readings obtained for up to twenty-five weeks post BM transplant demonstrated that these OT-I $Bim^{-/-}$→RIP-mOVA chimeras did not develop diabetes **(Table 4)** despite that on average, there were 6.5 x 10^7 $CD8^+$ OT-I T cells present in the spleen. This suggests that OT-I $Bim^{-/-}$ T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras are functionally impaired or unable to traffic into the pancreas.

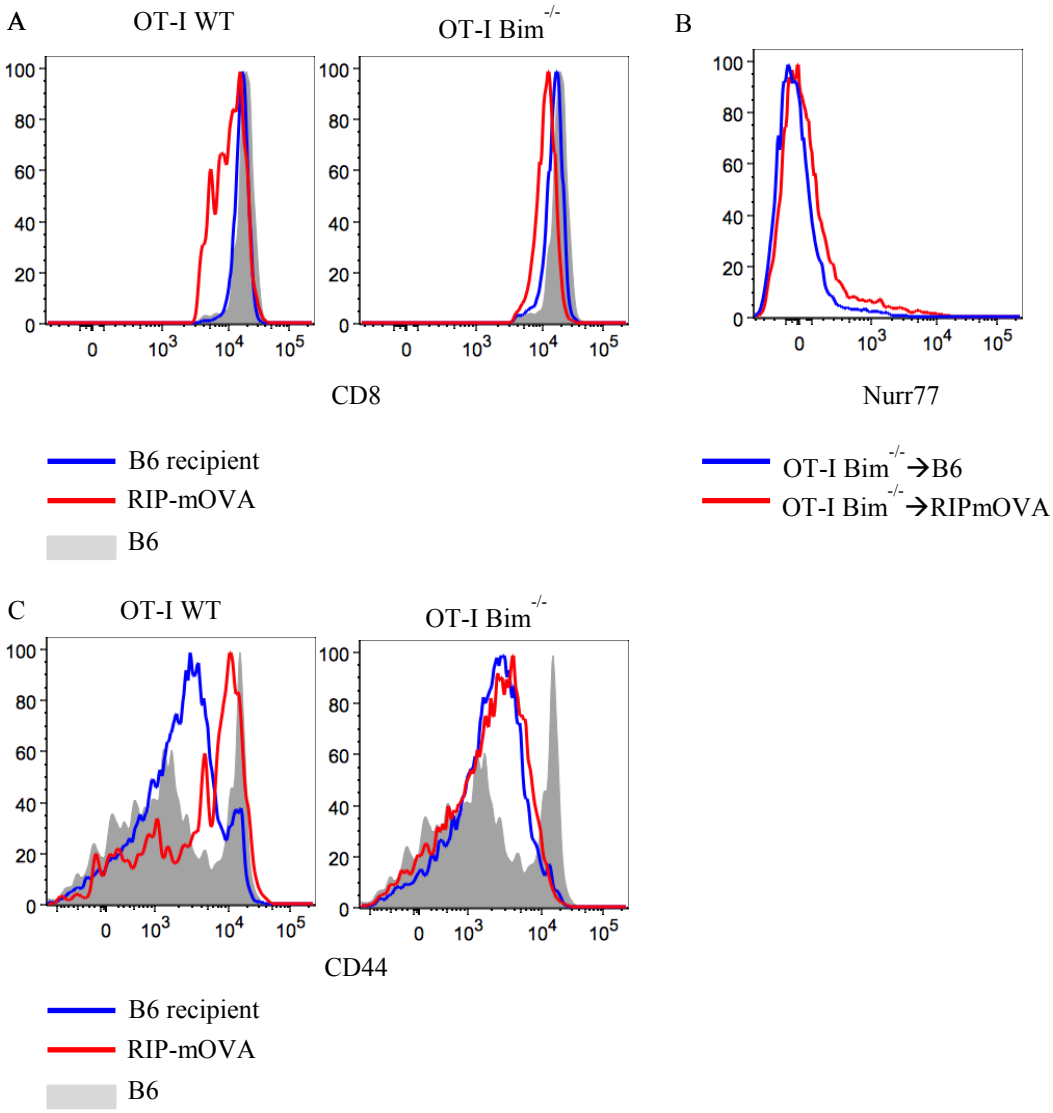


Figure 3-11: TRA specific T cells show evidence of antigen encounter but are not activated in the absence of Bim.

A. CD8 expression of Vα2$^+$ CD8$^+$ T cells from spleens of OT-I→B6 (n = 12), OT-I→RIPmOVA (n = 18), OT-I Bim$^{-/-}$→B6 (n = 46) and OT-I Bim$^{-/-}$→RIPmOVA (n = 46) chimeras. **B.** Nur77 expression of Vα2$^+$ CD8$^+$ T cells from spleen of OT-I Bim$^{-/-}$→B6 (n = 5) and OT-I Bim$^{-/-}$→RIPmOVA (n = 5) chimeras. **C.** CD44 expression of Vα2$^+$ CD8$^+$ T cells from spleens of OT-I→B6 (n = 4), OT-I→RIPmOVA (n =4), OT-I Bim$^{-/-}$→B6 (n = 8) and OT-I Bim$^{-/-}$→RIPmOVA (n = 8) chimeras. Data is representative of the indicated number of samples.

Table 4: Diabetes Incidence in BM Chimeras

Chimera	OT-I→B6	OT-I→ RIPmOVA	OT-I Bim$^{-/-}$ →B6	OT-I Bim$^{-/-}$ →RIPmOVA	OT-I→ ActmOVA	OT-I Bim$^{-/-}$ →ActmOVA
Incidence of Diabetes	0/12	0/18	0/46	0/46	0/4	0/4

Discussion

It was previously thought that Bim-mediated apoptosis was required for clonal deletion of thymocytes(96), however recent data demonstrates that in the case of clonal deletion to UbA, Bim is not required (99-101). Our data from the Act-mOVA chimeras supports the findings of these previous reports, where Bim is essential for thymocyte apoptosis but not clonal deletion to UbA, suggesting Bim-independent clonal deletion mechanisms exist **(Fig. 3-2: 3-3, 3-4)**. Thymocytes from OT-I→Act-mOVA chimeras were primarily deleted at the DN stage **(Fig. 3.3B)**. These results are consistent with the fact that the kinetics of the OT-I TCR expression is not physiological and the OT-I TCR is first expressed at the DN stage, where thymocytes will already be encountering OVA in the thymic cortex. Interestingly, there was a cleaved caspase 3^+ population in OT-I→B6 chimeras despite the absence of cognate antigen in the thymi of these mice **(Fig. 3.3 and 3.8)**. These cells were primarily DP^{dull} and $CD4^+CD8^{int}$ thymocytes. As we did not use Rag-deficient BM to generate the BM chimeras, it is likely these thymocytes that underwent deletion were expressing endogenous TCRβ chains and underwent deletion after positive selection. Whatever the mechanism of clonal deletion, it is efficient and fairly robust, with few OT-I $Bim^{-/-}$ T cells escaping into the periphery of Act-mOVA chimeras **(Fig. 3-5).**

While in the absence of Bim clonal deletion to UbA appears to be intact, the fact remains that Bim-deficient mice develop autoimmunity (94, 96-98). In this chapter, we demonstrated that unlike clonal deletion to UbA, Bim deficiency completely abrogates clonal deletion to TRA **(Fig. 3-6, 3-8 and 3-9)**. Similar to those from OT-I→B6 chimeras, thymocytes from OT-I→RIPmOVA chimeras were

primarily deleted at the DP^{dull} and $CD4^{+}CD8^{int}$ stage (**Fig. 3-9).** However in this case, it is likely that deletion is occurring in the medulla against OVA peptide being expressed as a TRA

While defective antigen encounter may explain the lack of deletion in OT-I $Bim^{-/-}$→RIPmOVA chimeras, we find this unlikely as CD8 dulling in the thymic $V\alpha2^{+}$ CD8SP compartment in OT-I $Bim^{-/-}$→RIP-mOVA chimeras suggests antigen encounter **(Fig. 3-7A).** Furthermore, Nur77, a gene induced by TCR stimulation, was up-regulated **(Fig. 3-7B)** in OT-I CD8SP thymocytes from OT-I $Bim^{-/-}$→RIP-mOVA chimeras which indicates there is TCR stimulation and activation of a transcriptional program, which is consistent with previous reports (49). Finally, it is unlikely that the abrogated clonal deletion resulted from the killing of mOVA expressing APC in the thymus (135) since we did not find $CD44^{hi}$ OT-I $Bim^{-/-}$ cells in the thymus of Rip-mOVA recipients **(Fig. 3-7C)** and the OT-I $Bim^{-/-}$ T cells from Rip-mOVA recipients did not induce diabetes **(Table 4)**. Collectively, these data demonstrate that, unlike clonal deletion to UbA, thymic clonal deletion to TRA is impaired in the absence of Bim. Importantly, this is the first report of differential thymocyte intrinsic molecular requirements for clonal deletion to UbA versus TRA downstream of the TCR. These data also demonstrate that other mediators of clonal deletion, such as Nur77 or Nor1, cannot compensate for the absence of Bim in clonal deletion to TRA.

While we have demonstrated that the absence of Bim allows escape of TRA-specific T cells from clonal deletion, we were unable to replicate autoimmune disorders associated with Bim deficiency (94, 96-98). Again, CD8 co-receptor dulling on peripheral OT-I T cells from OT-I $Bim^{-/-}$→RIP-mOVA chimeras **(Fig. 3-10B, 3-**

11A) suggested antigen encounter in the periphery and this is supported by elevated Nur77 expression in Vα2^{+} CD8^{+} T cells from OT-I Bim$^{-/-}$→RIP-mOVA chimeras **(Fig. 3-11B)**, consistent with a previous report (49). This suggests that in the absence of thymic clonal deletion, either a dominant or recessive mechanism of tolerance was established to control these TRA-reactive T cells in the OT-I Bim$^{-/-}$→RIP-mOVA chimeras.

There are several mechanisms by which OT-I Bim$^{-/-}$ T cells may be controlled in the periphery of RIP-mOVA mice. First, the OT-I Bim$^{-/-}$ T cells could be rendered anergic upon self-antigen encounter either in the thymus or periphery. These data do not demonstrate whether the OT-I Bim$^{-/-}$ thymocytes that encountered OVA as a TRA in the thymus, but were not deleted, are functional. This will be addressed in the next chapter. Since thymic anergy is considered a form of negative selection, we cannot discount the thymus as the location of anergy induction. Previous work has demonstrated that transfer of Bim-deficient DO11.10 T cells into mice that express a soluble form of OVA are anergized (136). However, in this situation, the anergic DO11.10 Bim$^{-/-}$ T cells proliferated and upregulated CD44 following adoptive transfer. Since the peripheral OT-I Bim$^{-/-}$ T cells were CD44^{lo} **(Fig. 3-7C)**, it is unlikely that they underwent antigen driven proliferation, and therefore the mechanism of tolerance induction may be different. We cannot discount lymphopenia-induced proliferation since it was recently demonstrated that self-specific CD8 T cells that underwent lymphopenia-induced proliferation remained CD44^{lo} (137). It is also possible that anergy was induced in the thymus but maintained by continuous cognate antigen encounter in the periphery.

Second, OT-I Bim$^{-/-}$ T cells from RIP-mOVA chimeras could become subject to regulation by T_{reg} cells. It has been shown that high affinity antigen encounter can result in differentiation into CD4^{+}Foxp3^{+}CD25^{+} T_{reg} cells. Therefore it is possible that survival of high affinity antigen encounter in the thymus could lead to the generation of an unknown CD8 regulatory population from the OT-I thymocytes. The Santamaria group has recently identified low affinity autoregulatory CD8^{+} T cells that can potently suppress diabetogenic T cells (138). The possibility exists that either a traditional CD4^{+} Treg or a CD8^{+} regulatory cell population was induced in the OT-I Bim$^{-/-}$→RIP-mOVA chimeras. Experiments designed to test for the presence of a dominant regulatory population are required to address this possibility.

Third, it was demonstrated that some thymocytes expressing the self-specific A18 transgenic TCR as well as a positively selecting BM3 transgenic TCR were rescued from negative selection (139). These thymocytes that were rescued from deletion did not mediate autoimmunity *in vivo*, but could be stimulated *in vitro* by a BM3-specific target cell and mediate killing of an A18 –specific target cell. As we did not use Rag deficient OT-I or OT-I Bim$^{-/-}$ BM donors, it is possible that there was endogenous TCRα chain rearrangement and the expression of dual TCRs on OT-I Bim$^{-/-}$→RIPmOVA thymocytes. One caveat of the dual TCR model is that the second transgenic TCR was a positively selecting TCR complete with both TCRα and TCRβ chains. In the case of OT-I and OT-I Bim$^{-/-}$ chimeras, the expression of a second TCR would require the generation of an endogenous TCRα chain that could pair with the OT-I Vβ5 TCRβ chain and also mediate positive selection. Given that between 20%-

25% of DP thymocytes are positively selected in a polyclonal population (140), these fortuitous TCRα rearrangements would be exceedingly rare occurrences.

The dual transgenic TCR model did not completely abrogate negative selection, as was observed in the OT-I Bim$^{-/-}$→RIPmOVA chimeras. Presumably, a similar proportion of OT-I→RIPmOVA thymocytes would also express dual OT-I and endogenous TCR as in OT-I Bim$^{-/-}$→RIPmOVA chimeras, yet there is significant deletion of thymocytes in OT-I→RIPmOVA chimeras.

Additionally, thymocytes escaping deletion in the dual transgenic TCR model downregulated the self-reactive TCR, (139), which is not evident in OT-I Bim$^{-/-}$→RIPmOVA thymocytes (Fig. 3-6). Given these differences in TCR expression and the significant rescue of CD8^{+} OT-I Bim$^{-/-}$→RIPmOVA T cells, it is unlikely that dual TCR expression on OT-I Bim$^{-/-}$→RIPmOVA chimeras could account for the impaired deletion in these mice.

Finally, the OT-I Bim$^{-/-}$ T cells may be signaled to further differentiate into another inactive subset (141). This possibility has recently been demonstrated for CD4+ T cells (141). Bim deficient mice were enriched for self-reactive CD25^{low}Foxp3^{+} CD4SP thymocytes and CD25^{low}Foxp3^{+}CD4^{+} T cells. These populations of thymocytes and T cells were impaired in their ability to proliferate and produced cytokines. Additionally, their suppressive capabilities were less robust than that of the CD25^{+}Foxp3^{+}CD4^{+} T_{reg} cell population. The CD25^{low}Foxp3^{+}CD4^{+} T cell population did not readily convert to the CD25^{+}Foxp3^{+}CD4^{+} phenotypes (141). It is possible that a similar CD8^{+} population is generated in OT-I Bim$^{-/-}$→RIPmOVA chimeras. To further explore this possibility, we will need to cross OT-I Bim$^{-/-}$ mice to a Foxp3 reporter

strain. The resulting cross can be used to generated BM chimeras. $CD25^{low}Foxp3^{+}$, $CD25^{+}Foxp3^{+}$ and $CD25^{-}Foxp3^{-}$ CD8 T cell populations can be isolated by fluorescence-activated cell sorting and assessed for functionality and suppressive capabilities. It is also possible that OT-I $Bim^{-/-}$ T cells differentiated into an as of yet uncharacterized inactive subset. However, further experiments are necessary to examine this possibility.

Collectively, our data demonstrate that while the pro-apoptotic molecule Bim is not required for clonal deletion to UbA, it is required for clonal deletion to TRA. This is the first demonstration of differences in the molecular requirements of clonal deletion to TRA versus UbA downstream of the TCR. Though clonal deletion to TRA is abrogated in the absence of Bim, the OT-I T cells appear to remain tolerant. These results have been replicated using OT-I and OT-II BM chimeras (142)(Gray) as well as OT-II RIPmOVA double transgenic mice (84)While the exact nature of this tolerance is unknown, understanding the regulatory mechanisms at play in this situation should provide insight into understanding how autoimmunity progresses beyond impaired clonal deletion.

Chapter 4:
Examining functional impairment of T cells escaping Bim mediated clonal deletion to TRA

Introduction

I clearly demonstrated in the previous chapter that in the absence of Bim, clonal deletion to TRA is abrogated. Despite the large numbers of OT-I $Bim^{-/-}$ T cells in the periphery of OT-I $Bim^{-/-}$→RIPmOVA chimeras, these mice did not develop diabetes **(Table 4)**. It is possible that Bim-deficient T cells are intrinsically impaired in their ability to function, however thymocytes and splenocytes from OT-I $Bim^{-/-}$→B6 and OT-I→B6 chimeras were fairly similar phenotypically. There have also been conflicting reports about the role of Bim in T cell function. It was demonstrated that $Bim^{-/-}$ $CD4^+$ T cells were unable to produce cytokines following activation with plate-bound αCD3/α28 antibodies (143). In contrast, pathogen-challenge experiments against *L. monocytogenes* secreting OVA **(LM-OVA)** demonstrated no such functional impairment (144). *In vitro* and *in vivo* examination of the functionality of T cells isolated from OT-I $Bim^{-/-}$→RIPmOVA and OT-I $Bim^{-/-}$→B6 chimeras may reconcile this conflicting data.

As T cells in OT-I $Bim^{-/-}$→RIPmOVA chimeras will encounter OVA persistently in the periphery, it is possible that T cells in these mice are exhausted. T cell exhaustion generally accompanies inflammation and chronic infection (145) and coincides with expression of many inhibitory receptors, including programmed death 1 **(PD-1)**, lymphocyte activation gene 3 **(Lag-3)**, T cell immuglobulin- and mucin domain-containing molecule 3 **(Tim-3)**, B- and T-lymphocyte attenuator

(BTLA), and cytotoxic T lymphocyte-associated antigen 4 **(CTLA-4)** (145, 146). However, exhausted T cells are generally $CD44^{hi}$ and chronically activated. Low CD44 expression suggests that OT-I $Bim^{-/-}$→RIPmOVA T cells and thymocytes have not been overtly activated despite upregulation of Nur77 expression, which suggests these cells have received a TCR stimulus **(Fig. 3-7 and 3-11)**. Given this information, it is unlikely T cell and thymocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras are exhausted, but it does not discount the possibility that they are intrinsically impaired in activation.

Another form a functional impairment that could be at play is T cell anergy. Anergy can be described as a tolerance mechanism whereby lymphocytes are intrinsically functionally impaired after encountering antigen, but remain alive in this state of dysfunction (41). Typically, anergy has been associated with sub-optimal co-stimulation (41, 145). There are many models used to study anergy ranging from *in vitro* stimulation to developmental models to adoptive transfer models (41). Generally, *in vitro* models of anergy involve stimulation via αCD3 plus APC in the absence of co-stimulation by αCD28 and CTLA-4 may play a role in this state (41). $CD8^+$ T cell models of *in vitro* anergy result in $CD8^+$ T cells suffering from proliferative impairment while maintaining IFNγ production and CTL activity. Anergy has also been induced *in vivo* using adoptive transfer experiments and developmental models using both $CD4^+$ and $CD8^+$ T cells. These various induction methods come with an equally varying list of characteristics including reversal by exogenous IL-2, antigen requirement for persistence, impaired proliferation and/or cytokine production, peripheral induction or thymic induction. The fact that the

various induction methods have different combinations of characteristics highlights the many different mechanisms available to modulate T cell responses.

In addition to T cell intrinsic mechanisms of tolerance, it is possible that we are observing a T cell extrinsic mechanism of control such as T_{reg} cells. As previously mentioned, $CD4^+CD25^+Foxp3^+$ are believed to be selected on high affinity interactions in the medulla (48). OT-I $Bim^{-/-}$→RIPmOVA thymocytes may (122) differentiate into a regulatory population after failing to be deleted in the thymus. While the majority of studies involving T_{reg} focus on traditional $CD4^+CD25^+$ T_{reg}, there have been reports of self-specific $CD8^+$ T_{reg} populations for non-classical MHC class I molecule Qa-1 in EAE, Herpes Stromal Keratitis and Type 1 diabetes **(T1D)** models and HLA-E in human T1D and multiple sclerosis patients (122). Qa-1 restricted CD8 T_{reg} cells target autoreactive $CD4^+$ T cells, but appear to require restimulation by antigen before exerting their regulatory nature (122). It is not yet clear how these $CD8^+$ T_{reg} populations are selected for, but a $CD8^+$ regulatory population may be generated in OT-I $Bim^{-/-}$→RIPmOVA chimeras.

While OT-I $Bim^{-/-}$→RIPmOVA chimeras appear to be protected from diabetes onset, it will be of interest to see how T cells from these chimeras respond to lymphopenic cues. As OT-I T cells are sensitive to lymphopenia induced proliferation **(LIP)** and lymphopenia has been associated with a variety of autoimmune disorders (147-150), it is possible that lymphopenia could facilitate diabetes onset. Lymphopenia and homeostatic expansion has also been associated with the rescue of T cells from an anergic state (151, 152).

There are a wide variety of mechanisms capable of protecting OT-I Bim$^{-/-}$ →RIPmOVA chimeras from developing diabetes. Identifying and understanding what these mechanisms are may further shed light on how immune responses can be modulated to affect disease outcomes.

Results

Bim deficiency does not affect the function of OT-I T cells from B6 chimeras

Recently, it was demonstrated that Bim$^{-/-}$ CD4^{+} T cells were unable to produce cytokines following activation by plate-bound αCD3/αCD28 (143). Therefore, if OT-I Bim$^{-/-}$ T cells were functionally impaired regardless of antigen encounter in the thymus, it would explain why the OT-I Bim$^{-/-}$→RIP-mOVA chimeras did not develop diabetes. However, pathogen challenge experiments with OT-I Bim$^{-/-}$ T cells showed no such impairment in effector function (144). Before examining the effector function of splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras, we sought to ensure that Bim deficiency did not affect T cell function in B6 chimeras.

To study the effector capacity of peripheral Vα2^{+} CD8^{+} T cells from the BM chimeras, we either stimulated CFSE-labeled bulk splenocytes from the chimeras with SIINFEKL-pulsed congenic splenocytes or with plate-bound αCD3/αCD28 antibodies and examined their ability to proliferate on day 2 and day 3 **(Fig. 4-1A)**. To analyse cytokine production, cells were rested for 2 days in the absence of stimulation after the initial 3 days of stimulation. Cells were then restimulated on day 5 with SIINFEKL-pulsed stimulators or challenged with 100nM of soluble SIINFEKL in the presence of Brefeldin A. Brefeldin A prevents protein transport from the endoplasmic reticulum to the Golgi apparatus, allowing for cytokines to be detected by intracellular staining (153-155).

Stimulation with SIINFEKL-pulsed splenocytes is a more physiologically relevant way of activating OVA specific OT-I or OT-I Bim$^{-/-}$ T cells compared to non-specific stimulation by plate-bound αCD3/αCD28 mAb. Co-stimulatory and co-

inhibitory ligands will be provided by the stimulators at physiological levels. In contrast, plate-bound αCD3/αCD28 antibody stimulation results in the crosslinking of TCR molecules and provides a strong co-stimulatory signal resulting in non-antigen specific activation of T cells of any TCR specificity. Despite the less physiological nature of this stimulation, it may nonetheless shed light on what tolerance or anergy mechanisms are at play in this model.

We first examined whether Bim deficiency had an effect on T cell function in the B6 chimeras. Proliferation was assessed qualitatively through CFSE dilution histograms, and quantitatively through division and proliferation indices. The division index is the average number of division of all cells in the original starting population whereas the proliferation index is the average number of divisions that only responding cells underwent.

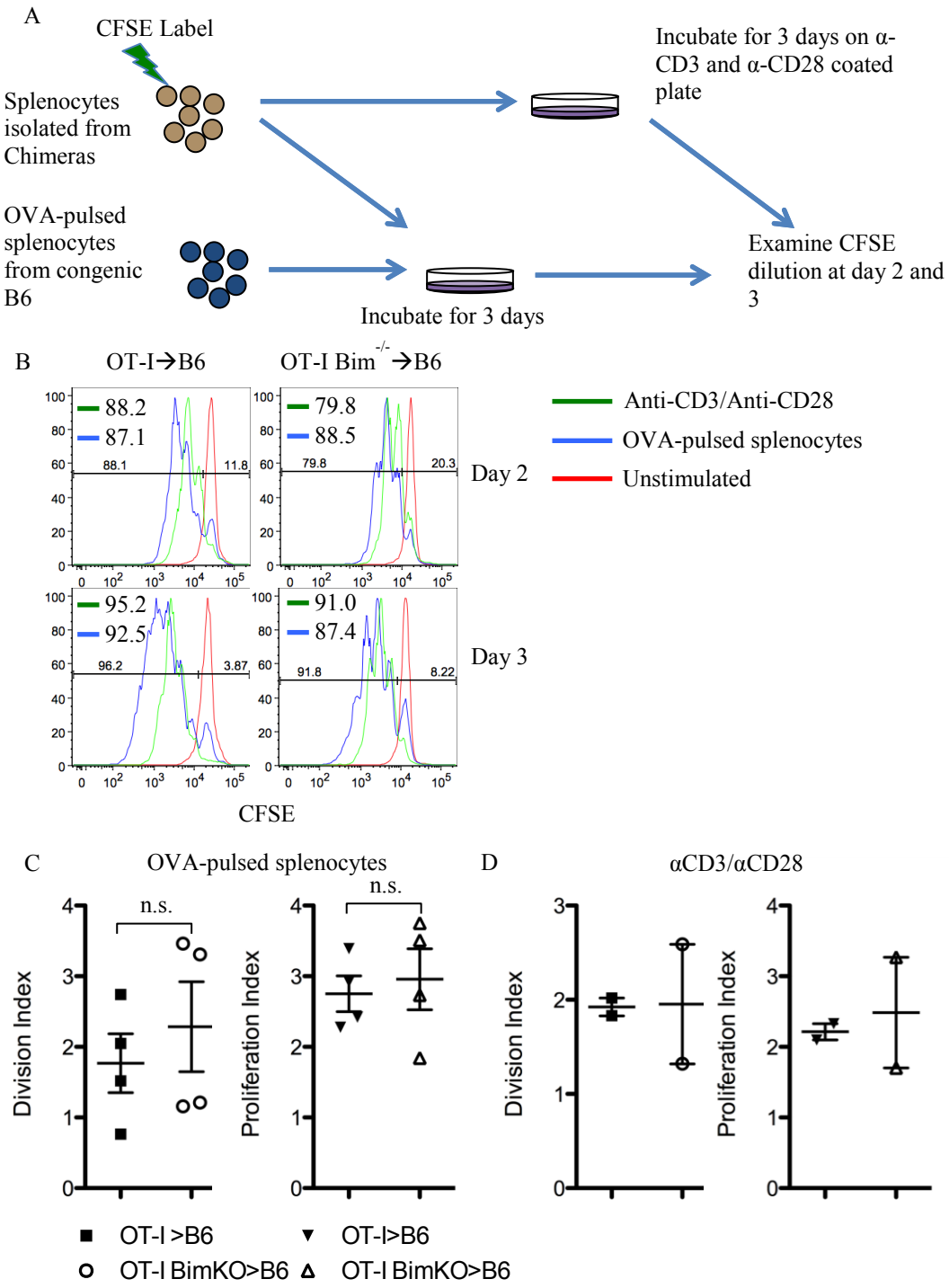


Figure 4-1: Bim deficiency does not affect proliferation of OT-I T cells from the spleens of B6 chimeras

A. Schematic of stimulation experiments. **B.** CFSE dilution of Vα2+CD8+ splenocytes on Day 2 and Day 3 from OT-I and OT-I Bim$^{-/-}$→B6 chimeras after stimulation with SIINFEKL pulsed splenocytes (n=4) or plate bound αCD3/αCD28 (n=2). **C.** Division and proliferation index day 3 following stimulation with SIINFKEL-pulsed splenocytes. **D.** Division and proliferation index day 3 following stimulation with plate-bound αCD3/αCD28.

A similar proportion of OT-I and OT-I Bim$^{-/-}$ splenocytes from B6 chimeras had entered cycle on day 2 and day 3 post-stimulation with both SIINFEKL-pulsed splenocytes as well as plate-bound αCD3/αCD28 antibodies **(Fig. 4-1B)**. There was no significant difference in the division index or proliferation index at day 3 post-stimulation with SIINFEKL-pulsed splenocytes between splenocytes from OT-I→B6 and OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 4-1C).** While we lack the replicates to establish statistical significance, splenocytes from OT-I→B6 and OT-I Bim$^{-/-}$→B6 chimeras showed a similar trend day 3 post-stimulation with plate bound αCD3/αCD28 stimulation **(Fig. 4-1D)**.

Continuing with this trend, OT-I and OT-I Bim$^{-/-}$ T cells from B6 chimeras had a similar distribution of IFNγ and TNFα producing cells with either initial stimulation condition **(Fig. 4-2A and B).**

These data show that the proliferative and cytokine production capacities of OT-I T cells are unaffected by Bim deficiency in B6 chimeras. This suggests that any differences observed in OT-I Bim$^{-/-}$→RIPmOVA chimeras are not a result of Bim deficiency alone, but are likely a direct a result of OT-I Bim$^{-/-}$ T cells surviving clonal deletion.

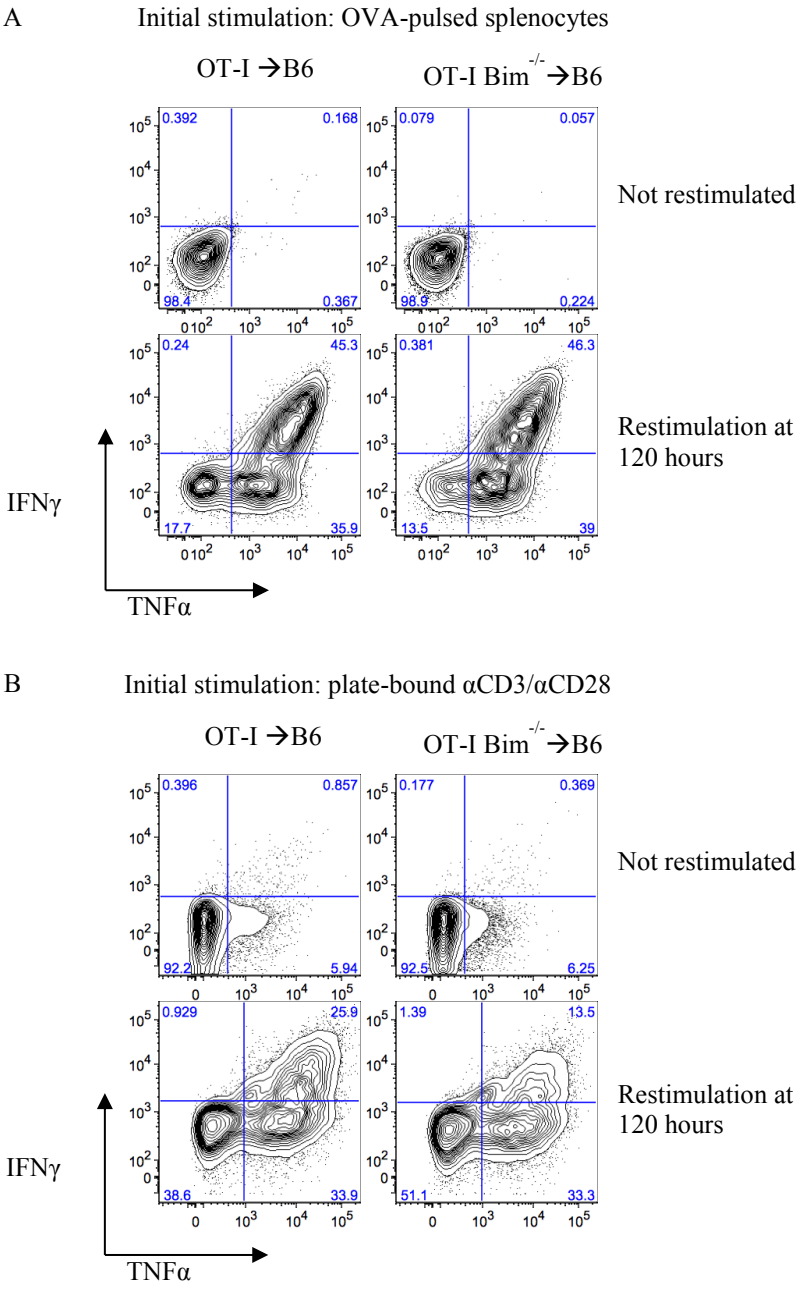


Figure 4-2: Bim deficiency does not affect cytokine production of OT-I T cells from B6 chimeras

A. IFNγ and TNFα production by restimulated and non-restimulated Vα2+CD8+ splenocytes from OT-Bim→B6 and OT-I Bim-/-→B6 chimeras on Day 5 following initial stimulation by SIINFELK-pulsed splenocytes. **B.** IFNγ and TNFα production by restimulated and non-restimulated Vα2+CD8+ splenocytes from OT-Bim→B6 and OT-I Bim-/-→B6 chimeras on Day 5 following initial stimulation by plate-bound αCD3/αCD28.

T cells escaping Bim mediated clonal deletion to TRA are functionally impaired

While there have been conflicting reports as to the effect of Bim deficiency on T cell function (143, 144), our data demonstrate that OT-I T cells are not intrinsically functionally impaired by Bim deficiency in B6 chimeras. However, we have not addressed whether T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras are functionally impaired.

To study the functionality of Vα2^{+}CD8^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras, splenocytes were stimulated as in **Figure 4-1A**, however they were stained with Celltrace Violet instead of CFSE. Proliferation and cytokine production were examined as previously described and compared to Vα2^{+}CD8^{+} T cells from OT-I Bim$^{-/-}$→B6 chimeras.

In contrast to those from OT-I Bim$^{-/-}$→B6, only a minority of splenocytes from the OT-I Bim$^{-/-}$→RIP-mOVA chimeras proliferated in response to SIINFEKL-pulsed splenocytes **(Fig. 4-3A).** There is a significant reduction in both division and proliferation indices of OT-I Bim$^{-/-}$→RIPmOVA chimeras compared to OT-I Bim$^{-/-}$→B6 after SIINFEKL-pulsed splenocytes stimulation **(Fig. 4-3B)**. Interestingly, proliferation of CD8 T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras was comparable to B6 controls upon stimulation with plate bound αCD3/αCD28 **(Fig. 4-3A)**. This proliferative rescue is reflected in there being no significant difference in the division and proliferation indices between OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras after stimulation with plate-bound αCD3/αCD28 stimulation **(Fig. 4-3C).**

These data clearly demonstrate that $V\alpha2^{+}CD8^{+}$ splenocytes from OT-I $Bim^{-/-}$ →RIPmOVA chimeras suffer from a proliferative defect *in vitro* after stimulation with SIINFEKL-pulsed splenocytes, which can be rescued by stimulation with plate-bound αCD3/αCD28 antibodies.

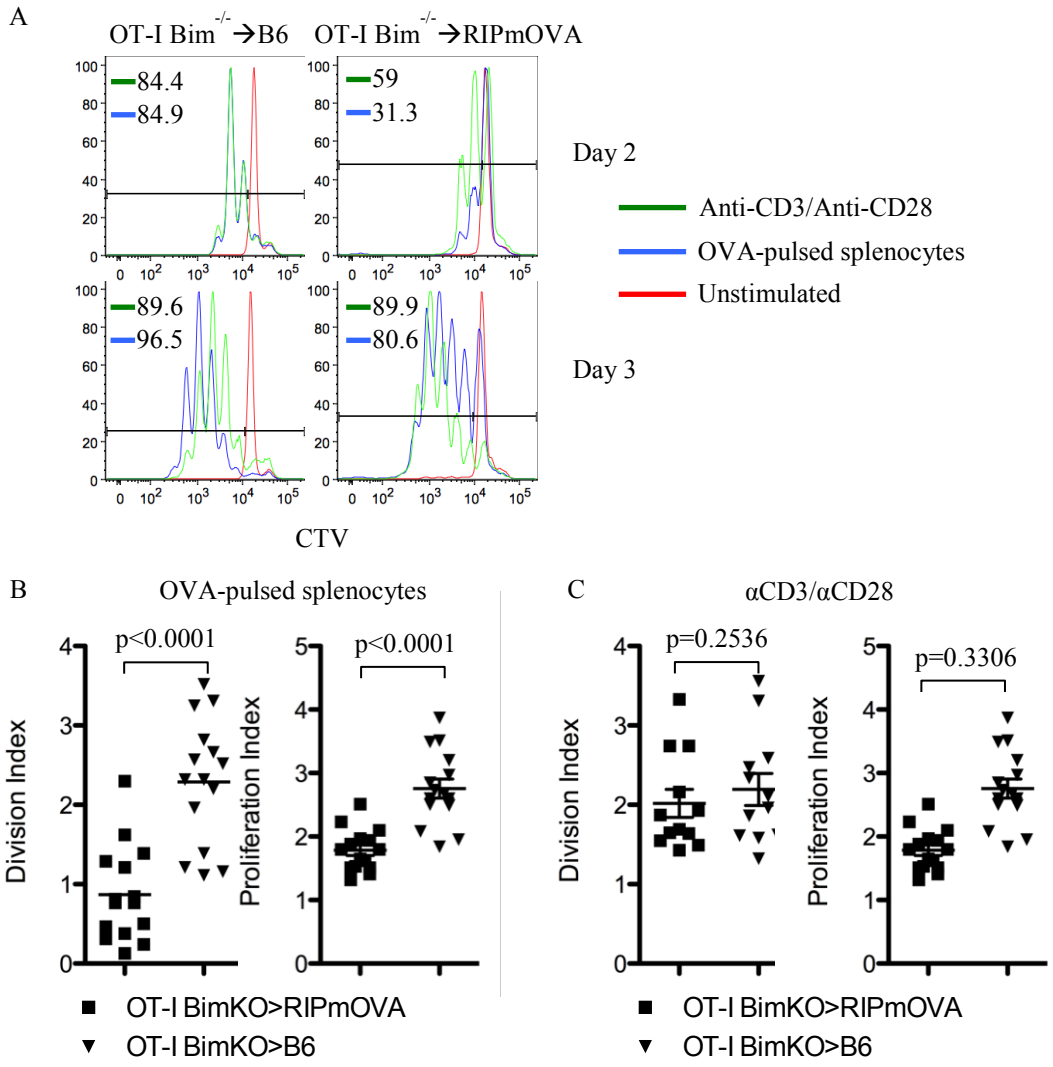


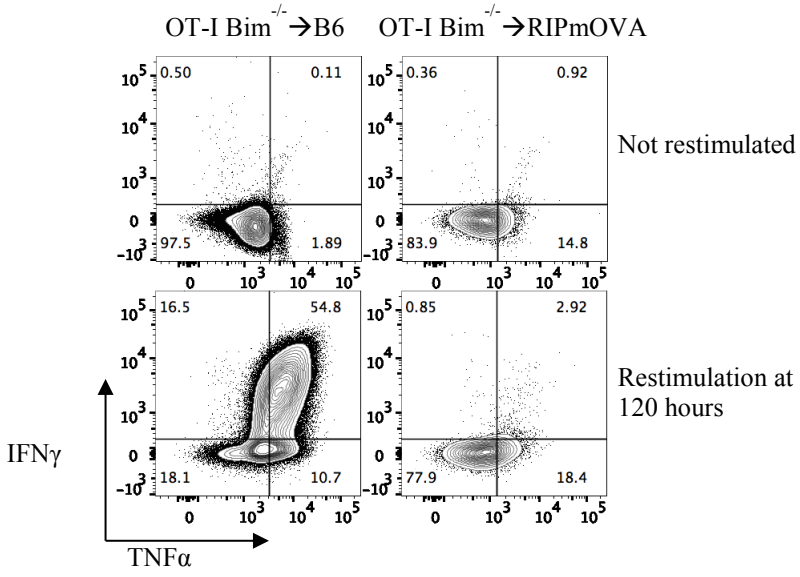
Figure 4-3: T cells escaping Bim mediated clonal deletion to TRA are proliferatively impaired
A. Celltrace Violet dilution of Vα2⁺CD8⁺ splenocytes from OT-I Bim$^{-/-}$→B6 and RIPmOVA chimeras after stimulation with SIINFEKL pulsed splenocytes (n=15) or plate bound αCD3/αCD28 (n=11). Percentage of cells that have diluted CTV indicated in the top left of each panel for indicated population. **B.** Division and proliferation index day 3 following stimulation with SIINFKEL-pulsed splenocytes. **C.** Division and proliferation index day 3 following stimulation with plate-bound αCD3/αCD28.

To further examine the effector function of the OT-I Bim$^{-/-}$ T cells, following 3 days of stimulation with SIINFEKL-pulsed splenocytes or plate-bound αCD3/αCD28 antibodies, we rested splenocytes for 2 days before restimulating them with 100nM SIINFEKL for another 4 hours in the presence of brefeldin A. After restimulation, the majority of Vα2^{+} CD8^{+} splenocytes from OT-I Bim$^{-/-}$→B6 chimeras produced cytokines. In contrast, very few or almost none of the OT-I Bim$^{-/-}$ splenocytes from the OT-I Bim$^{-/-}$→RIP-mOVA chimeras produced cytokines after initial stimulation with SIINFEKL pulsed splenocytes **(Fig 4-4A)**. While initial stimulation with plate-bound αCD3/αCD28, rescued proliferation, it did not rescue cytokine production in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras **(Fig 4-4B).** This points to the presence of multiple mechanisms of anergy at work in this model. The mechanism or mechanisms involved with proliferation can be overcome with a strong non-specific stimulus, whereas those involved with cytokine production cannot.

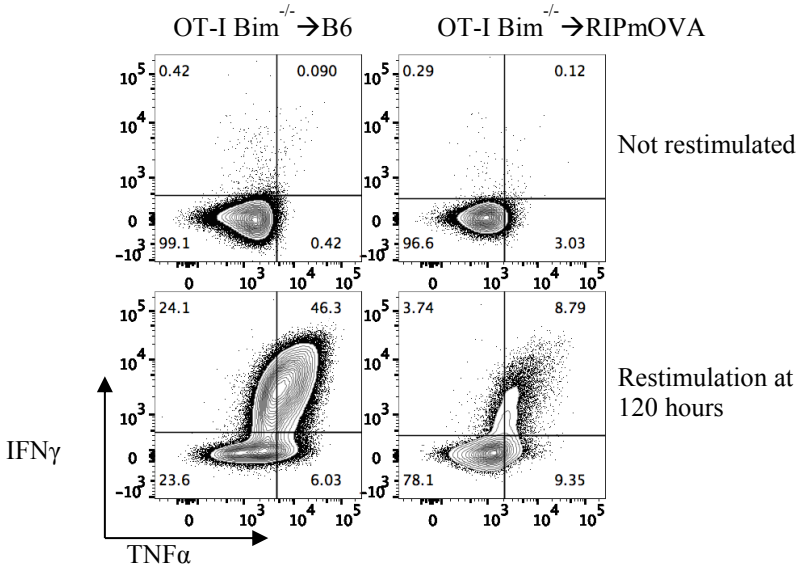
It has been demonstrated in CD4^{+} OVA reactive DO11 T cells, that in a model of peripheral tolerance, cytokines can be regulated post-transcriptionally through AU-rich elements **(ARE)** in the 3' untranslated region **(UTR)** of the cytokine mRNA (156). In particular, IFN-γ has a large 3'UTR with a prominent ARE and high IFN-γ mRNA content can be found in anergic CD4^{+} DO11 T cells after antigen stimulation. We isolated RNA from unstimulated, magnet bead purified CD8^{+} T cells from Day 0 and from CD8^{+} T cells stimulated for 3 days with plate bound αCD3/αCD28 and restimulated on day 5 with OVA peptide from OT-I Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$ →RIPmOVA chimeras. RNA was examined by qRT-PCR for TNFα and IFNγ message. Both chimeras demonstrated an increase in TNFα and IFNγ message following

restimulation on day 5 **(Fig. 4-4C)**. Preliminary results showed that the fold increase was less prominent in T cells from OT-I Bim$^{-/-}$→RIPmOVA compared to OT-I Bim$^{-/-}$→B6 chimeras, however there was still a significant amount of IFNγ and TNFα message. This suggests that IFNγ and TNFα transcription is not completely impaired in OT-I Bim$^{-/-}$→RIPmOVA chimeras, but that the block in cytokine activity may at least partly be post transcriptional **(Fig 4-4C)**.

A Initial stimulation: OVA-pulsed splenocytes



B Initial stimulation: plate-bound αCD3/αCD28



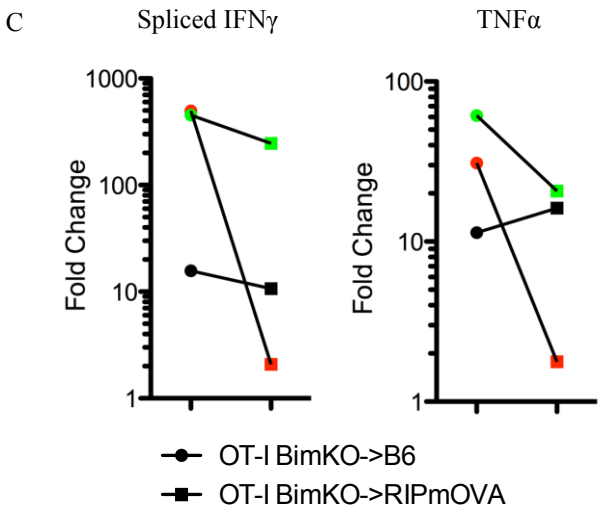


Figure 4-4: T cells escaping Bim mediated clonal deletion to TRA have impaired cytokine production

A. IFNγ and TNFα production by restimulated and non-restimulated Vα2^{+}CD8^{+} splenocytes from OT-Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$→RIPmOVA chimeras on Day 5 following initial stimulation by SIINFELK-pulsed splenocytes. **B.** IFNγ and TNFα production by restimulated and non-restimulated Vα2^{+}CD8^{+} splenocytes from OT-Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$→RIPmOVA chimeras on Day 5 following initial stimulation by plate-bound αCD3/αCD28. **C.** Fold change in spliced IFNγ and TNFα message following restimulation on day 5 after initial stimulation with plate-bound αCD3/αCD28 relative to unstimulated samples as measured by qRT-PCR. Lines and colours indicate samples from the same experiments, stimulated in wells coated by the same antibody mix.

OT-I Bim$^{-/-}$→RIPmOVA CD8^{+} T cells are unable to induce diabetes in lymphopenic RIPmOVA recipients

As previously stated, it has been shown that adoptive transfer of 5 x 10^6 OT-I T cells into a RIP-mOVA mouse will result in rapid onset of diabetes (134). To see if this holds true for cells isolated from the chimeras, we adoptively transferred 5 x10^6 magnetic bead purified CD8^{+} T cells from OT-I Bim$^{-/-}$→B6 or OT-I Bim$^{-/-}$→RIPmOVA chimeras into sub-lethally irradiated RIPmOVA recipients **(Fig. 4-5A)**. Recipients were sub-lethally irradiated because lymphopenia has been associated with the induction of autoimmunity (147-149) as well as the rescue of T cells from an anergic state (151). We felt that doing so would maximize the chance of inducing autoimmunity in the recipients.

While recipients of CD8^{+} T cells from OT-I Bim$^{-/-}$→B6 chimeras rapidly developed diabetes, recipients of CD8^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras did not develop diabetes up to 30 days post injection **(Fig. 4-5B).**

The lack of diabetes induction demonstrates that CD8^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras are functionally impaired *in vivo* even with the presence of cognate antigen in a lymphopenic environment. This suggests that the tolerance mechanism is intrinsic to the CD8^{+} T cells themselves, or that there may be a CD8^{+} regulatory population within the T cell compartment of OT-I Bim$^{-/-}$→RIPmOVA chimeras.

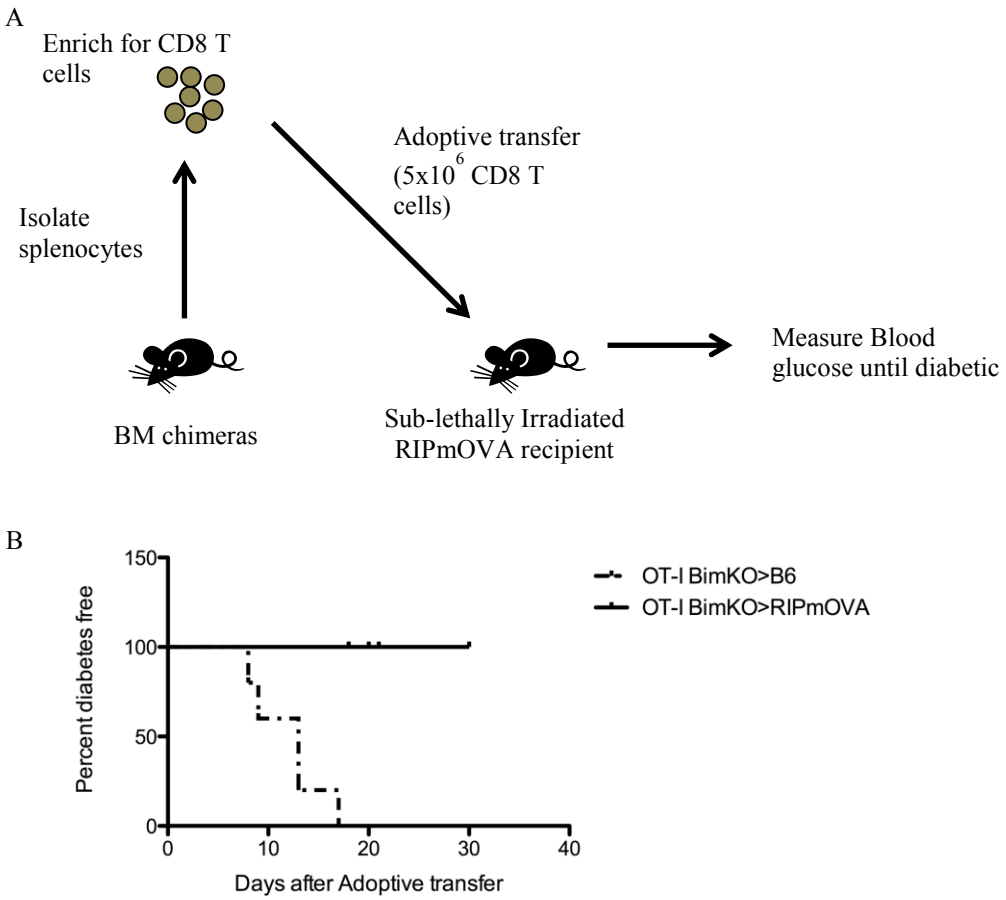


Figure 4-5: Vα2⁺CD8⁺ splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras cannot induce diabetes

A Schematic of adoptive transfer experiments. **B.** Diabetes induction in sublethally irradiated RIPmOVA mice after transfer for 5 x 10^6 CD8⁺ T cells from OT-I Bim$^{-/-}$→B6 (n=5) or OT-I Bim$^{-/-}$→RIPmOVA (n=7) chimeras.

IL-2 potentiates proliferation of only responding T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras

As bone marrow donors were not Rag-deficient, the BM chimeras would also contain T cells expressing endogenous TCR. Stimulation of bulk splenocytes from the chimeras by plate-bound αCD3/αCD28 would also result in the activation of T cells expressing endogenous TCRs. This may have a resulted in the production of IL-2, which has been shown to reverse certain forms of anergy (41) and may be responsible for the rescue of proliferation after stimulation of OT-I Bim$^{-/-}$ →RIPmOVA splenocytes with plate-bound αCD3/αCD28.

To examine this possibility, CFSE or CTV labeled splenocytes were stimulated SIINFELK-pulsed splenocytes as in **Figure 4-1A,** in the presence or absence of exogenous IL-2 during the first 3 days of stimulation. Proliferation was examined by CFSE or CTV dilution.

Even with the addition of exogenous IL-2, the division index of OT-I Bim$^{-/-}$ →RIPmOVA CD8^{+} T cells on day 3 post-stimulation continued to be lower than that of OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 4-6).** In contrast, with addition of exogenous IL-2, the proliferation index of OT-I Bim$^{-/-}$→RIPmOVA CD8^{+} T cells reached a similar value to that of CD8^{+} T cells from OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 4-6)**.

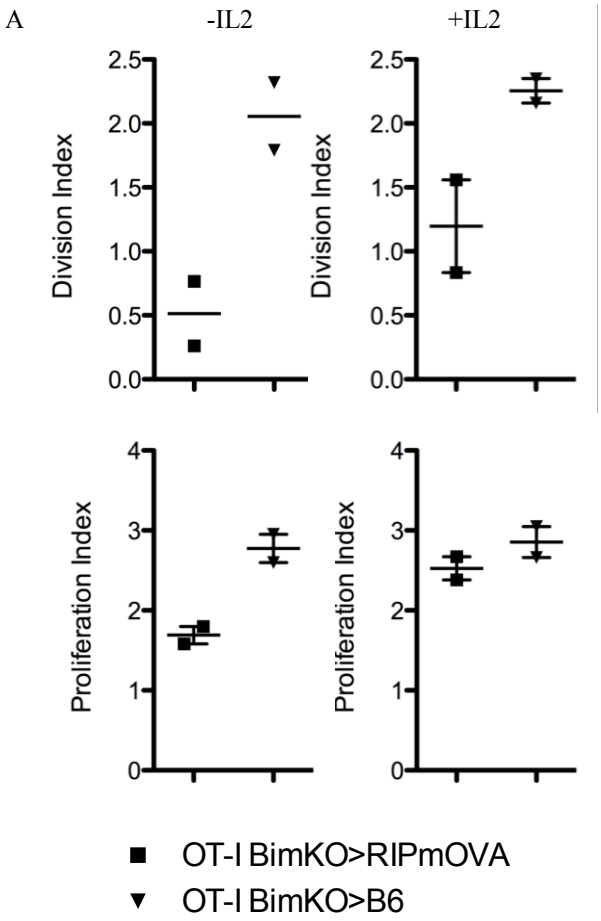


Figure 4-6: IL-2 potentiates division of responding cells but cannot rescue proliferation in non-responders

A. Division and proliferation indices of splenocytes from OT-I Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$→RIPmOVA chimeras after 3 days of stimulation with SIINFELK-pulsed splenocytes with or without exogenous IL-2.

As exogenous IL-2 did not completely rescue the division index of $CD8^+$ T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras to match those from OT-I $Bim^{-/-}$→B6 chimeras, this suggests that IL-2 cannot rescue proliferation in non-responding OT-I $Bim^{-/-}$→RIPmOVA T cells. However, addition of exogenous IL-2 increased the proliferation index of $CD8^+$ T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras to a similar value seen for OT-I $Bim^{-/-}$→B6 T cells, suggesting that IL-2 can potentiate the proliferative capacity of responding T cells from the RIPmOVA chimeras to a similar level seen in the B6 chimeras **(Fig. 4-6)**. These data demonstrate that exogenous IL-2 alone is not able to replicate the proliferative rescue observed with plate-bound αCD3/αCD28 stimulation. Additional IL-2 production with plate-bound αCD3/αCD28 antibody stimulation may play a role in the observed proliferative rescue; however, there must be other mechanisms at play in this rescue.

Potential mechanisms of anergy in T cells escaping Bim mediated clonal deletion to TRA

It is possible that the impaired function of OT-I $Bim^{-/-}$→RIPmOVA T cells could be a result of increase expression of co-inhibitory receptors or anergy factors, or possibly impaired expression of co-stimulatory molecules.

To attempt to identify potential mechanisms of anergy at play in this system, we examined *ex* vivo expression of several cell markers associated with anergy or co-inhibition of T cell activity (BTLA, CTLA4, Lag3, PD-1, Tim-3, Egr2) and two associated with T cell activation or co-stimulation (Helios and GITR).

B and T lymphocyte attenuator **(BTLA)** is a member of the Ig superfamily and acts a co-inhibitory molecule after interacting with its ligand tumor necrosis factor

receptor superfamily, member 14 **(TNFRSF14)**, also known as herpes virus entry mediator **(HVEM)**(157). In addition to being highly expressed on anergic $CD4^+$ T cells *in vivo* (158), BTLA has been implicated in $CD8^+$ T cell proliferation and memory formation (159). However, BTLA is slightly down regulated in CD8SP thymocytes, $CD8^+$ splenocytes and pancreatic lymphocytes of OT-I $Bim^{-/-}$→RIPmOVA chimeras, contrary to what we would have expected given the functional data **(Fig. 4-7A)**. This suggests that BTLA has little role to play in the observed anergy in OT-I $Bim^{-/-}$→RIPmOVA chimeras.

Cytotoxic T lymphocyte antigen 4 **(CTLA-4)** is an inhibitor of the prominent CD28 co-stimulatory molecule by competing for its ligand B7 and thus inducing anergy or apoptosis. Unlike CD28, CTLA-4 is not constitutively expressed on the cell surface of resting T cells, but is maintained intracellularly (160). In thymus, spleen and pLN of OT-I $Bim^{-/-}$→RIPmOVA chimeras, CTLA-4 intracellular expression was not significantly changed compared to OT-I $Bim^{-/-}$→B6 or intact B6 mice and not detectable on the cell surface in any mouse. These data suggest that CTLA-4 does not play a prominent role in the observed anergy of OT-I $Bim^{-/-}$→RIPmOVA T cells **(Fig. 4-7A and B).**

Lag-3 is a CD4-related activation-induced cell surface molecule that binds to MHC class II molecules with high affinity (161, 162). Lag-3 has been shown to regulate expansion of activated T cells and T cell homeostasis (161, 163) and to be involved in $CD8^+$ T cell exhaustion (146) and tolerance (164). Contrary to what we would expect, Lag3 expression was lower both on the cell surface and intracellularly in OT-I $Bim^{-/-}$→RIPmOVA T cells from the thymus, spleen and pLN compared to B6

chimeras and intact B6 mice **(Fig. 4-7A and B).** This data suggests that Lag-3 is not one of the tolerance mechanisms at work in OT-I Bim$^{-/-}$→RIPmOVA chimeras.

Programmed death 1 **(PD-1)** is an inhibitory receptor of the CD28 superfamily of T cell regulators (165) and is typically expressed on activated T cells but not unstimulated ones (166). Additionally, PD-1 has been associated with CD8^{+} T cell exhaustion (167) and CD8^{+} T cell anergy in transplant tolerance (168). Interestingly, PD-1 expression was higher in thymocytes from OT-I Bim$^{-/-}$ →RIPmOVA chimeras compared to OT-I Bim$^{-/-}$→B6 chimeras, but it was similar to what was observed in an intact B6 thymus **(Fig. 4-7A)**. PD-1 expression was not detectable in splenocytes from either chimera or an intact B6 mouse, but there was a slight upregulation of PD-1 in the pLN of OT-I Bim$^{-/-}$→RIPmOVA chimeras **(Fig. 4-7A)**. This expression pattern coincides with where thymocytes or T cells would encounter OVA, and suggest that PD-1 may play a role in the observed anergy.

T cell Ig- and mucin-domain–containing molecule–3 **(Tim-3)** has been associated with T cell exhaustion in tumor and chronic viral infection models (169). Interestingly, Tim-3 has also been associated with improved bactericidal activity in T cells during tuberculosis infection (169). Tim-3 was not detectable on the surface of T cells from either chimera and likely does not play a role in the functional impairment of OT-I Bim$^{-/-}$→RIPmOVA T cells **(Fig .4-7A)**.

Integrin α_E (CD103) is important for the retention of lymphocytes in epithelial tissues (170). It has also been implicated in Foxp3 expression in T_{reg} (171) and associated with a CD8^{+}Foxp3^{-} T_{reg} population (172). If it were an important tolerance mechanism in OT-I Bim$^{-/-}$→RIPmOVA chimeras, we would expect it to be

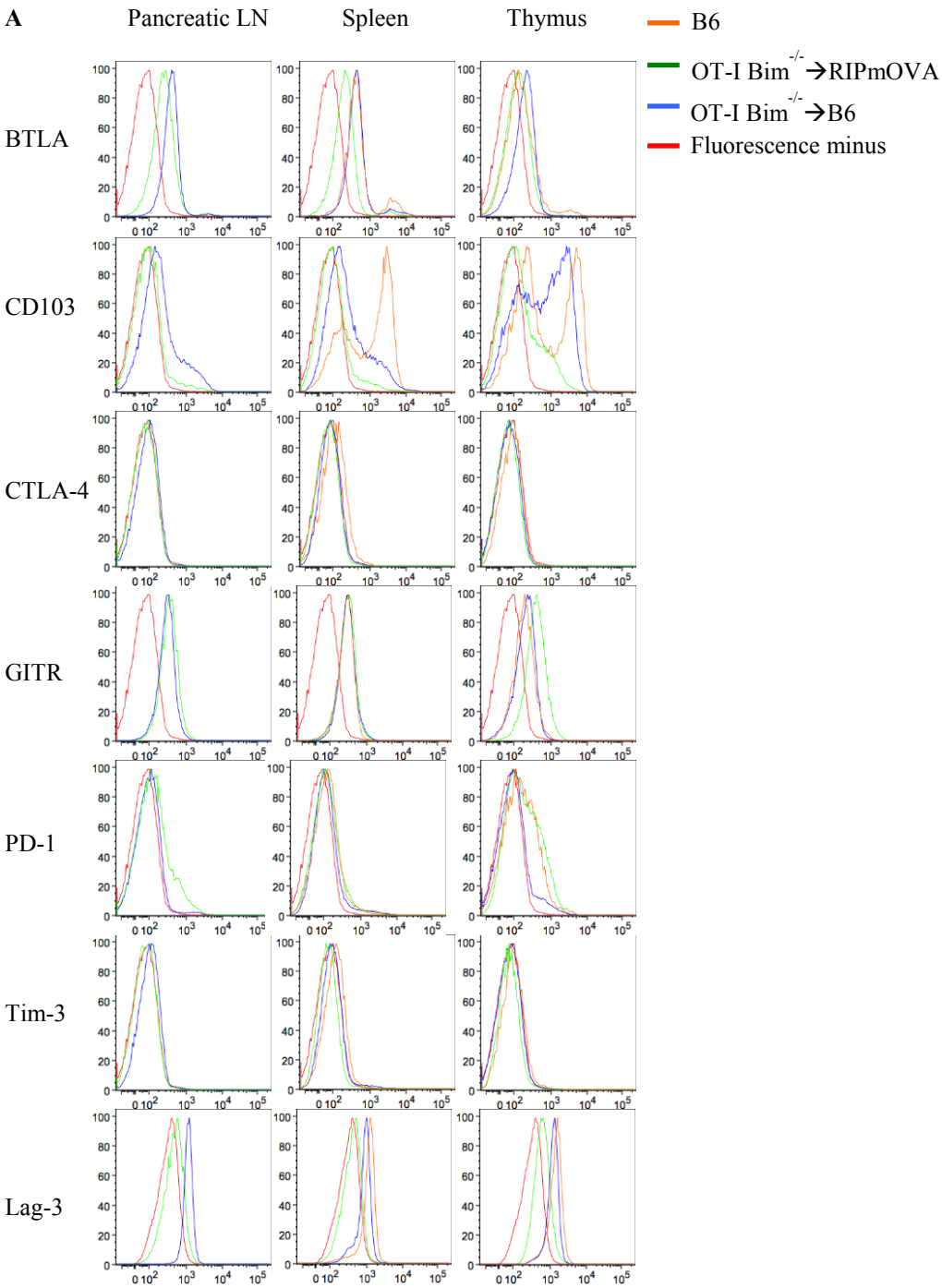
more highly expressed in these chimeras. However, OT-I Bim$^{-/-}$→RIPmOVA T cells from the spleen and pLN had very little CD103 expression **(Fig. 4-7A).** Thymocytes from these mice also had much lower CD103 expression compared to OT-I Bim$^{-/-}$→B6 and intact B6 mice **(Fig. 4-7A)**. This data suggests that if a regulatory population is at work in OT-I Bim$^{-/-}$→RIPmOVA chimeras, it does not involved one of the CD103^{+} regulatory populations previously described.

The transcription factor early growth response gene 2 **(Egr2)** has been shown to be essential in certain SA and tumor-induced anergy models (173). Surprisingly, Egr2 expression was lower in the thymus and pLN of OT-I Bim$^{-/-}$→RIPmOVA chimeras compared to OT-I Bim$^{-/-}$→B6 chimeras and not significantly different in the spleen **(Fig 4-7B)**. The thymus and pLN are where we would expect Egr2 would be more highly expressed in OT-I Bim$^{-/-}$→RIPmOVA chimeras, as that is where T cells would encounter OVA. The fact that the trend is the opposite suggests the anergy mechanism observed in this mice is different that previously described anergic states.

The tumor necrosis factor receptor **(TNFR)** superfamily member glucocorticoid-induced TNFR-related protein **(GITR)** is highly expressed on CD4^{+}CD25^{+}Foxp3^{+} T_{reg} cells (174). However it has been also shown to be cell-intrinsically important for potentiating the clonal expansion of CD8^{+} T cells in viral infection models (175, 176). As such, we would expect GITR expression to be lower in the anergic OT-I Bim$^{-/-}$→RIPmOVA T cells. In contrast, GITR expression in thymocytes from OT-I Bim$^{-/-}$→RIPmOVA was higher than thymocytes from OT-I Bim$^{-/-}$→B6 chimeras, while being not significantly different in the periphery **(Fig. 4-7A)**.

The expression pattern of GITR appears to contradict what is known about its function and the anergic state observed in OT-I Bim$^{-/-}$→RIPmOVA chimeras. However, this may point to the complexities and multiple mechanisms involved in this anergic state.

Helios (Ikzf2) is a member of the Ikaros family zinc finger transcription factors that is upregulated during negative selection and downregulated during positive selection (86, 87). There have been conflicting reports on role of Helios in T cells, some suggesting it has minimal impact on development and function (85), others demonstrating that Helios is essential for stable inhibitory activity of CD4$^+$ and CD8$^+$ T_{reg} (88, 93). Helios was slightly upregulated in the thymus of OT-I Bim$^{-/-}$→RIPmOVA chimeras compared to OT-I Bim$^{-/-}$→B6 chimeras and intact B6 mice **(Fig. 4-7B).** While Helios expression returned to background levels in the spleen and pLN of OT-I Bim$^{-/-}$→B6 chimeras and intact B6 mice, it remained elevated in OT-I Bim$^{-/-}$→RIPmOVA chimeras. This could point to CD8$^+$ T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras gaining regulatory function; it may be a result of antigen encounter; or both mechanisms could be involved.


A
Pancreatic LN
Spleen
Thymus
B6
OT-I Bim-/-→RIPmOVA
OT-I Bim-/-→B6
Fluorescence minus
BTLA
CD103
CTLA-4
GITR
PD-1
Tim-3
Lag-3

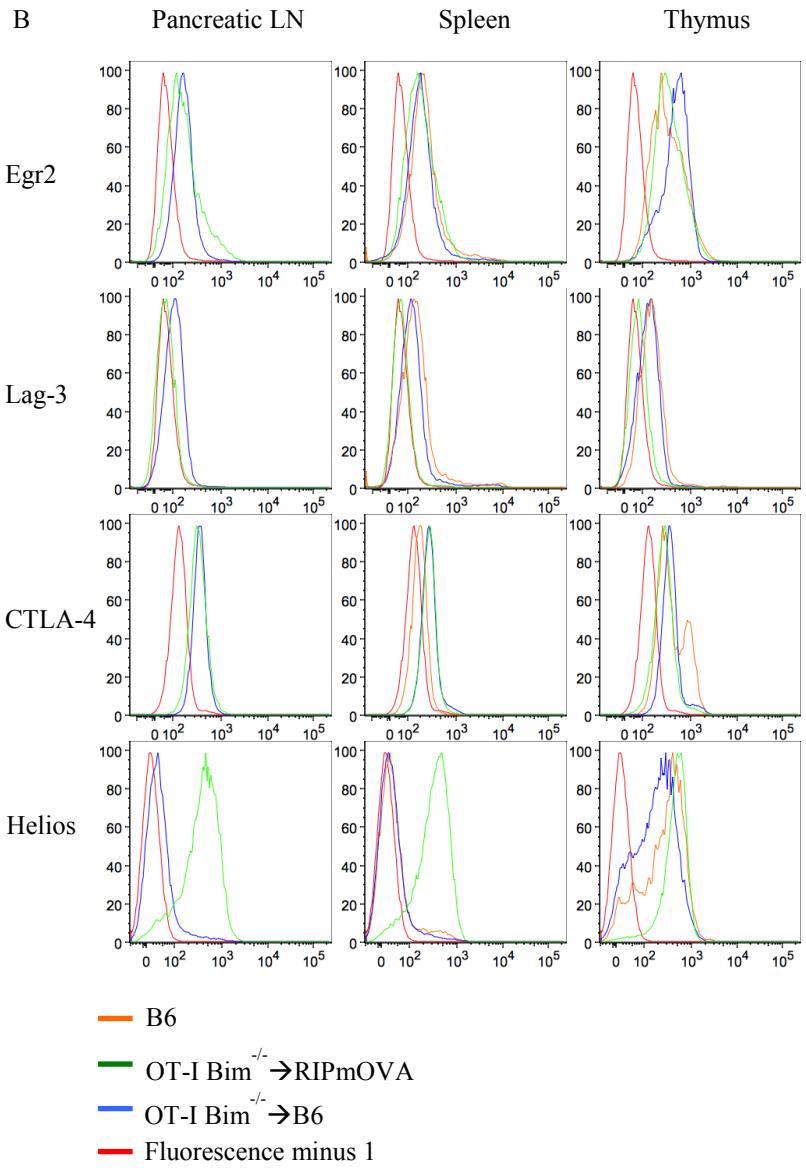
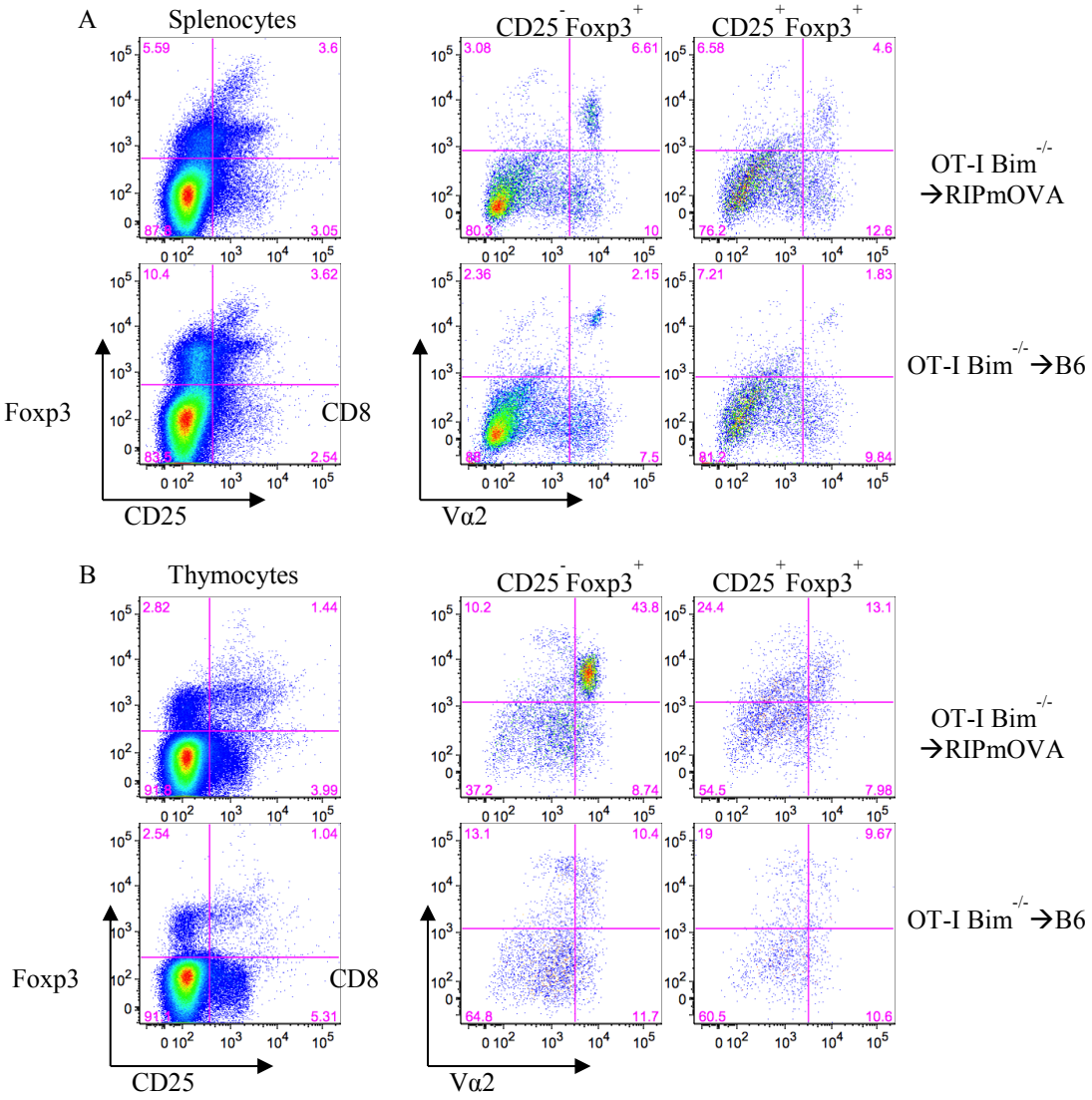


Figure 4-7: Few molecular markers of T cell activation or T cell inhibition coincide with functional data.
A. Cell surface staining of indicated markers *ex vivo*. BTLA (n=7), CD103 (n=7), CTLA-4 (n=7), GITR (n=8), PD-1 (n=7), Tim-3 (n=6), Lag-3 (n=5). **B**. Intracellular staining of indicated markers *ex vivo.* Egr2 (n=3), Lag-3 (n=5), CTLA-4 (n=4), Helios (n=2).

To examine the possibility of a regulatory population, cells from OT-I $Bim^{-/-}$ →B6 and OT-I $Bim^{-/-}$→RIPmOVA chimeras were examined for Foxp3 and CD25 expression. In the pancreatic lymph nodes, spleen and thymus of both chimeras $CD25^{-}Foxp3^{+}$ and $CD25^{+}Foxp3^{+}$ cells were detected **(Fig. 4-8A, B and C)**. Interestingly, in the OT-I $Bim^{-/-}$→RIPmOVA chimeras, the $CD25^{-}Foxp3^{+}$ population was enriched for $V\alpha2^{+}CD8^{+}$ cells. The thymus and pancreatic lymph nodes, both sites of antigen encounter, showed a more pronounced enrichment that was absent in the $CD25^{+}Foxp3^{+}$ population and OT-I $Bim^{-/-}$→B6 chimeras **(Fig. 4-8 A, B and C)**. This suggests the possibility of an antigen specific, $CD8^{+}$ regulatory population at work in this model as has been described in other models (122, 177, 178), however, none of the cell surface markers examined were exclusively co-expressed with the $Foxp3^{+}$ population.


A
Splenocytes
CD25- Foxp3+
CD25+ Foxp3+
OT-I Bim-/- →RIPmOVA
OT-I Bim-/- →B6
Foxp3
CD8
CD25
Vα2
B
Thymocytes
CD25- Foxp3+
CD25+ Foxp3+
OT-I Bim-/- →RIPmOVA
OT-I Bim-/- →B6
Foxp3
CD8
CD25
Vα2

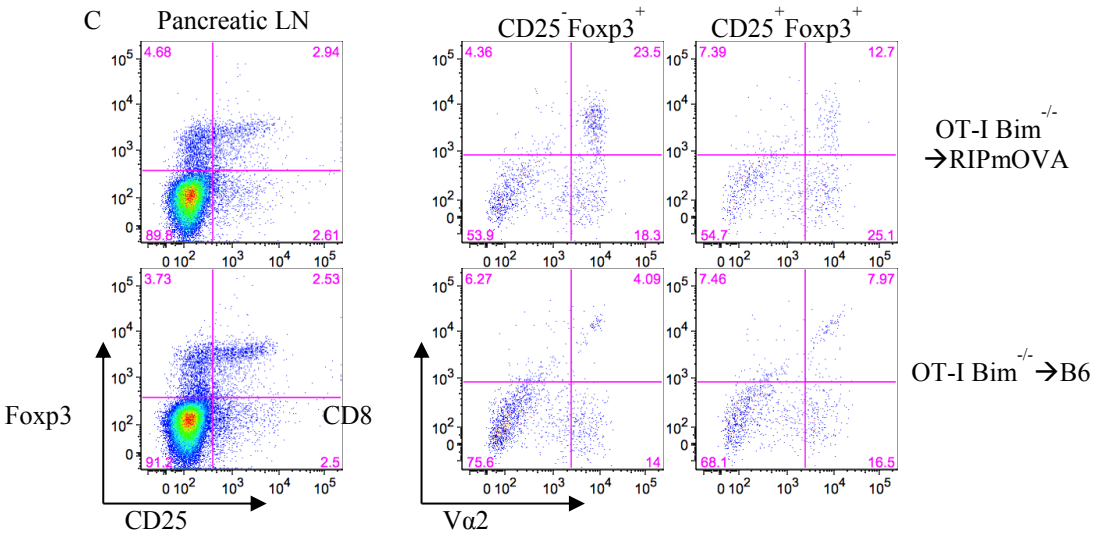


Figure 4-8: The Foxp3⁺CD25⁻ population is enriched for Vα2⁺CD8⁺ T cells
A. Foxp3 by CD25 profile of splenocytes from indicated chimeras (left panel), Vα2 by CD8 profiles of indicated populations from splenocytes (right panel). **B.** Foxp3 by CD25 profile of thymocytes from indicated chimeras (left panel), Vα2 by CD8 profiles of indicated populations from thymocytes (right panel. **C.** Foxp3 by CD25 profile of pancreatic lymphocytes from indicated chimeras (left panel), Vα2 by CD8 profiles of indicated populations from pancreatic lymphocytes (right panel). Representative of at least 7 experiments

Examining T cell activation markers following stimulation

While many co-stimulatory and co-inhibitory molecules did not have any conclusive expression patterns *ex vivo* that could account for the impaired function of T cell from OT-I Bim$^{-/-}$→RIPmOVA chimeras, it is possible that stimulation might uncover such expression patterns.

To examine this possibility, CTV labeled splenocytes from OT-I Bim$^{-/-}$→RIPmOVA and B6 chimeras were stimulated as outlined in **Figure 4.1A**. Vα2^{+}CD8^{+} splenocytes from both chimeras were examined for PD-1, CD28 and CD69 expression on day 2 and 3 following stimulation.

PD-1 was examined because it was slightly upregulated *ex vivo* in sites with cognate antigen encounter **(Fig. 4-7A)** and has been implicated in T cell exhaustion and tolerance.

As several forms of *in vitro* CD4^{+} T cell anergy can be induced by TCR stimulation without CD28 co-stimulation, changes in CD28 expression after stimulation may uncover a role for co-stimulation in this model (41).

CD69 upregulation following stimulation is a TCR proximal event. Following its expression kinetics may shed light on whether there is a block in proximal TCR signaling.

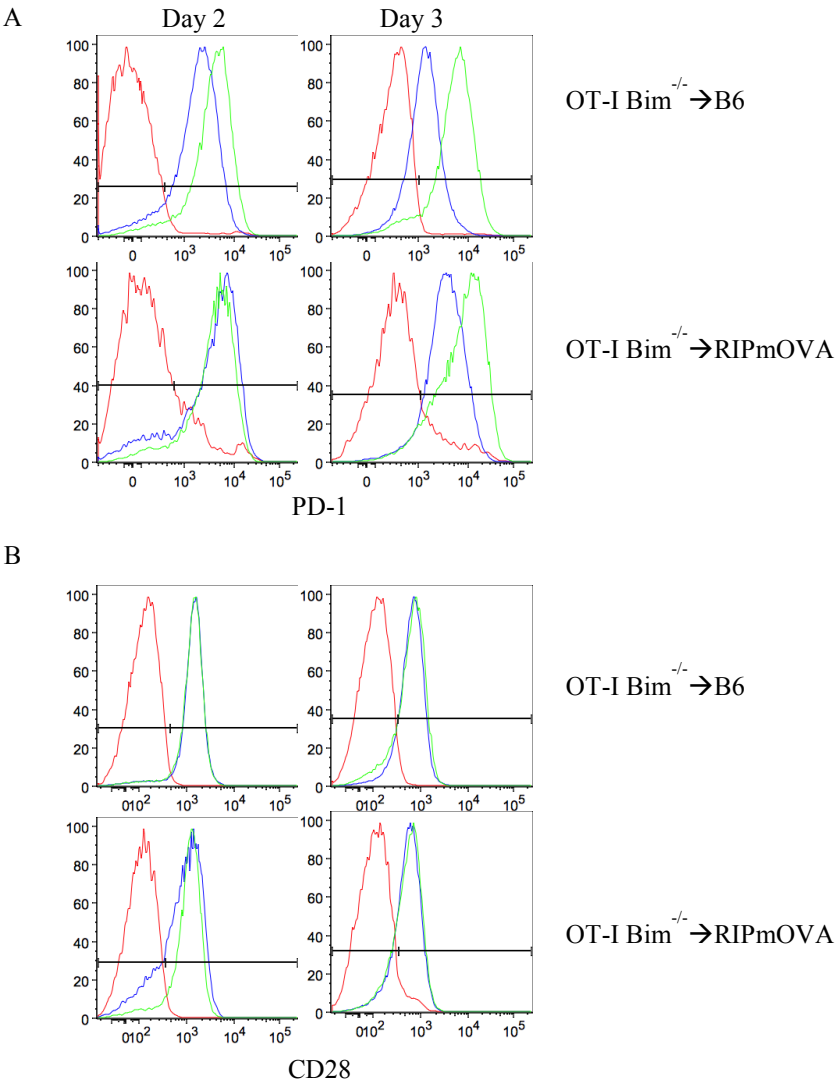
As observed *ex* vivo in the thymus and pLN, PD-1 was more highly expressed on day 2 and 3 in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras after stimulation with SIINFELK-pulsed splenocytes compared to OT-I Bim$^{-/-}$→B6 chimeras and after plate bound αCD3/αCD28 stimulation **(Fig. 4-9A)**. This further

suggests that PD-1 may be playing a role in the observed anergy in OT-I Bim$^{-/-}$ →RIPmOVA T cells

CD28 expression on splenocytes from OT-I Bim$^{-/-}$→RIPmOVA appeared to lag behind on day 2 following SIINFELK-pulsed splenocyte stimulation, but recovers on day 3 to similar levels seen in splenocytes from OT-I Bim$^{-/-}$→B6 chimeras **(Fig 4-9B)**. CD28 expression did not appear to be impaired in splenocytes from OT-I Bim$^{-/-}$ →RIPmOVA chimeras following stimulation with plate bound αCD3/αCD28 when compared to splenocytes from OT- I Bim$^{-/-}$→B6 chimeras **(Fig 4-9B).** These data suggest that impaired co-stimulation by CD28 is not a factor in the establishment or maintenance of anergy in this model.

CD69 expression following stimulation with SIINFELK-pulsed splenocytes was lower on day 2 in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras but higher on day 3 when compared to splenocytes from OT-I Bim$^{-/-}$→B6 chimeras, suggesting that CD69 upregulation lagged behind in OT-I Bim$^{-/-}$→RIPmOVA splenocytes. Despite this, the maximum expression levels of CD69 remained lower in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA than those from OT-I Bim$^{-/-}$→B6 splenocytes **(Fig. 4-9C)**. While the frequency of cells upregulating CD69 following plate bound αCD3/αCD28 stimulation was similar in splenocytes from both chimeras, the magnitude of CD69 expression in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras remains lower compared to OT-I Bim$^{-/-}$→B6 splenocytes **(Fig. 4-9C)**. As CD69 upregulation was impaired and not blocked, it suggests that TCR signaling is intact albeit slightly impaired proximally. This points to mechanisms targeting points further downstream of TCR signaling or co-stimulation as being responsible for the

functional impairment observed in OT-I Bim$^{-/-}$→RIPmOVA chimeras and maybe related to increase cell surface PD-1 in T cells following antigen encounter in OT-I Bim$^{-/-}$→RIPmOVA chimeras.


A
Day 2
Day 3
OT-I Bim-/-→B6
OT-I Bim-/-→RIPmOVA
PD-1
B
OT-I Bim-/-→B6
OT-I Bim-/-→RIPmOVA
CD28
Anti-CD3/Anti-CD28
OVA-pulsed splenocytes
Unstimulated

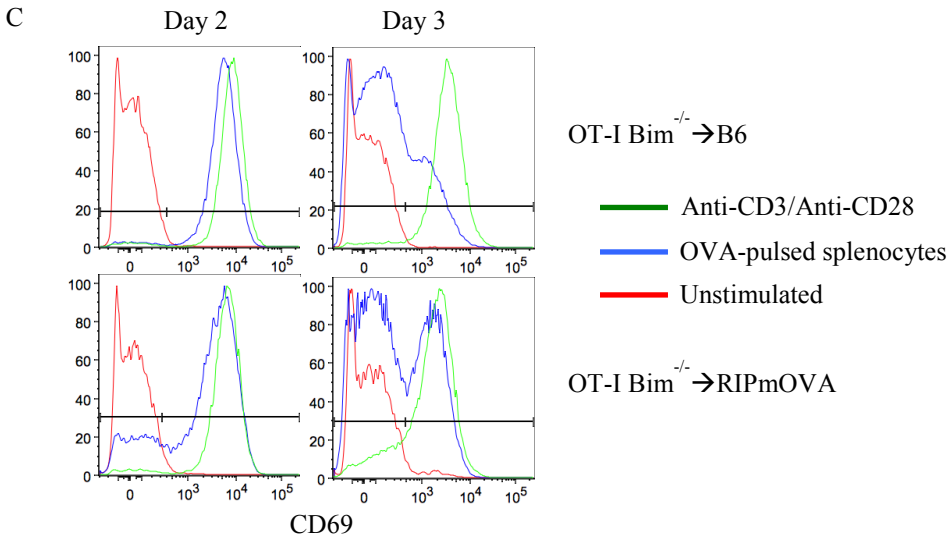


Figure 4-9: Impaired activation
A. PD-1 expression two and three days following stimulation with SIINFEKL-pulsed splenocytes or plate bound αCD3/αCD28 on splenocytes from indicated chimeras. **B.** CD28 expression two and three days following stimulation with SIINFEKL-pulsed splenocytes or plate bound αCD3/αCD28 on splenocytes from indicated chimeras. **C.** CD69 expression two and three days following stimulation with SIINFEKL-pulsed splenocytes or plate bound αCD3/αCD28 on splenocytes from indicated chimeras. Representative of 4 experiments

Impaired lymphopenia induced proliferation

Lymphopenia has been associated with the onset of autoimmune diseases as well as the rescue of function in anergic $CD4^+$ T cells (151, 152).

To determine how lymphopenia may affect the observed functional impairment, we first looked at CD127 expression *ex vivo*. CD127 is the alpha chain of the receptor for IL-7, the primary homeostatic cytokine, and changes in expression may point to functional defects. CD127 was downregulated in $V\alpha2^+CD8^+$ splenocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras compared to intact B6 mice and OT-I $Bim^{-/-}$→B6 chimeras **(Fig. 4-10A)**. $V\alpha2^+CD8^+$ SP thymocytes from both OT-I $Bim^{-/-}$→B6 and RIPmOVA chimeras had reduced CD127 expression compared to intact B6 mice **(Fig. 4-10A)**. These data suggest that thymocytes and splenocytes from OT-I $Bim^{-/-}$→RIPmOVA chimera may be impaired in their ability to respond to IL-7.

To determine if CD127 expression translated into functional impairment, we assessed the ability of splenocytes and thymocytes from OT-I $Bim^{-/-}$→B6 and OT-I $Bim^{-/-}$→RIPmOVA chimeras to undergo lymphopenia induced proliferation **(LIP)**. Splenocytes or thymocytes from OT-I $Bim^{-/-}$→B6 and OT-I $Bim^{-/-}$→RIPmOVA chimeras were CFSE labeled and 2×10^6 were adoptively transferred into sub-lethally irradiated B6 recipients and were examine for LIP seven days later.

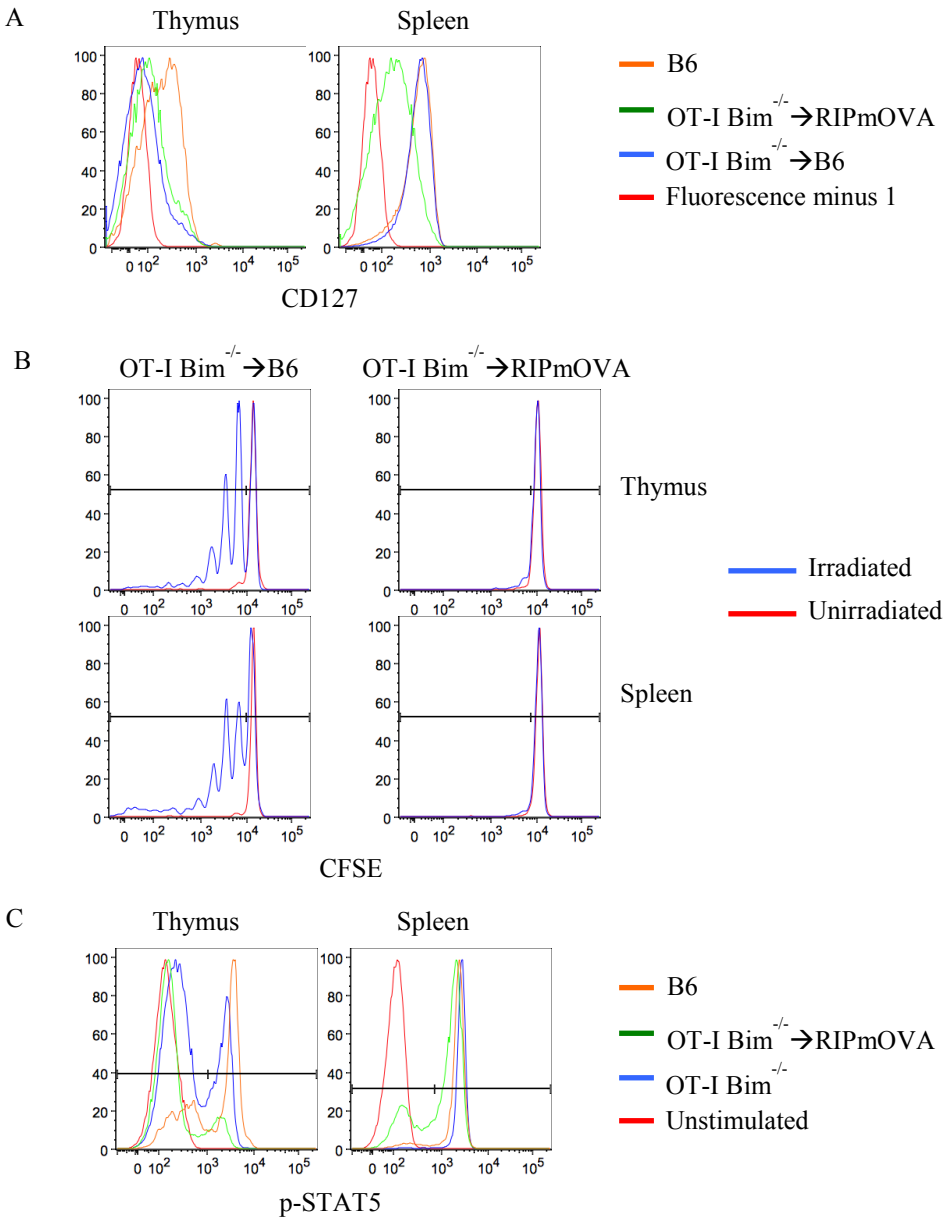


Figure 4-10: Thymocytes and T cells escaping Bim-mediated clonal deletion have impaired LIP
A. Cell surface CD127 expression (n=5). **B.** CFSE dilution 7 days after adoptive transfer of 2 x 10^6 CFSE labeled cells from the indicated source into sub-lethally or unirradiated B6 recipients. (n=3) **C.** STAT5 phosphorylation 20 minutes after stimulation with 25 ng/mL IL-7 in serum free media (n=3).

While OT-I Bim$^{-/-}$→B6 splenocytes were able to undergo robust LIP compared to an unirradiated control, those from OT-I Bim$^{-/-}$→RIPmOVA chimeras were completely unable to undergo LIP **(Fig 4-10B)**. While expressing low levels of CD127, similar to OT-I Bim$^{-/-}$→RIPmOVA chimeras, thymocytes from OT-I Bim$^{-/-}$→B6 chimeras were able to undergo robust LIP much like splenocytes whereas those from OT-I Bim$^{-/-}$→RIPmOVA were not **(Fig. 4-10C)**.

The lack of LIP by thymocytes and splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras may or may not be directly related to signaling through CD127. CD127 signals through the phosphorylation of STAT5.

To examine if signaling through CD127 is impaired in OT-I Bim$^{-/-}$→RIPmOVA thymocytes and splenocytes, cells were stimulated with 25 ng/mL IL-7 for 20 minutes. Very few OT-I Bim$^{-/-}$→RIPmOVA thymocytes phosphorylated STAT5 after stimulation compared to OT-I Bim$^{-/-}$→B6 chimeras and intact B6 mice. Compared to an intact B6, thymocytes from the OT-I Bim$^{-/-}$→B6 chimera had impaired STAT5 phosphorylation, but not to the same degree as OT-I Bim$^{-/-}$→RIPmOVA thymocytes **(Fig 4-10C)**. Splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras also showed impaired STAT5 phosphorylation in terms of frequency and MFI compared to intact B6 mice and OT-I Bim$^{-/-}$→B6 chimeras, but not to the extent seen in thymocytes **(Fig. 4-10C)**.

These data suggest that impaired LIP in thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras may be due to impaired signaling through CD127, but not solely as a result of reduced CD127 expression, as thymocytes from OT-I Bim$^{-/-}$→B6 chimeras express similar levels of CD127, but have markedly better STAT5

phosphorylation. While there is a drastic reduction in CD127 expression in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras, the impairment in STAT5 phosphorylation does not appear as impressive. It is difficult to conclude if the impairment in LIP in splenocytes is solely due to impaired expression and signaling through CD127 or blocks further downstream in signaling.

Discussion

There have been conflicting reports on the role of Bim in T cell function. Ludwinski *et al.* examined Bim deficiency in mouse models of experimental autoimmune encephalitis **(EAE)** and diabetes (143). Mice were immunized with myelin oligodendrocyte glycoprotein 38–50 (MOG 38–50) peptide to generate a model of EAE**.** Diabetes was examined in NOD.CD57BL/6 mice and streptozotocin-treated C57BL/6 mice. Bim^{-}deficient mice were protected from the onset of EAE or autoimmune diabetes (143). While MOG specific T cells were still able to proliferate to antigen, Bim$^{-/-}$ CD4^{+} T cells were deficient in cytokine mRNA expression after stimulation with plate-bound αCD3 and soluble αCD28. This was attributed to Bim-mediated control of the IP_3R/calcium/NFAT pathway. In contrast, in pathogen challenge experiments against *L. monocytogenes* secreting ovalbumin **(LM-OVA)**, OT-I Bim$^{-/-}$ T cells showed no such impairment in effector function (144). Bim-deficient and Bim-sufficient OT-I T cells were able to expand equally to LM-OVA challenge, and expressed similar levels of KLRG-1, CD62L, CD127 and CD27. They were also able to produce similar levels of cytokines and respond similarly to IL-2 complexes *in vivo* and IL-7 *in vitro*. The data from OT-I→B6 and OT-I Bim$^{-/-}$→B6 chimeras clearly supports the findings of pathogen challenge experiments, as Bim deficiency had no effect of the proliferation or cytokine production of OT-I T cells **(Fig. 4-1 and 4-2)**. However, LM infection causes infected DCs to become potent IL-12 producers, which important for the activation of CD8^{+} T cells (179). This is not necessarily reproduced by stimulation with plate-bound αCD3/αCD28 or OVA-pulsed splenocytes. This will warrant further examination of stimulated thymocytes and T

cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras under inflammatory conditions to determine if functionality is affected.

Incidentally, my data can also reconcile some of the observations made by Ludwinski *et al* (143). All three *in vivo* models were models of autoimmunity to TRA. Bim deficiency in those mouse models may have resulted in the similar abrogation of clonal deletion in the thymus and the establishment of a similar anergic phenotype **(Fig. 4-3 and 4-4)**. This may explain why Bim deficiency appeared to protect mice from those autoimmune models. While cytokine production is impaired in the EAE model and OT-I Bim$^{-/-}$→RIPmOVA chimeras, unlike T cells isolated from the EAE model, T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras did not proliferate to antigen stimulation **(Fig. 4-3)(143).** This discrepancy may be the result of the immunization method utilized to induce EAE, as I did not perform any similar immunizations with OT-I Bim$^{-/-}$→RIPmOVA chimeras. This also mirrors the observation that stimulation of splenocytes from OT-I Bim$^{-/-}$→RIPmOVA with plate bound αCD3/α28 rescued proliferation but not cytokine production when compared to splenocyte stimulations **(Fig. 4-3 and 4-4)**. This suggests that regulatory populations or co-inhibitory receptors may inhibit proliferation whereas intrinsic mechanisms may inhibit cytokine production and reinforces the fact that the immune system has developed 'fail-safe' mechanisms to control auto-reactive T cells and limit the potential for autoimmunity. While, Bim$^{-/-}$ CD4^{+} T cells showed significant impairment of cytokine mRNA production(143), stimulated CD8^{+} OT-I Bim$^{-/-}$→RIPmOVA T cells were able to induce cytokine mRNA expression to a similar but slightly reduced extent as OT-I Bim$^{-/-}$→B6 chimeras **(Fig.4-4C)**. It is difficult to directly compare these results as

Bim deficiency may have differing effects on $CD4^+$ versus $CD8^+$ T cells. Additionally, different sets of cytokines were examined. Ludwinski *et al.* did not examine IFNγ or TNFα mRNA expression, while I did not examine IL-2, IL-6 or IL-17A cytokine expression (143).

Lymphopenia has been associated with the induction of autoimmunity (147-150) as well as the rescue of T cells from an anergic state (151, 152). Despite the high sensitivity of OT-I T cells to LIP, OT-I T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras, did not demonstrate any sort of disease induction or reversal of anergy due to lymphopenia **(Fig. 4-5 and 4-10)**. These conflicting results may have resulted from differences in the model systems. King *et al*. utilized NOD mice, which suffer slightly from lymphopenia, but perhaps not to the same extent as would be induced by irradiation (147). Additionally, in this model system, both $CD4^+$ and $CD8^+$ T cells are involved in pathogenesis. Deshpande *et al*. demonstrated that the homeostatic cytokines IL-7 and IL-15 enhanced T cell responsiveness to low-affinity antigen, however the OT-I transgenic TCR used in this chimeric models has a high affinity for its cognate antigen OVA (150). Knoechel *et al*. used the DO11 TCR transgenic model, which generates $CD4^+$ T cell as opposed to the $CD8^+$ T cells generated by the OT-I TCR (148). Additionally, neither Knoechel *et al.* nor Kisand *et al.* examined T cells that would have encountered high affinity antigen in the thymus or were necessarily anergic (148, 149). While Brown *et al.* examined anergic tumor-infiltrating $CD8^+$ T cells, the nature of that anergy may differ from that induced in this model again due to the lack of high affinity antigen encounter in the thymus and perhaps the environmental context of anergy induction (151). These observations

highlight the complexity and different levels of control in T cell activation. While the mechanisms involved in the anergy observed in this model appear to be more robust with respects to lymphopenia than those observed in other models, they may nonetheless provide insight into the many avenues available for modifying T cell activity.

While the lack of functional rescue by exogenous IL-2 contradicts what is normal observed in traditional *in vitro* models of anergy **(Fig 4-6)**(41), it does mirror what has been observed in several developmental models of anergy (180, 181). In both these models, autoreactive thymocytes are not completely deleted. Those that escape to the periphery are anergic and are also not completely rescued by exogenous IL-2. The similarities in response to IL-2 between these two models and OT-I Bim$^{-/-}$→RIPmOVA chimeras suggests perhaps we are observing a more physiological anergic state than that induced *in vitro* through sub-optimal co-stimulation (41).

After examining a variety of co-stimulatory, co-inhibitory and anergy related factors *ex vivo* and after *in vitro* stimulation, its is clear that anergic state imposed on CD8^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA is not enforced by commonly observed anergy, exhaustion or regulatory mechanisms **(Fig 4-7, and 4-9)**. This could stem from the fact that many studies involved CD4^{+} T cells as opposed to CD8^{+} T cells; some studied *in vitro* induced anergy; and most did not involve high affinity antigen encounter in the thymus. This also applies to the lack of functional rescue after the addition of exogenous IL-2 **(Fig 4-6)**. Of all the molecules queried, only PD-1 and Helios demonstrated an expression pattern that correlates with impaired T cell

function **(Fig 4-7)**. The maintenance of Helios expression suggests a $CD8^+$ T cells from OT-I $Bim^{-/-}$→RIPmOVA chimera may take on a regulatory role, and the enrichment of $V\alpha2^+CD8^+$ OT-I T cells in the $Foxp3^+CD25^-$ compartment of OT-I $Bim^{-/-}$→RIPmOVA chimera would support notion. However, neither Helios nor any other of the markers examined were not exclusively co-expressed with Foxp3. As such as I was unable to sort $Foxp3^+CD25^-$ T cells from chimeras to test their regulatory potential.

It must be noted that in these experiments only single concentrations of SIINFEKL peptide and plate-bound αCD3/αCD28 were used for stimulations. It is possible that increasing SIINFEKL peptide during pulsing could result in some functional rescue; experiments with increasing concentration of SIINFEKL during pulsing will need to be done to determine if this is the case. Likewise, the concentration of αCD3 and αCD28 used to coat the plates for stimulation may already be at or above saturating levels. Titration of both antibodies may reveal functional differences between T cells from OT-I $Bim^{-/-}$→RIPmOVA and OT-I $Bim^{-/-}$→B6 chimeras.

Interestingly, while many of the co-stimulatory, co-inhibitory and anergy related factors examined *ex vivo* did not follow expression patterns associated with impaired function, differential expression between OT-I $Bim^{-/-}$→RIPmOVA and OT-I $Bim^{-/-}$→B6 chimeras began in the thymus for many of the markers **(Fig. 4-7).** This observation combined with the more pronounced enrichment for $V\alpha2^+CD8^+$ OT-I $Bim^{-/-}$→RIPmOVA thymocytes in the $Foxp3^+CD25^-$ compartment in the thymus compared to peripheral organs **(Fig. 4-8)** and the impaired LIP by thymocytes **(Fig.**

4-10) from OT-I Bim$^{-/-}$→RIPmOVA chimeras suggests that whatever anergy or tolerance mechanisms being induced begin in the thymus. This is further supported by the fact a key difference between this study and previous studies, is high affinity antigen encounter in the thymus.

Chapter 5: Functional impairment by Bim insufficiency is induced in the thymus

Introduction

In this model system, splenocytes that have escaped Bim-mediated clonal deletion are functionally impaired. T cell anergy has largely been studied as a peripheral mechanism after antigen encounter (41, 181). However, in OT-I Bim$^{-/-}$ →RIPmOVA chimeras, the site of first high affinity antigen encounter is in the thymic medulla. Additionally, many of the changes in *ex vivo* expression of co-inhibitory and co-stimulatory molecules began in the thymus **(Fig. 4-7 and 4-8)** and impaired LIP and IL-7 signaling was already established in thymocytes. This begs the question: is the observed functional impairment a result of peripheral mechanisms, thymic mechanisms or a combination of both? While the notion of thymically induced anergy is not new (180, 182), it has not been as widely studied as peripheral and *in vitro* anergy systems. However many of the development models used to examine anergy involve antigen encounter in the thymus, as a UbA or TRA though that aspect may not have been thoroughly examined (180-189). While these models are not identical, there are many aspects that are shared between several models. Partial and non-response to exogenous IL-2 is one characteristic shared with splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras **(Fig. 4-6)(180-182)**. In contrast, unlike some models of anergy (151, 152), splenocytes and thymocytes from OT-I Bim$^{-/-}$ →RIPmOVA chimeras do not expand homeostatically **(Fig. 4-10)**.

As previously mentioned, OT-I Bim$^{-/-}$→RIPmOVA T cells first encounter antigen in the thymus. This encounter results in changes in *ex vivo* expression of

many co-inhibitory and co-stimulatory molecules that starts on thymocytes. However, the thymus is not the only site of OVA encounter; T cells can also encounter OVA in the pancreatic lymph nodes and pancreas of RIPmOVA mice. PD-1 expression *ex vivo* is more pronounced in pLN compared to splenocytes **(Fig. 4-7)**, as is the enrichment of $V\alpha2^{+}CD8^{+}$ T cells in the $CD25^{-}Foxp3^{+}$ subset **(Fig. 4-9)**. This difference between pancreatic lymphocytes and splenocytes in OT-I $Bim^{-/-}$ →RIPmOVA chimeras suggests that peripheral antigen may play a role in the observed anergy. In fact, persistent antigen encounter has been shown to be important for the maintenance of some anergy models (110, 111, 113, 152).

In this chapter, I will examine where anergy is induced in OT-I $Bim^{-/-}$ →RIPmOVA chimeras as well as the importance of antigen in maintaining this state in the periphery.

Results

Proliferative impairment induced in the thymus

From the data presented in the previous chapter, we saw that many changes in *ex vivo* expression of molecules related to T cell activation, such as CD103, GITR, PD-1, Lag3, and Helios were first detected in thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras **(Fig 4-7).** Additionally, impairments in LIP and STAT5 phosphorylation after IL-7 stimulation were also observable in thymocytes **(Fig 4-10)**. Together these data suggests that the functional impairment observed in peripheral cells may be established in the thymus.

To examine this possibility, thymocytes from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras were harvested and CTV labeled. They were then stimulated with SIINFEKL-pulsed splenocytes as outlined in **Figure 4-1A**.

CTV dilution was almost completely absent on day 3 in Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras, whereas there was abundant dilution observed in Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→B6 chimeras **(Fig 5-1A)**. Both division and proliferation indices of Vα2^{+}CD8SP thymocytes were significantly reduced in OT-Bim$^{-/-}$→RIPmOVA chimeras when compared to OT-I Bim$^{-/-}$→B6 chimeras **(Fig 5-1B).** These data show that the proliferative impairment observed in peripheral cells is induced in the thymus

The proliferation indices were similar for thymocytes and splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras as well as those from OT-I Bim$^{-/-}$→B6 chimeras **(Fig 5-1C and D)**. While not statistically significant, and with the exception of one outlier, thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras appeared to have a lower

division index than splenocytes from the same mouse. The similar proliferation indices may suggest that responding cells from the thymus and spleen of OT-I Bim$^{-/-}$ →RIPmOVA chimeras have similar proliferative capacities. The difference in the division indices may suggest that non-responders from the thymus suffer from a more robust proliferative impairment than those from the spleen.

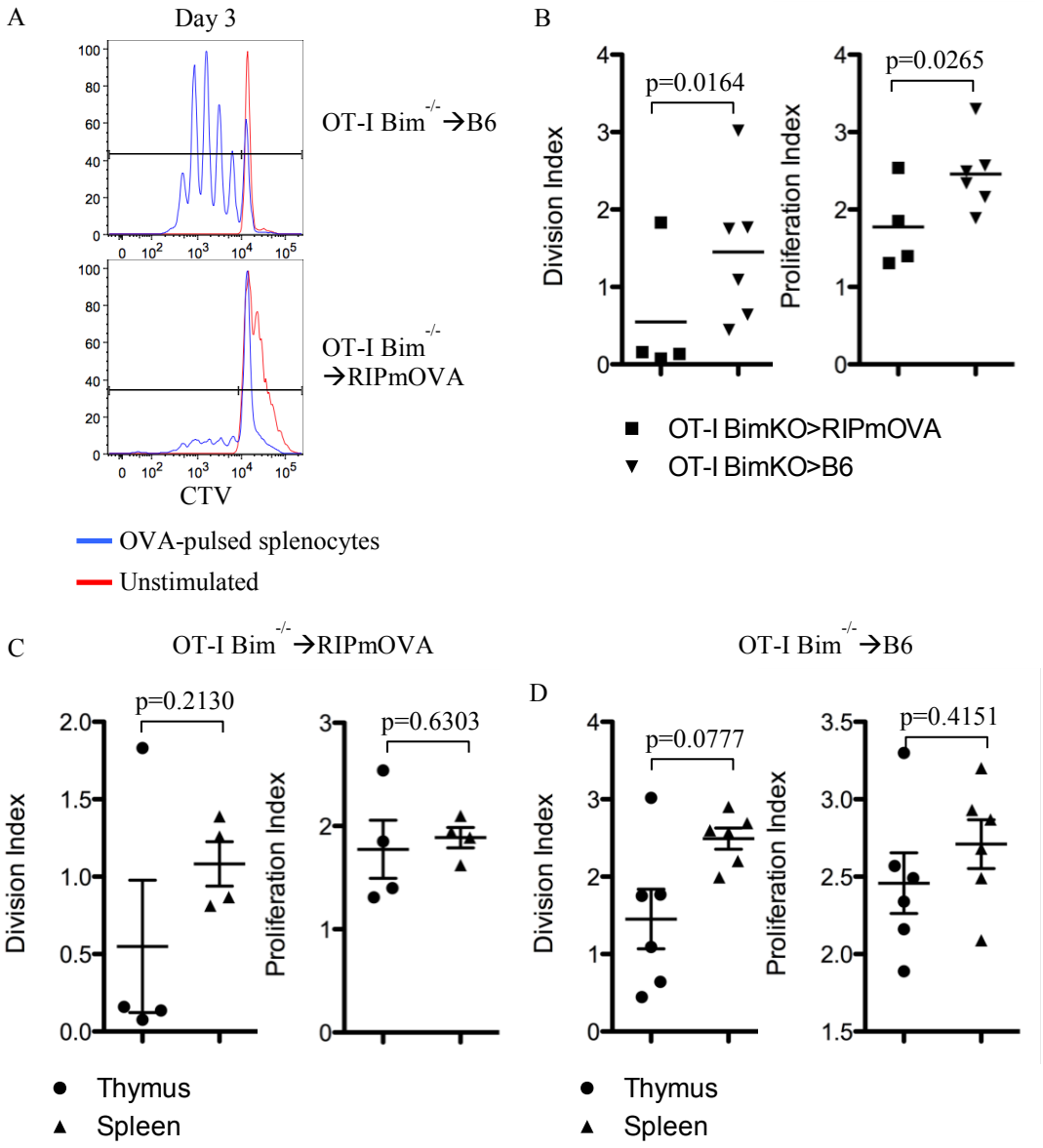


Figure 5-1: Thymocytes are proliferatively impaired
A. CTV dilution of Vα2+CD8SP thymocytes from OT-I Bim-/-→RIPmOVA (n=4) and OT-I Bim-/-→B6 (n=6) chimeras day 3 after stimulation with SIINFEKL-pulsed splenocytes. **B.** Division and proliferation indices of thymocytes from OT-I Bim-/-→RIPmOVA (n=4) and OT-I Bim-/-→B6 (n=6) chimeras day 3 after stimulation with SIINFEKL-pulsed splenocytes. **C.** Division and proliferation indices of thymocytes and splenocytes from OT –I Bim-/-→RIPmOVA chimeras. **D.** Division and proliferation indices of thymocytes and splenocytes from OT –I Bim-/-→B6 chimeras Data are representative of the indicated number of samples.

Examining T cell activation/inhibition markers following stimulation of thymocytes

The proliferative defect appears to be similar, if not more robust in thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras compared to splenocytes from the same mouse. To further examine this possibility, we examined the ability of thymocytes to upregulate PD-1, CD28 and CD69 following stimulation.

Thymocytes from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras were harvested and stimulated with SIINFEKL-pulsed splenocytes as outlined in **Figure 4-1A** and analysed on day 2 and day 3 post stimulation for PD-1, CD28 and CD69 expression.

Similar to Vα2^{+}CD8^{+} splenocytes, Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras upregulated PD-1 to a greater degree than their OT-I Bim$^{-/-}$→B6 counterparts **(Fig. 5-2A)**. Interestingly, unstimulated Vα2^{+}CD8SP thymocytes OT-I Bim$^{-/-}$→RIPmOVA had higher PD-1 expression compared to unstimulated splenocytes from the same chimeras **(Fig. 4-9A, Fig. 5-2A)**. There was also a population of thymocytes expressing high levels of PD-1 in OT-I Bim$^{-/-}$→B6 chimeras that is absent in splenocytes from the same chimera **(Fig. 4-9A, Fig. 5-2A)**. It is not clear why this is the case. In OT-I Bim$^{-/-}$→RIPmOVA chimeras, this may be a result of thymocytes having more recently encountered OVA. In OT-I→B6 chimeras, there was a small population undergoing caspase 3 mediated deletion (**Fig. 3-4 and 3-9**). Perhaps the PD-1^{high} thymocytes in OT-I Bim$^{-/-}$→RIPmOVA represent the subset of thymocytes that would have undergone this deletion process were Bim present. Thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras had higher PD-1 expression than their counterparts from OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 5-2A)**. The more robust

upregulation of PD-1 in stimulated thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras compared to OT-I Bim$^{-/-}$→B6 chimeras reinforces the idea that anergy is induced in the thymus. It also suggests that PD-1 may play a role in the observed anergy in OT-I Bim$^{-/-}$→RIPmOVA chimeras.

CD69 upregulation following stimulation was also impaired in Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras though to a higher degree at day 2 post-stimulation when compared to Vα2^{+}CD8^{+} splenocytes **(Fig. 5-2B)**. By day 3 post-stimulation, CD69 expression in Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA was similar to what is seen in splenocytes from the same mouse **(Fig. 4-9C, Fig. 5-2B).** However, when compared to the unstimulated control, impairment of CD69 upregulation in thymocytes appeared to be more complete with little difference between stimulated and unstimulated thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras. In contrast, CD69 downregulation on day 3 post-stimulation in Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→B6 chimeras was not as pronounced as what is seen in Vα2^{+}CD8^{+} splenocytes from the same mouse **(Fig. 4-9C, Fig. 5-2B)**. The impairment of CD69 induction in thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras suggests that the mechanisms driving anergy are established in the thymus and maintained in the periphery. The more complete impairment in CD69 upregulation further supports the notion that anergy is more robust in thymocytes than splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras.

Interestingly, CD69 expression in unstimulated Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras was higher at day 2 and day 3 post-stimulation compared to those from OT-I Bim$^{-/-}$→B6 chimeras and splenocytes from both

chimeras **(Fig. 4-9C, Fig. 5-2B).** This combined with the increase in PD-1 and in unstimulated Vα2^{+}CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras maybe a result of the thymocytes having recently encountered OVA in the thymus prior to stimulation **(Fig. 5-2)**. PD-1 expression was elevated in Vα2^{+}CD8SP thymocytes and T cells from the pLN of OT-I Bim$^{-/-}$→RIPmOVA chimeras *ex vivo*, both sites of OVA encounter, further suggesting that these increases could be due to OVA encounter in the thymus **(Fig. 4-7A).**

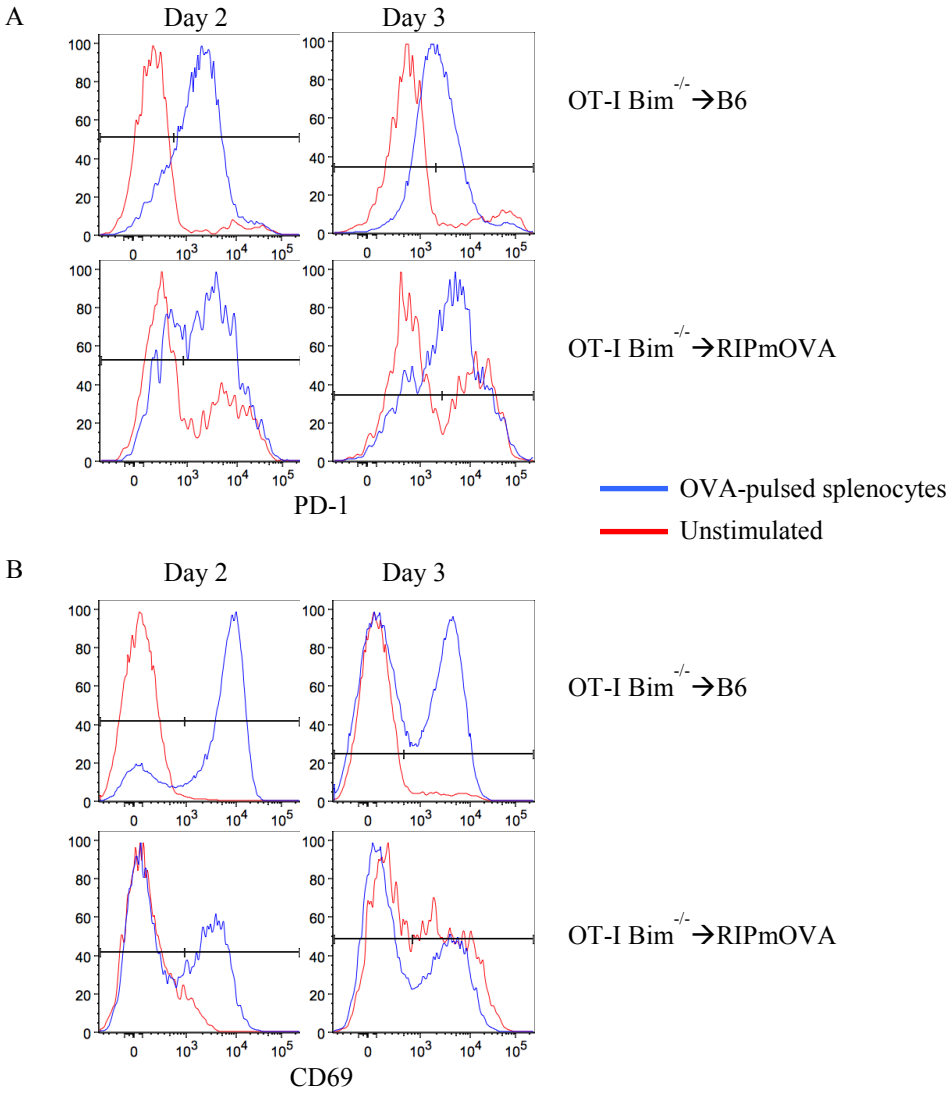


Figure 5-2: Expression of PD-1, CD28 and CD69 post-stimulation in thymocytes
A. PD-1 expression on Vα2⁺CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA (n=4) and OT-I Bim$^{-/-}$→B6 (n=4) chimeras on day 2 and day 3 post-stimulation with SIINFELK-pulsed splenocytes. **B.** CD69 expression on Vα2⁺CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA (n=3) and OT-I Bim$^{-/-}$→B6 (n=3) chimeras on day 2 and day 3 post-stimulation with SIINFELK-pulsed splenocytes.

Generating a model of negative selection to TRA present only in the thymus and not the periphery

Based on the data from the previous sections, it is clear that anergy is induced in thymocytes after high affinity antigen encounter in the thymus of OT-I Bim$^{-/-}$→RIPmOVA chimeras, perhaps to an even greater degree than in splenocytes **(Fig. 5-1 and 5-2)**. If this more robust anergic state is due to more recent OVA encounter in thymus, it would suggest that persistent antigen encounter in the periphery is important for maintaining anergy. Additionally, several models of anergy are reversible in the absence of cognate antigen (110, 111, 113, 152).

To examine this possibility we generated a mouse model where high affinity antigen would be encountered as a TRA in the thymus but would not be present in the periphery **(Fig. 5-3)**. Neonatal thymic lobes from RIPmOVA or B6 mice were transplanted under the kidney capsules of athymic nude B6 mice. These recipients lack a thymus and the T cell compartment will be reconstituted through the transplanted thymic lobes. These mice will then be used as recipients to generate bone marrow chimeras with OT-I Bim$^{-/-}$ BM. OT-I T cells in OT-I Bim$^{-/-}$→RIPmOVA thymus transplanted **(OT-I Bim$^{-/-}$→RT)** mice will encounter high affinity OVA in the thymus, but unlike intact chimeras, they will not encounter OVA in the periphery. OT-I T cells in OT-I Bim$^{-/-}$→B6 thymus transplanted **(OT-I Bim$^{-/-}$→BT)** chimeras will not encounter high affinity OVA similarly to an intact OT-I Bim$^{-/-}$→B6 chimera.

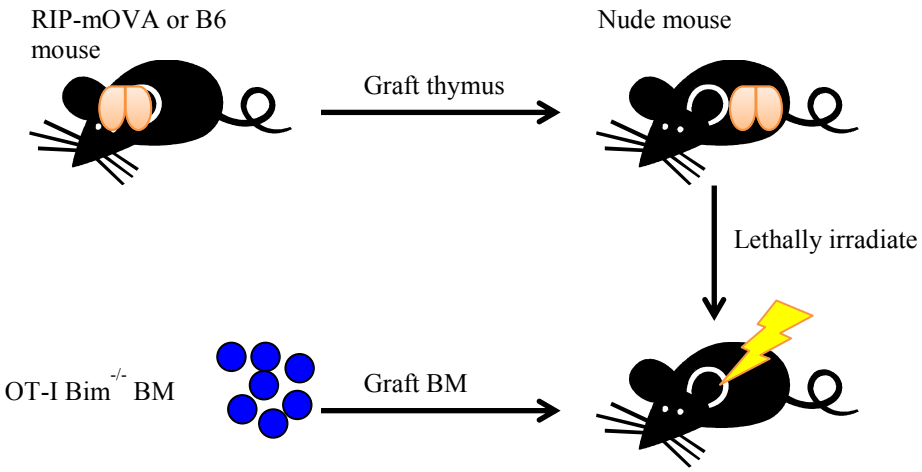


Figure 5-3: Setting up thymus transplanted BM chimeras
Neonatal thymic lobes were harvest from RIPmOVA or B6 mice and transplanted into nude mice. These mice were then used as recipients to generate BM chimeras with OT-I $Bim^{-/-}$ BM.

Examining CD4 by CD8 expression in Vα2^{+} thymocytes isolated from intact and thymus-transplanted chimeras showed that thymocytes development was normal in the thymus-transplanted chimeras **(Fig 5-4A)**. The frequency of thymocytes in each compartment was similar between the intact chimeras and thymus-transplanted chimeras **(Fig 5-4A)**. Examining CD8 SP thymocytes for Vα2 and CD24 expression, it was evident that in both the B6 and RIPmOVA thymus-transplanted chimeras, the majority of CD8 SP thymocytes were mature Vα2^{+}CD24^{lo} OT-I thymocytes, with a slight reduction in the frequency of Vα2^{+}CD24^{lo} thymocytes in the OT-I Bim$^{-/-}$→RIPmOVA thymus-transplanted chimera **(Fig. 5-4B)**. These data demonstrate that thymocyte development is reconstituted in the nude mice that have been transplanted with neonatal RIPmOVA or B6 thymic lobes.

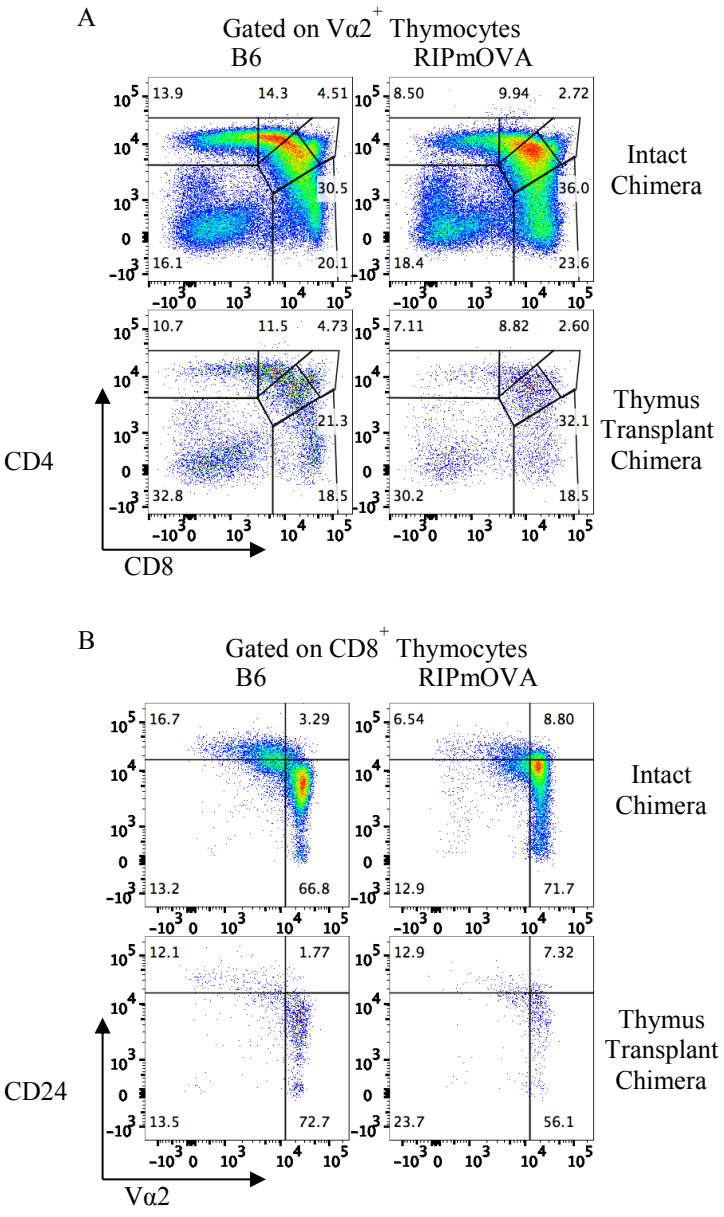


Figure 5-4: Thymocyte development is normal in thymus transplant chimeras
A. CD4 by CD8 profile of Vα2+ thymocytes from the indicated chimeras. **B.** Vα2 by CD24 profile of CD8SP thymocytes from the indicated chimeras. OT-I Bim$^{-/-}$→RIPmOVA (n=46), OT-I Bim$^{-/-}$→B6 (n=46), OT-I Bim$^{-/-}$→RT (n=4), OT-I Bim$^{-/-}$→RT (n-4).

While thymocyte development in the thymus-transplanted chimeras closely mirrored what was observed in the intact chimeras, this was not the case with splenocytes from the thymus-transplanted chimeras. The frequency of $V\alpha2^+$ splenocytes in the spleens of thymus-transplanted chimeras was lower than in intact chimeras **(Fig. 5-5A).** However, this is to be expected, as the transplanted neonatal thymic lobes are much smaller than the thymi of intact chimeras. This would result in a smaller output of T cells from the transplanted thymic lobes, accounting for the lower frequency of antigen-specific $V\alpha2^+$ splenocytes. Focusing on the CD4 by CD8 profiles of $V\alpha2^+$ splenocytes compartment from the intact and thymus-transplanted chimeras, it is clear there were a similar frequency and distribution $CD4^+$ and $CD8^+$ splenocytes in all the chimeras (**Fig 5-5B).**

CD8 dulling can be associated with high affinity antigen encounter and was present in CD8 SP thymocytes from both OT-I $Bim^{-/-}$→RIPmOVA and OT-I $Bim^{-/-}$→RT chimeras when compared to their B6 counterparts **(Fig. 5-6A).** However, the CD8 dulling that was observed in $V\alpha2^+CD8^+$ splenocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras was not present in $V\alpha2^+$ $CD8^+$ splenocytes from OTI $Bim^{-/-}$→RT chimeras **(Fig. 5-6A).** CD8 expression in $V\alpha2^+CD8^+$ splenocytes from OT-I $Bim^{-/-}$→RT chimeras returned to similar levels observed in control OT-I $Bim^{-/-}$→BT chimeras.

Additionally, Nur77 is known to be induced following TCR activation and thus cognate antigen encounter (49). Nur77-GFP was clearly induced to a greater degree in $V\alpha2^+$CD8 SP thymocytes from OT-I $Bim^{-/-}$→RIPmOVA and OT-I $Bim^{-/-}$→RT chimeras compared to the B6 control chimeras **(Fig. 5-6B).** In contrast, Nur77-GFP was only induced in $V\alpha2^+CD8^+$ splenocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras.

$V\alpha2^+CD8^+$ splenocytes from OT-I $Bim^{-/-}$→RT chimeras expressed little Nur77-GFP, much like splenocytes from the OT-I $Bim^{-/-}$→BT chimeras **(Fig. 5-6B)**.

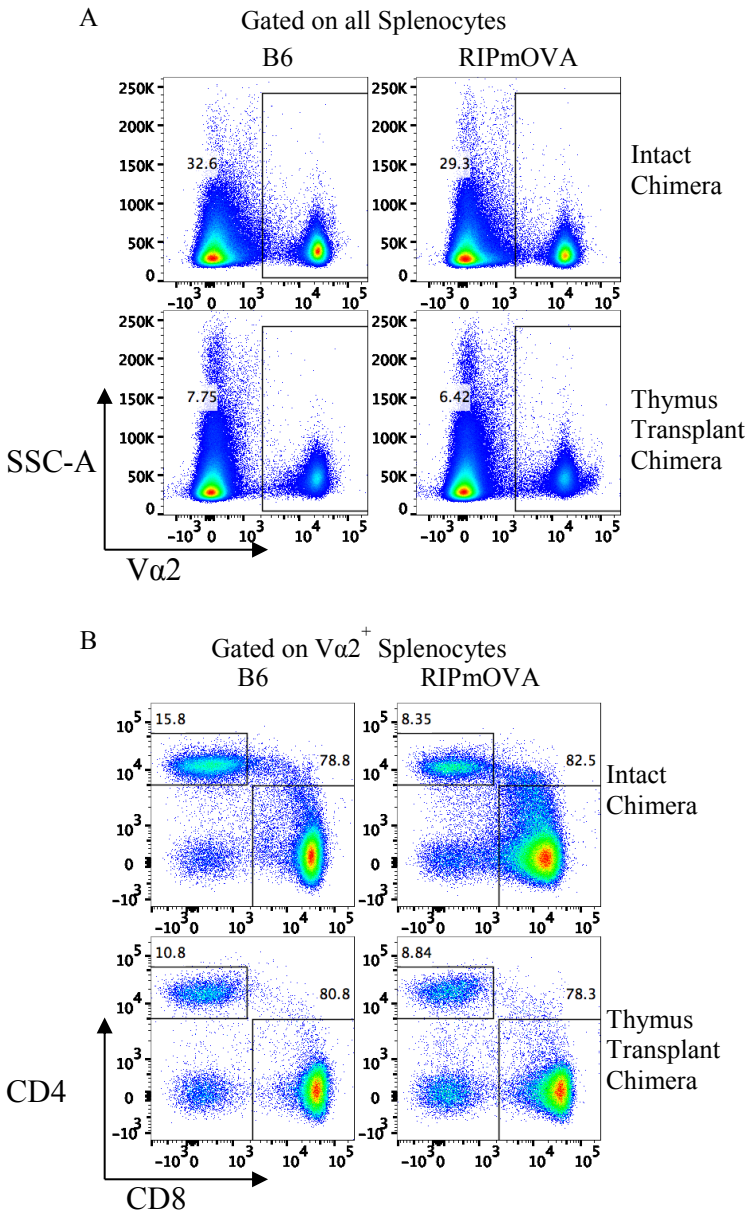


Figure 5-5: Splenocytes from RIPmOVA thymus transplant chimeras phenotypically resemble those from control B6 chimeras rather than an intact OT-I Bim$^{-/-}$→RIPmOVA chimera
A. Vα2 expression within splenocytes from the indicated chimeras. **B.** CD4 by CD8 profile of Vα2^{+} splenocytes from the indicated chimeras. OT-I Bim$^{-/-}$→RIPmOVA (n=46), OT-I Bim$^{-/-}$→B6 (n=46), OT-I Bim$^{-/-}$→RT (n=4), OT-I Bim$^{-/-}$→BT (n-4).

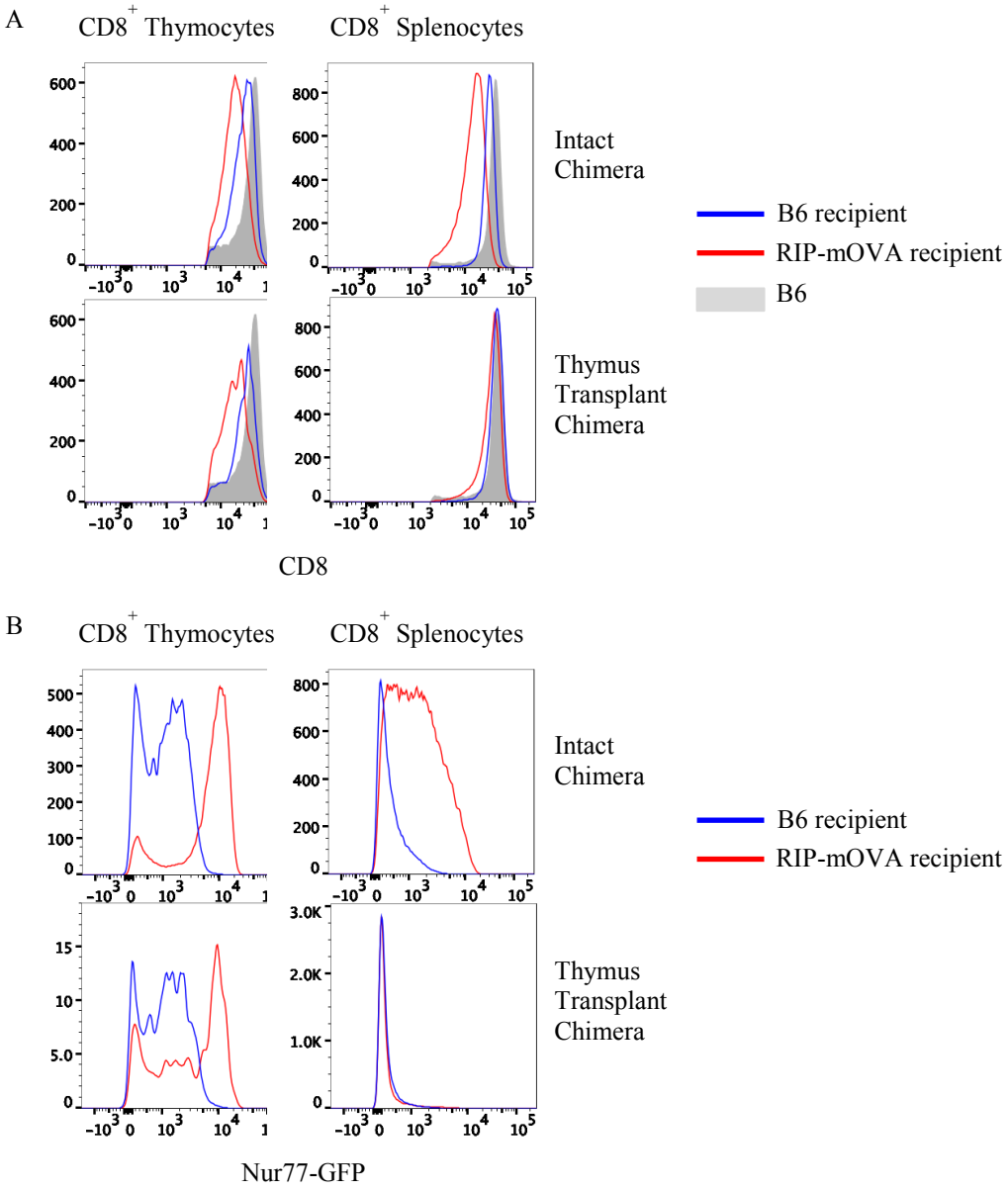


Figure 5-6: CD8 dulling and Nur77 induction absent in splenocytes from RIPmOVA thymus transplant chimeras

A. CD8 expression in Vα2+CD8+ thymocytes and splenocytes from the indicated chimeras. OT-I Bim-/- →RIPmOVA (n=46), OT-I Bim-/-→B6 (n=46), OT-I Bim-/-→RT (n=4), OT-I Bim-/-→BT (n-4). **B.** Nur77-GFP expression in Vα2+CD8+ thymocytes and splenocytes from the indicated chimeras. OT-I Bim-/- →RIPmOVA (n=3), OT-I Bim-/-→B6 (n=3), OT-I Bim-/-→RT (n=4), OT-I Bim-/-→BT (n=4). Data are representative of the indicated number of samples.

As CD8 dulling and Nur77-GFP induction was observed in the thymi of OT-I Bim$^{-/-}$→RT and OT-I Bim$^{-/-}$→RIPmOVA chimeras, it suggest that this phenotype results from OVA encounter by thymocytes in the thymic medulla of these chimeras.

The lack of CD8 dulling and Nur77 induction in splenocytes from OT-I Bim$^{-/-}$→RT chimeras suggests that they are not encountering OVA in the periphery, and that OVA is present only in the thymi of these chimeras. It also suggests that thymically induced molecular patterns may require peripheral antigen encountered to be maintained. By extension, this raises the possibility that the functional impairment observed in OT-I Bim$^{-/-}$→RIPmOVA chimeras requires peripheral antigen encounter to be maintained.

Thymocyte anergy induced in thymus transplant chimeras is similar to that observed in intact chimeras

Before assessing whether anergy in peripheral cells is intact, it must be determined if thymocytes from thymus transplant chimeras behave in a similar fashion to those from intact chimeras upon stimulation.

Thymocytes from intact and thymus-transplanted chimeras were harvested and stimulated as outlined in **Figure 4-1.** Cells were analysed for functionality through proliferation and CD69. Due to small numbers, thymocytes were analysed qualitatively for proliferation through c-Myc staining. The expression of c-Myc is rapidly, but transiently induced following T cell activation through the TCR and IL-2R (190).

Looking at CTV dilution and c-Myc expression in stimulated splenocytes from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras, it is clear that the induction

and reduction of c-Myc expression can be used to qualitatively assess proliferation of T cells **(Fig. 5-7).** On day 1, there were already differences in c-Myc expression between Vα2^{+}CD8^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras, before any proliferation occurred. A smaller frequency of OT-I Bim$^{-/-}$→RIPmOVA Vα2^{+}CD8^{+} T cells upregulated c-Myc day 1 after stimulation and to a lesser degree than OT-I Bim$^{-/-}$→B6 counterparts **(Fig 5-7)**. By day 2 post-stimulation, while the frequency of Vα2^{+}CD8^{+} OT-I Bim$^{-/-}$→RIPmOVA T cells inducing c-Myc expression remained lower, the level of c-Myc induced in responding cells was similar to corresponding cells from OT-I Bim$^{-/-}$→B6 chimeras **(Fig 5-7)**. Similarly, at day 3 post-stimulation, frequency of Vα2^{+}CD8^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras inducing c-Myc was lower than those from OT-I Bim$^{-/-}$→B6 chimeras. However, the level of c-Myc expression in the entire responding population was similar in cells from the two chimeras **(Fig 5-7).** However, with successive divisions, Vα2^{+}CD8^{+} OT-I Bim$^{-/-}$→RIPmOVA T cells showed increasing c-Myc levels. In contrast, those from OT-I Bim$^{-/-}$→B6 chimeras showed decreasing c-Myc levels **(Fig 5-7).** Based on this, examining c-Myc expression, two days after stimulation will best allow us to assess proliferation.

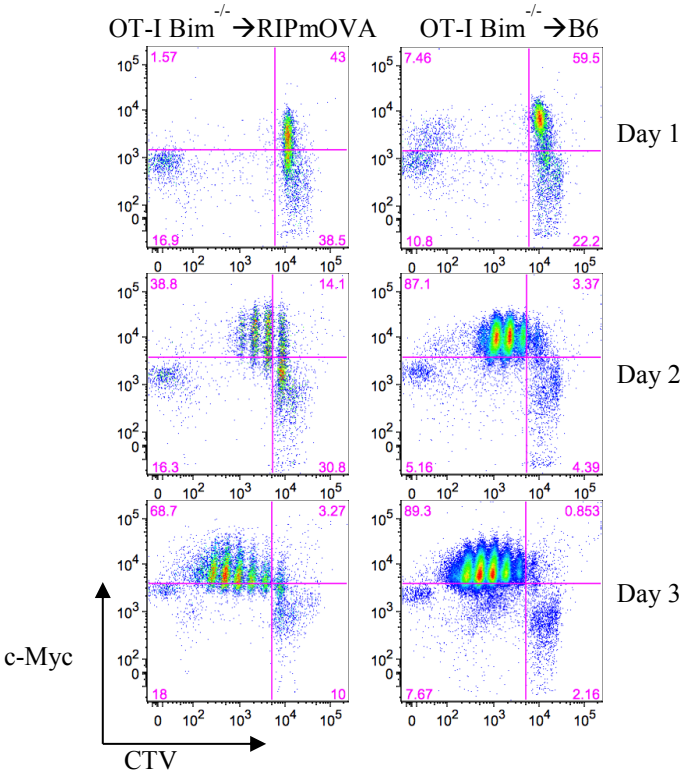


Figure 5-7: c-Myc expression correlates with proliferative capacity of T cells
CTV dilution by c-Myc expression in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras after stimulation with SIINFELK-pulsed splenocytes at day 1, 2 and 3.

CD69 induction was impaired in thymocytes from OT-I Bim$^{-/-}$→RT chimeras compared to those from OT-I Bim$^{-/-}$→BT and intact chimeras **(Fig. 5-8A)**. Unstimulated cells from both the intact and thymus transplant RIPmOVA chimeras had increase CD69 expression when compared to their B6 counterparts **(Fig 5-8A)**.

Much like the data from intact RIPmOVA chimeras, thymocytes from OT-I Bim$^{-/-}$→RT chimeras were impaired in their ability to induce c-Myc day 2 post-stimulation when compared to the appropriate B6 chimera **(Fig. 5-8B).**

These data suggest that the functional impairment induced in the thymic lobes of OT-I Bim$^{-/-}$→RT chimeras, is similar to that observed in intact OT-I Bim$^{-/-}$→RIPmOVA chimeras **(Fig. 5-2, 5-8).** Additionally thymocytes from OT-I Bim$^{-/-}$→BT chimeras behave similarly to their intact chimera counterparts **(Fig. 5-2, 5-8)**. This establishes that thymocyte development and function in the thymus transplant chimeras are similar to that of intact chimeras.

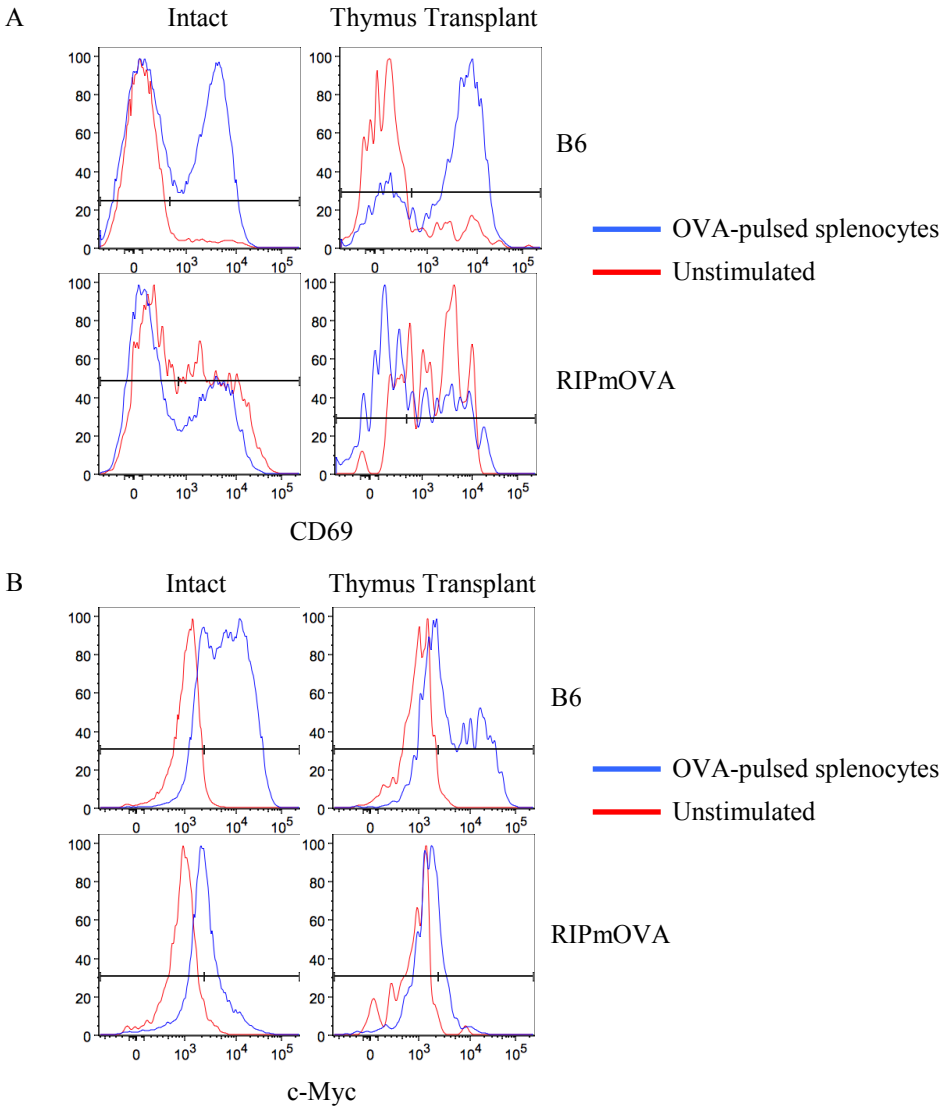


Figure 5-8: OVA encounter induces anergy in OT-I Bim$^{-/-}$→RT chimeras
A. CD69 expression day 3 post-stimulation in indicated chimeras. OT-I Bim$^{-/-}$→RIPmOVA (n=3), OT-I Bim$^{-/-}$→B6 (n=3), OT-I Bim$^{-/-}$→RT (n=2), OTI-I Bim$^{-/-}$→BT (n=2). **B.** c-Myc expression day 2 post-stimulation in indicated chimeras. OT-I Bim$^{-/-}$→RIPmOVA (n=4), OT-I Bim$^{-/-}$→B6 (n=4), OT-I Bim$^{-/-}$→RT (n=4), OTI-I Bim$^{-/-}$→BT (n=4). Gated on Vα2^{+}CD8^{+} SP thymocytes.

Peripheral anergy is maintained by cognate antigen encounter

Since peripheral T cells appear to require peripheral antigen encounter to maintain the phenotypic changes established in the thymus of OT-I Bim$^{-/-}$→RIPmOVA chimeras, it stands to reason that the thymically-induced anergic state induced in the thymus of OT-I Bim$^{-/-}$→RIPmOVA chimeras also requires persistent antigen encounter. In the previous section, it was demonstrated that the anergic phenotype also develops in the thymic lobes of RIPmOVA thymus transplant chimeras.

To determine if anergy requires persistent antigen encounter, splenocytes from intact and thymus transplanted chimeras were harvest and stimulated as outlined in **Figure 4-1.** Cells were analysed for functionality through proliferation, cytokine production and the ability to induce PD-1 and CD69.

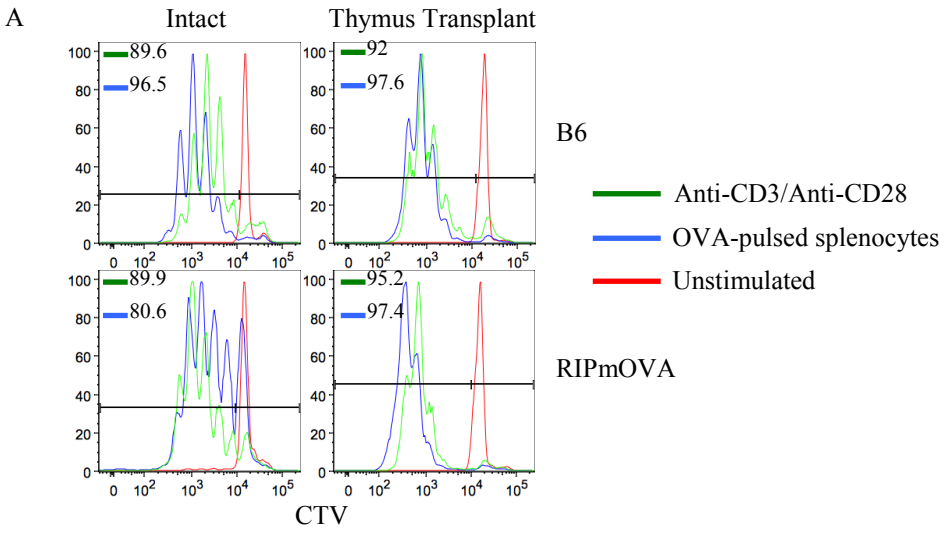
Splenocytes from OT-I Bim$^{-/-}$→RT chimeras divided more robustly on day 3 post-stimulation with SIINFEKL-pulsed splenocytes or plate bound αCD3/αCD28 than those from OT-I Bim$^{-/-}$→RIPmOVA chimeras, as assessed by CTV dilution **(Fig. 5-9A)**. While splenocytes from OT-I Bim$^{-/-}$→BT chimeras also proliferated more extensively than OT-I Bim$^{-/-}$→B6 chimeras, their proliferative capacity was on par with those from OT-I Bim$^{-/-}$→RT chimeras **(Fig. 5-9A)**. This is more evident upon examining the division and proliferation indices for SIINKFEKL-pulsed splenocyte and plate-bound αCD3/αCD28 stimulation **(Fig. 5-9B and C)**. While there was a significant reduction in both the division and proliferation indices for OT-I Bim$^{-/-}$→RIPmOVA chimeras compared to OT-I Bim$^{-/-}$→B6 chimeras on day 3 post-stimulation with SIINFEKL-pulsed splenocytes, there was no significant difference

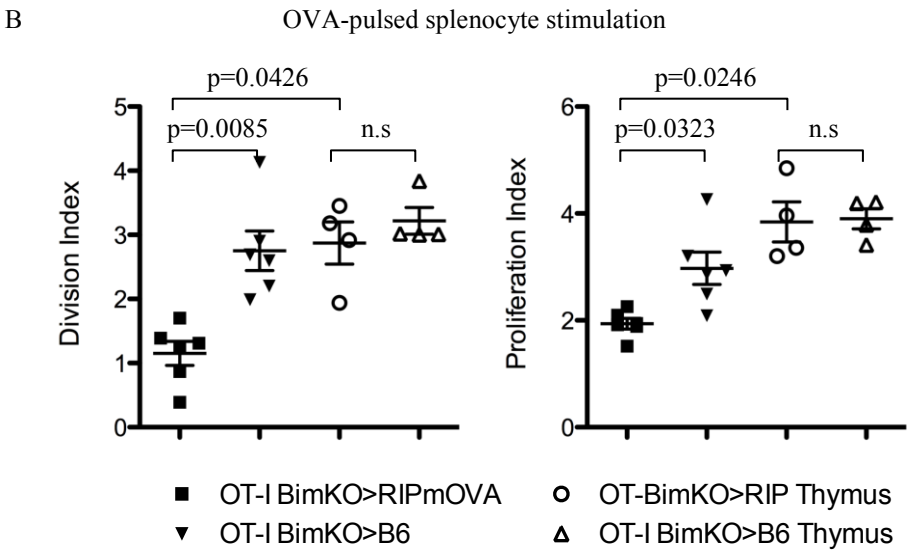
between both division and proliferation indices of OT-I Bim$^{-/-}$→RT and OT-I Bim$^{-/-}$→BT chimeras **(Fig. 5-9B)**. Much like in the intact chimeras, splenocytes from OT-I Bim$^{-/-}$→RT and OT-I Bim$^{-/-}$→BT chimeras proliferated similarly to plate-bound αCD3/αCD28 stimulation. These data demonstrate that in the absence of peripheral OVA, the proliferative defect established in the thymus of OT-I Bim$^{-/-}$→RT chimeras is not maintained in the periphery.

Interestingly, PD-1 and CD28 induction in splenocytes from OT-I Bim$^{-/-}$→RT and OT-I Bim$^{-/-}$→BT were very similar to what is seen in intact chimeras **(Fig 5-10A and B)**. Splenocytes from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→RT chimeras induced PD-1 to similar levels. When compared to OT-I Bim$^{-/-}$→B6 and OT-I Bim$^{-/-}$→BT chimeras respectively, they had slightly enhanced PD-1 induction day 3 after SIINFEKL-pulsed splenocyte and plate-bound αCD3/αCD28 stimulation **(Fig. 5-10A).** CD28 was induced to a similar degree in all four chimeras **(Fig. 5-10B)**. This suggests that PD-1 and CD28 may not play a role in the anergic state observed in OT-I Bim$^{-/-}$→RIPmOVA chimeras.

As previously shown, CD69 induction lagged behind in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras. They had reduced CD69 expression at day 2 post-stimulation with SIINFEKL-pulsed splenocytes and by day 3 post-stimulation, while splenocytes from OT-I Bim$^{-/-}$→B6 were down regulating CD69 expression, those from OT-I Bim$^{-/-}$→RIPmOVA chimeras were still expressing CD69 **(Fig. 5-10C and D)**. Splenocytes from both OT-I Bim$^{-/-}$→BT and OT-I Bim$^{-/-}$→RT chimeras induced CD69 to similar levels and kinetics to those from OT-I Bim$^{-/-}$→B6 chimeras **(Fig. 5-10C and D)**. As CD69 upregulation is a TCR proximal event, this data suggests that

mechanisms modulating TCR signaling may mediate the functional defect observed in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras as well as the functional rescue of splenocytes from OT-I Bim$^{-/-}$→RT chimeras.


A
Intact
Thymus Transplant
89.6
96.5
92
97.6
89.9
80.6
95.2
97.4
B6
RIPmOVA
CTV
Anti-CD3/Anti-CD28
OVA-pulsed splenocytes
Unstimulated


B
OVA-pulsed splenocyte stimulation
p=0.0426
p=0.0085
n.s
p=0.0246
p=0.0323
n.s
Division Index
Proliferation Index
OT-I BimKO>RIPmOVA
OT-I BimKO>B6
OT-BimKO>RIP Thymus
OT-I BimKO>B6 Thymus

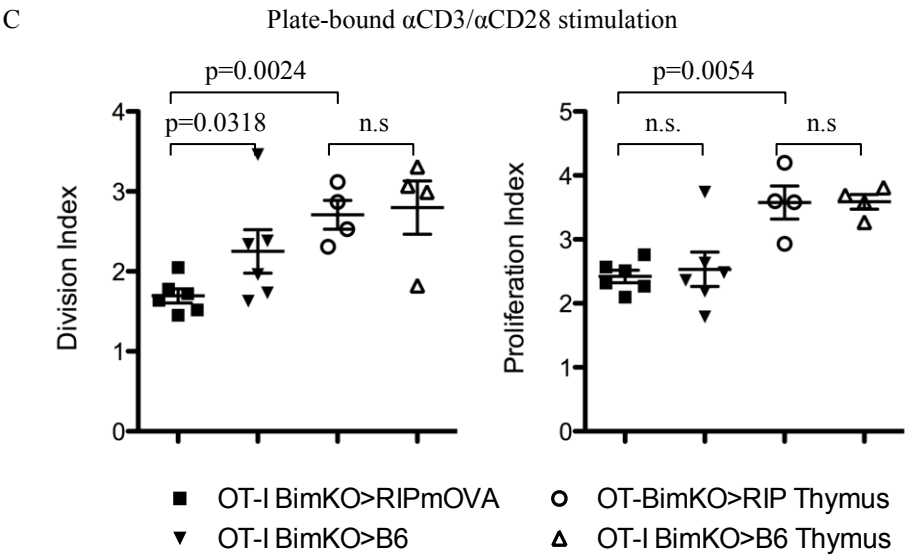
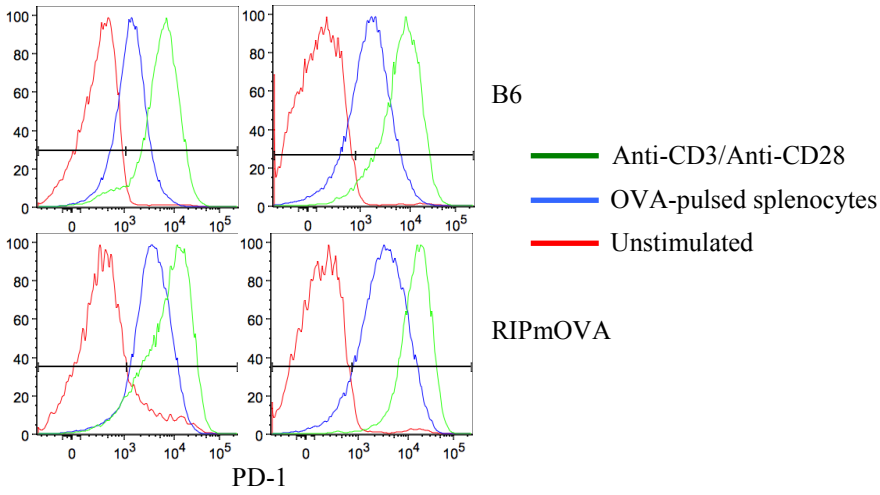


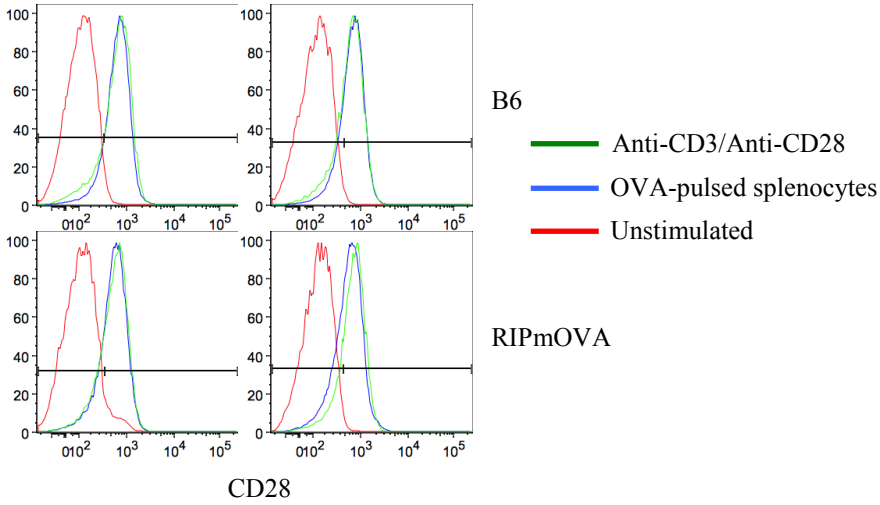
Figure 5-9: Peripheral antigen is required to maintain proliferative impairment in OT-I Bim$^{-/-}$→RIPmOVA chimeras

A. CTV dilution of the Vα2^{+}CD8^{+} T cells from indicated samples day 3 after stimulation with SIINFEKL-pulsed splenocytes or plate-bound αCD3/αCD28. **B.** Division and proliferation indices day 3 post-stimulation with SIINFEKL-pulsed splenocytes. **C.** Division and proliferation indices day 3 post-stimulation with plate-bound αCD3/αCD28. OT-I Bim$^{-/-}$→RIPmOVA (n=6), OT-I Bim$^{-/-}$→B6 (n=6), OT-I Bim$^{-/-}$→RT (n=4), OTI-I Bim$^{-/-}$→BT (n=4).

A


Intact
Thymus Transplant
B6
RIPmOVA
Anti-CD3/Anti-CD28
OVA-pulsed splenocytes
Unstimulated
PD-1

B


B6
RIPmOVA
Anti-CD3/Anti-CD28
OVA-pulsed splenocytes
Unstimulated
CD28

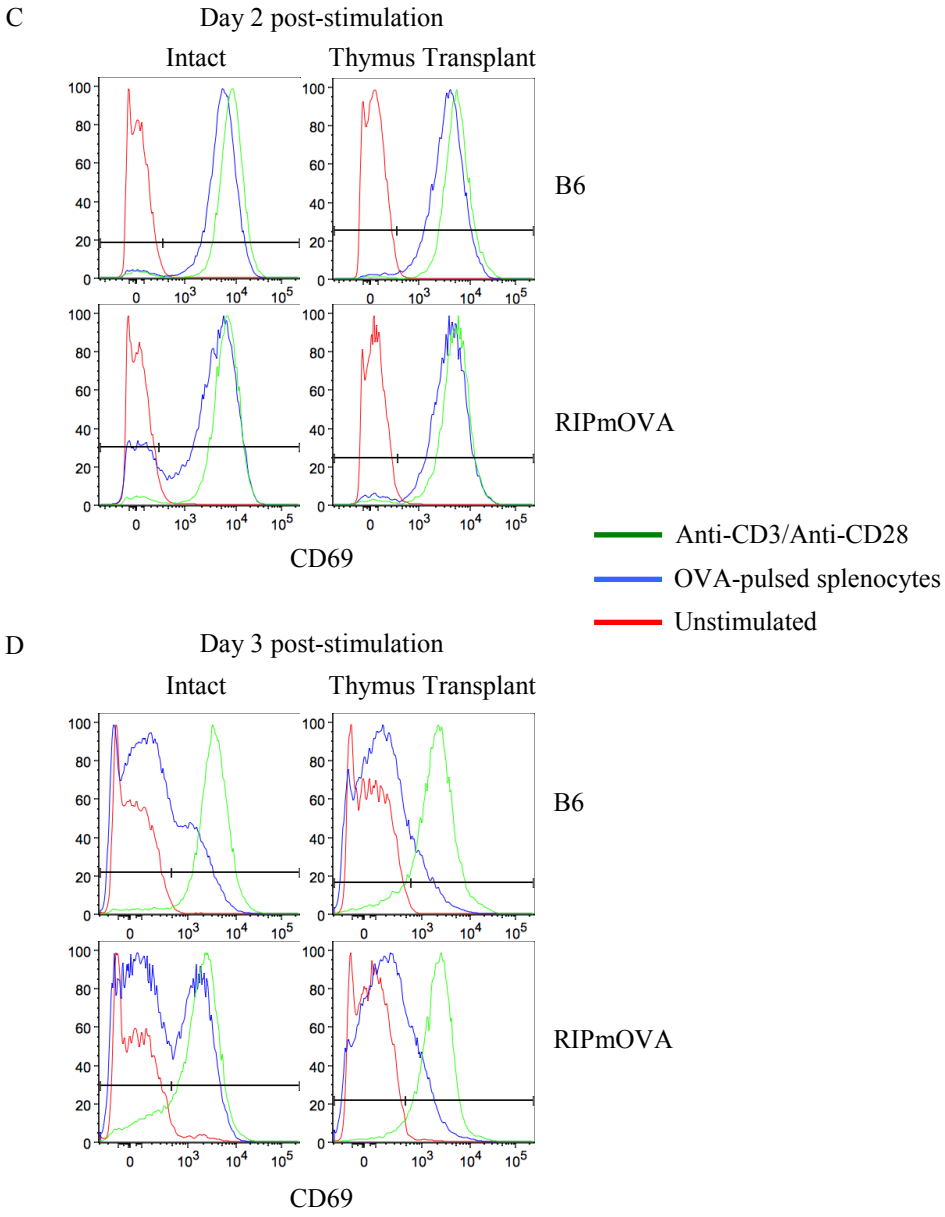


Figure 5-10: CD69 induction but not PD-1 or CD28 expression is rescued in OT-I Bim$^{-/-}$→RT chimeras

A. PD-1 expression day 3 after stimulation with SIINFEKL-pulsed splenocytes or plate-bound αCD3/αCD28. **B.** CD28 expression day 3 after stimulation with SIINFEKL-pulsed splenocytes or plate-bound αCD3/αCD28. **C.** CD69 expression day 2 after stimulation with SIINFEKL-pulsed splenocytes or plate-bound αCD3/αCD28, *D,* CD69 expression day 3 after stimulation with SIINFEKL-pulsed splenocytes or plate-bound αCD3/αCD28. OT-I Bim$^{-/-}$→RIPmOVA (n=4), OT-I Bim$^{-/-}$→B6 (n=4), OT-I Bim$^{-/-}$→RT (n=4), OTI-I Bim$^{-/-}$→BT (n=4). Gated on Vα2^{+}CD8^{+} splenocytes.

Compared to splenocytes from OT-I Bim$^{-/-}$→B6 chimeras, those from OT-I Bim$^{-/-}$→BT chimeras showed an increase of frequency of cells capable of producing both TNFα and IFNγ at the expense of single positive cells whether initial stimulation was with SIINFEKL-pulsed splenocytes or plate-bound αCD3/αCD28 **(Fig. 5-11A and B)**. While stimulation with plate-bound αCD3/αCD28 was able to rescue proliferation in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras, it did not rescue cytokine production upon restimulation on day 5 **(Fig. 4-3A and B, Fig. 5-11A and B)**. In contrast, the absence of OVA in the periphery of OT-I Bim$^{-/-}$→RT chimera not only rescued proliferation in splenocytes, but also resulted in the robust production of cytokines, similar to that seen in the OT-I Bim$^{-/-}$→BT chimeras with either initial stimulation condition **(Fig. 5-9A, Fig 5-11A and B).**

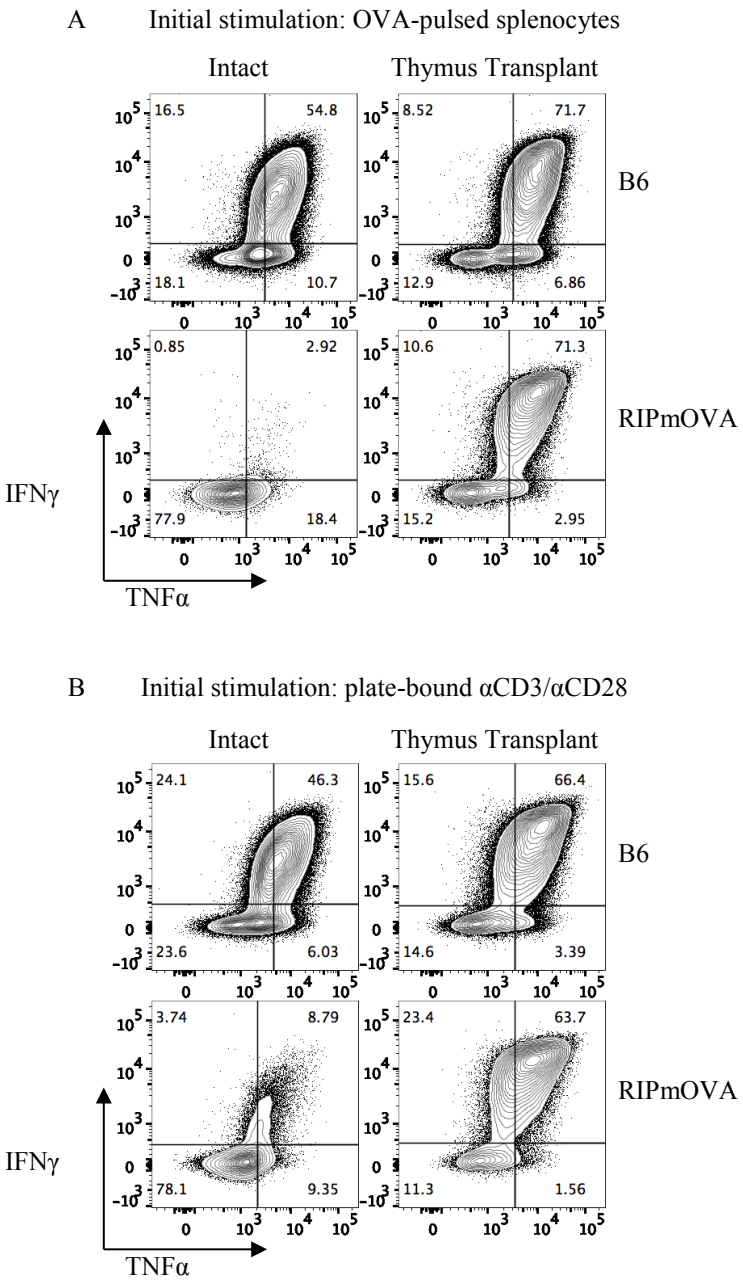


Figure 5-11: Cytokine production is rescued in OT-I Bim$^{-/-}$→RT chimeras
A. IFNγ and TNFα production by restimulated and non-restimulated Vα2^{+}CD8^{+} splenocytes from OT-Bim$^{-/-}$→B6 (n=4), OT-I Bim$^{-/-}$→RIPmOVA (n=4), OT-I Bim$^{-/-}$→BT (n=4) and OT-I Bim$^{-/-}$→RT (n=4) chimeras on Day 5 following initial stimulation by SIINFELK-pulsed splenocytes. **B.** IFNγ and TNFα production by restimulated and non-restimulated Vα2^{+}CD8^{+} splenocytes from OT-Bim$^{-/-}$→B6 (n=4), OT-I Bim$^{-/-}$→RIPmOVA (n=4), OT-I Bim$^{-/-}$→BT (n=4) and OT-I Bim$^{-/-}$→RT (n=4) chimeras on Day 5 following initial stimulation by plate-bound αCD3/αCD28 antibodies.

Discussion

In this chapter, I have demonstrated that the anergic state observed in peripheral OT-I Bim$^{-/-}$→RIPmOVA T cells is induced in the thymus **(Fig. 5-1 and 5-2).** Many of the previously described developmental models of anergy also involved thymic antigen encounter though in some this aspect was not thoroughly examined (180-189). However, unlike OT-I Bim$^{-/-}$→RIPmOVA chimeras, these models involved or would likely have involved clonal deletion to some extent.

Similar to what was observed in OT-Bim$^{-/-}$→RIPmOVA chimeras **(Fig. 4-2, 4-3, 5-1, 5-2)**, some models described an anergic state that was more robust in thymocytes compared to splenocytes (182)**.** Other models did not demonstrate this relaxation of anergy in the periphery despite using similar model to the previous two studies (180) or a MHC Class II restricted TRA model (183-185).

Studies using intact HY and non-deletional HY TCR models reported that antigen experienced thymocytes and peripheral T cells exhibited CD8 dulling (186, 189) much like we observe in OT-I Bim$^{-/-}$→RIPmOVA chimeras and the thymi of OT-I Bim$^{-/-}$→RT chimeras **(Fig. 5-4 and 5-5).** While thymocyte function was not observed, T cells isolated from the LN of these HY models were proliferatively impaired to male antigen, however similarly to splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras **(Fig. 5-9)**, proliferation was rescued when stimulated with the HY α-chain specific mAb T3.70 (186).

The importance of peripheral antigen encounter in maintaining thymically induced anergy is highlighted by the complete reversal of anergy in the periphery of OT-I Bim$^{-/-}$→RT chimeras **(Fig. 5-9, 5-10 and 5-11).** This is in agreement with some

studies of anergy, using developmental models as well as other *in vivo* models that highlight the transient nature of anergy in some models (110, 111, 113, 152). As recent antigen encounter in the thymus appears to enforce a more robust anergic state, it suggests that the same may hold true in the pLN of OT-I $Bim^{-/-}$→RIPmOVA chimeras. This would further support the importance of persistent antigen to maintain anergy.

Nude thymus transplant OT-I $Bim^{-/-}$→RT and OT-I $Bim^{-/-}$→BT chimeras remain lymphopenic even after reconstitution with OT-I $Bim^{-/-}$ BM. This lymphopenia could explain the enhanced proliferation and cytokine production in splenocytes from these chimeras compared to those from OT-I $Bim^{-/-}$→B6 chimeras as lymphopenia can enhance T cell function (151, 152).

PD-1 has been implicated as a regulator in $CD8^+$ T cell anergy in transplant tolerance (168). Increased PD-1 expression *ex vivo* in thymocytes and pancreatic lymphocytes **(Fig 4-7)** suggested a role for PD-1 in the observed anergy. This is further supported by the enhanced PD-1 upregulation in splenocytes and thymocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras when compared to those from OT-I Bim/-→B6 chimeras **(Fig. 4-9, 5-2)**. However, PD-1 upregulation following stimulation of splenocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras was comparable to that seen in splenocytes rescued from anergy in OT-I $Bim^{-/-}$→RT chimeras **(Fig 5-10).** The similar expression pattern and levels despite the marked change in proliferative capacity and cytokine production capacity between splenocytes from these two chimeras suggest that PD-1 is not involved in the observed anergy in OT-I $Bim^{-/-}$→RIPmOVA chimeras at least in an *in vitro* stimulation system. It was not

addressed in these experiments and it is not clear if PD-1 is relevant functionally *in vivo*.

These data clearly demonstrate that high affinity antigen encounter in the thymus can induce an anergic state in antigen specific T cells, though antigen encounter is required to maintain this state. While the OT-I Bim$^{-/-}$→RIPmOVA phenotype does not perfectly match any particular model of anergy previously describe, it does encompass many different aspects of anergy from different models. Thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras are protected from clonal deletion, unlike many previously studied models; however, it may be the lack of clonal deletion that unmasks many of the tolerance mechanisms induced by antigen encounter in the thymus. It is possible these mechanisms are normally induced in the thymus, but clonal deletion eliminates the majority of these thymocytes before they can be properly examined.

Chapter 6: General Discussion

Research Summary

In this thesis I have shown that Bim is required for clonal deletion to TRA. There is a significant rescue of $CD8^{+}CD24^{lo}$ thymocytes in OT-I $Bim^{-/-}$→RIPmOVA chimeras compared to OT-I→RIPmOVA chimeras in both frequency and numbers **(Fig. 3-7, 6-1).** As $V\alpha2^{+}$ cleaved caspase 3^{+} thymocytes from OT-I→RIPmOVA chimeras were primarily DP^{dull}, this indicates that deletion is occurring after thymocytes have undergone positive selection **(Fig. 3-7)**. This is consistent with TRA encounter and clonal deletion in the medulla and confirms that OT-I→RIPmOVA chimeras are a model of negative selection to TRA (46). The requirement for Bim during clonal deletion to TRA is likely not a feature of the OT-I model, as clonal deletion was intact in OT-I →ActmOVA and OT-I→RIPmOVA chimeras **(Fig. 3-2, 3-3, 3-4, 3-6 and 3-8)**. This is consistent with other models of negative selection to UbA (99, 100). Furthermore, soon after our initial report of the requirement for Bim in clonal deletion to TRA, other groups reported similar findings using OT-I and OT-II BM chimeras as well as OT-II RIPmOVA double Tg mice (84, 142). Thymocytes that escaped clonal deletion in the OT-I $Bim^{-/-}$→RIPmOVA chimera were not deleted by peripheral mechanisms and persist in the periphery **(Fig. 3-8, 6-1).** This is to be expected as peripheral deletion involves Bim (191). Both CD8 co-receptor dulling and Nur77 induction in $V\alpha2^{+}CD8^{+}$ SP thymocytes and splenocytes suggests Ag encounter in both the thymus and periphery while low CD44 expression suggests that they are not overtly activated by this Ag encounter **(Fig. 3-6 and 3-9).**

While mature $CD8^+CD24^{lo}$ thymocytes escaped clonal deletion in OT-I $Bim^{-/-}$ →RIPmOVA chimeras due the absence of Bim, they showed impaired proliferation when stimulated *in vitro* with OVA-pulsed splenocytes **(Fig 5-1, 5-2, 6-1)**. Similarly, they were unable to proliferate when transferred into lymphopenic C57BL/6 mice **(Fig. 4-10)**.

These thymocytes that survived clonal deletion egressed into the periphery and persisted in large numbers. However, contrary to expectation, the presence of $V\alpha2^+CD8^+$ OT-I splenocytes in OT-I $Bim^{-/-}$→RIPmOVA chimeras, did not induce diabetes (134) **(Table 4)**. This suggests that the functional defect persists *in vivo* in the periphery and extends beyond proliferation to T cell functionality as well. Further confirmation of this *in vivo* functional defect was obtained from experiments where $V\alpha2^+CD8^+$ T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras were adoptively transferred into sublethally irradiated RIPmOVA mice. These recipients did not develop diabetes, whereas transfer of $V\alpha2^+CD8^+$ T cells from OT-I $Bim^{-/-}$→B6 chimeras rapidly induced diabetes, similar to previous studies with intact RIPmOVA recipients **(Fig. 4-5)**. To further examine this functional impairment in peripheral T cells, splenocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras were stimulated *in vitro* with OVA-pulsed splenocytes, which resulted in impaired proliferation and cytokine production **(Fig. 4-3, 4-4, 6-1).** Interestingly, proliferation but not cytokine production was rescued by stimulation with plate-bound αCD3/αCD28 stimulation, indicating there are multiple mechanisms at play driving this anergic state **(Fig. 4-3, 4-4, 6-1).** This rescue of proliferation when stimulating with αCD3/αCD28 can only be partially explained by additional IL-2 production from non-OT-I T cells from the

spleens of OT-I Bim$^{-/-}$→RIPmOVA, as the addition of exogenous IL-2 potentiated the proliferation of responding T cells **(Fig. 4-6, 6-1).** The impairment in cytokine production may be post-transcriptional as OT-I Bim$^{-/-}$→RIPmOVA splenocytes could still induce cytokine mRNA transcription after re-stimulation **(Fig. 4-4)**. Similar to thymocytes, splenocytes from OT-I Bim$^{-/-}$→RIPmOVA were unable to proliferate in a lymphopenic environment, which may be a result of impaired STAT5 signaling which could stem from impaired surface expression of CD127 **(Fig. 4-10, 6-1)**. Thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras demonstrated a more robust impairment of function in terms of proliferative capacity and CD69 upregulation post-stimulation than splenocytes from the same mouse **(Fig. 5-1 and 5-2).** This might be a result of more recent Ag encounter by thymocytes than splenocytes. This apparent relaxing of the non-responsive state in splenocytes suggested that peripheral Ag encounter in the pancreatic LN might be important for maintaining the non-responsive state

To better understand the role of peripheral Ag in maintaining tolerance, I generated a model of negative selection to TRA where OVA was only expressed in the thymus and not the periphery. OT-I Bim$^{-/-}$ BM was transplanted into nude mice that had received RIPmOVA thymus grafts (OT-I Bim$^{-/-}$→RT) or control B6 thymus grafts (OT-I Bim$^{-/-}$→BT). Thymocytes from OT-I Bim$^{-/-}$→RT chimeras were phenotypically similar to those from OT-I Bim$^{-/-}$→RIPmOVA chimeras and shared a similar defect in proliferation *in vitro* **(Fig. 5-4, 5-6 and 5-8).** In contrast, splenocytes from OT-I Bim$^{-/-}$→RT chimeras were able to proliferate and produce cytokines equivalently to OT-I Bim$^{-/-}$→BT control chimeras **(Fig. 5-9, 5-10, 5-11, 6-1)**. This complete reversal of the impairment of proliferation and cytokine

production confirmed that peripheral antigen was required to maintain the functional impairment established in the thymi of OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→RT chimeras **(Fig. 6-1)**.

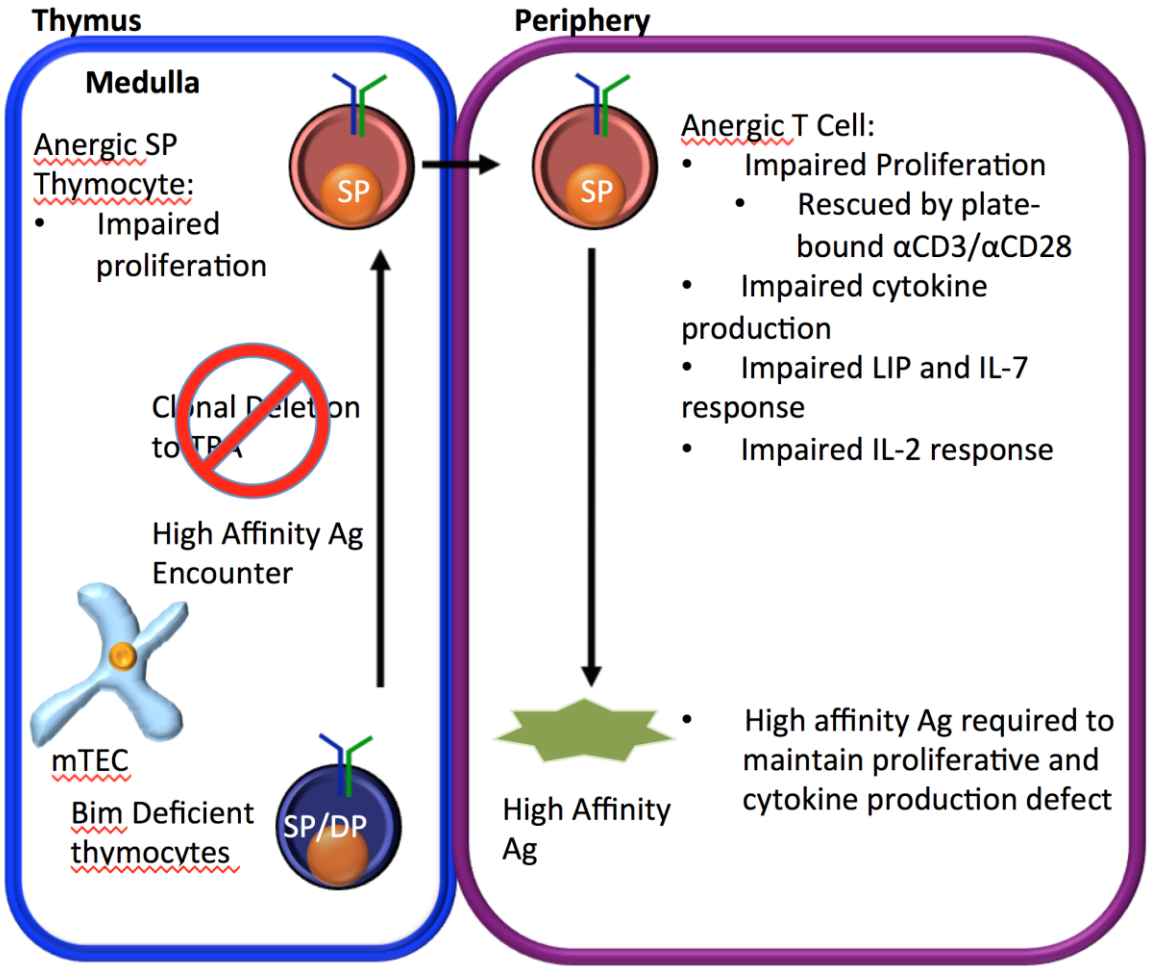


Figure 6-1: Model of Anergy in OT-I Bim$^{-/-}$→RIPmOVA chimeras
In OT-I Bim$^{-/-}$→RIPmOVA chimeras, OT- I T cells develop normally to the DP stage. After trafficking into the medulla, transitional DP thymocytes encounter their high affinity antigen, OVA, expressed on mTECs and cross-presented on DCs, however they are not clonally deleted. OT-I CD8SP thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras are proliferatively impaired when challenged with antigen and APC. This state persists in the periphery characterized by impaired proliferation and cytokine production to antigen and APC. While plate-bound αCD3/αCD28 can rescue proliferation, it does not rescue cytokine production. OT-I CD8^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras showed impaired response to lymphopenia, IL-7 and IL-2. This anergic state requires peripheral high affinity antigen encounter to be maintained.

Anergy Models and Mechanisms

Anergy can be described as a tolerance mechanism whereby lymphocytes are intrinsically functionally impaired after encountering antigen, but remain alive in this state of dysfunction (41). There are many models used to study anergy. This has led to many molecular mechanisms of anergy being identified. These models can be broadly separated into two groups: *in vitro* and *in vivo* models*. In vitro* anergy, or clonal anergy, has typically been used to describe the functional state of $CD4^+$ T cell clones that have been stimulated through the TCR in the absence of co-stimulation (108, 192-194). In addition to the models used to examine anergy in CD4+ T cells, there have been several studies of clonal anergy using $CD8^+$ T cell clones. In one study, stimulation of $CD8^+$ T cell clones with APC that lack co-stimulatory molecules led to anergy (195). The second form is referred to as activation-induced non-responsiveness **(AINR)** (196). In this case $CD8^+$ T cells stimulated with αTCR monoclonal antibodies or peptide/MHC complexes with B7 co-stimulation proliferate and produce IL-2, but rapidly lose the ability to proliferate or produce IL-2.

In vivo anergy can be divided into peripheral and developmental anergy **(Table 5)**. Peripheral models generally involved the transfer of TCR transgenic T cells into hosts constitutively expressing the cognate Ag (110, 111, 113, 114, 197). Developmental models generally involve thymic antigen encounter, though not all studies thoroughly investigated the role of the thymus in anergy induction or the characteristics of thymocyte non-responsiveness. I further segregate developmental models into 4 categories; models involving SA; models involving MHC mismatches as

TRA and UbA; TCR transgenic models against UbA; and finally TCR transgenic models against TRA **(Table 5)**.

Table 5: Characteristics of Anergy

Characteristic Trait	OT-I Bim$^{-/-}$ →RIPmOVA	In Vitro		In vivo				
		CD4	CD8	Peripheral	Developmental			
					SuperAg	UbA and TRA MHC Mismatch	TCR Tx UbA	TCR Tx TRA
Thymic Ag Encounter	Yes	-	-	No[110, 111, 113, 114]	Yes[152, 180]	Yes[182-185]	Yes[181, 186, 189]	Yes[145, 198]
Thymocyte impairment	Yes	-	-	-	Yes[180]	Yes[182-185]	Yes[186]	Yes[198]
Deletion/ Contraction	No	-	-	Yes[112]	No[180]	Partial[182]	Yes[186, 189]	Partial[145]/ No[198]
CD8 Dulling	Yes	-	No	No	No[152, 180]	-	Yes[186, 189]	No[145, 198]
Activated T cells	No	Yes	Yes	Yes[110, 111, 113]	-	-	-	Yes[145]
Impaired Proliferation	Yes	Yes	Yes	Yes[110, 111, 113, 114]	Yes[152, 180]	Yes[182-185]	Yes[181, 186, 189]	Yes[145]
Proliferation rescued by Ab stimulation	No	-	-	No[112]	Yes[180]	-	No[181]/ Yes[186]	-
Impaired effector function	Yes	-	-	Yes[110, 112]	Yes[180]	Yes[182-184]	Yes[186, 189]	Yes[145, 198]
Impaired Cytokine Production	Yes	No	No	Yes[111, 113]			-	Yes[145]
Rescued by IL-2	No	Yes	Yes	-	Partial[152]	No[182]	No[181]	-
Ag Persistence required	Yes	No	-	Yes[110, 111, 113, 114]	Yes[152]	-	-	No[145]

Thymic Ag Encounter and impaired thymocyte proliferation

In examining our OT-I Bim$^{-/-}$→RIPmOVA model, it is clear that there are many similarities with these anergy models; however, often one key feature that has been overlooked is the involvement of the thymus. The non-responsive state is clearly induced in the thymus of OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→RT chimeras **(Fig 5-1, 5-2, 6-1)**. *In vitro* anergy models and *in vivo* peripheral models do not even involve the thymus (41, 110-114, 195, 196). Developmental models by their nature involve antigen encounter in the thymus, however few studies actually investigated thymocyte functionality (145, 181, 189). Several noted impaired proliferation in thymocytes but most did not fully characterize the nature of this anergy (152, 180, 182-186) **(Table 5)**. One of the more extensively characterized models examined T cells specific for minor lymphocyte stimulatory (Mls) antigens derived from the mouse mammary tumor virus (MMTV) superantigen **(SA)** in the context of I-E in a polyclonal repertoire by transplanting SJL (H-2^{s}) BM into (B10.SxAKR)F1 (H-2^{sxk}, Mls^{axb}) recipients. In this model, both the Mls and I-E molecules are only expressed on host tissues. Vβ6^{+} and Vβ17^{+} TCRβ chain bearing thymocytes, which are enriched for populations specific for Mls^{a} and I-E, are not deleted in these mice, however they are unable to proliferate to host-type Ag (180). In a UbA against MHC mismatch model, Gao *et al.* transplanted B6.PL (H-2^{b}) BM into (B6xCBA/J)F1 (H-2^{b}xH-2^{k}) recipients (182). Here too, thymocytes show impaired proliferation to mixed lymphocyte reactions (MLR) against host H-2 molecules (182).

Similar to our model, Lo *et al.* generated two mouse models where the MHC class II gene I-E^{b} was expressed as a TRA in the pancreas of mice (184, 185). The first

model used a rat insulin promoter that drove expression on pancreatic β islets (Ins-I-E). While these mice developed diabetes, it was not due to I-E^b reactive T cells as stimulation of T cells and thymocytes by MLR against I-E^b did not result in proliferation (185). To better examine this phenomenon in the absence of diabetes, Lo *et al.* generated another model where I-E^b was expressed in the acinar cells of the pancreas under control of the elastase promoter (EL-I-E mice). Again, thymocytes were unable to proliferate in response to MLR against host-type Ag (184). Unlike OT-I $Bim^{-/-}$→RIPmOVA chimeras, these models involved MHC mismatches (152, 180, 182, 184, 185) and while thymocyte proliferative impairment was noted in these models, these models examined polyclonal populations and not specific TCR and antigen pairs.

In a study using the male antigen specific HY transgenic TCR, Carlow *et al* demonstrated that transplanting female HY transgenic TCR H-$2D^d$ BM into male H-$2D^b$ hosts resulted in the generation of HY specific thymocytes and peripheral T cells that did not proliferate to male stimulators *in vitro* (186). Similar to our thymus grafting experiments, they went on to transplant HY TCR transgenic female H-$2D^b$ BM into thymectomized female H-$2D^b$ recipients, which then received male H-$2D^b$ thymus transplants (186). They showed that directly *ex vivo,* the CD4 by CD8 profile of thymocytes was similar to what was observed in the first set of chimeras, however they did not assess proliferation or function of thymocytes or splenocytes from these particular mice.

While several studies have reported anergy being induced in the(186) thymus, they did not do so in the context of a transgenic TCR specific for a neo-TRA

(180, 182-186). However, by crossing mice expressing the alloantigen K^b under control of the bovine keratin IV promoter to mice expressing a transgenic K^b-specific TCR (0.8 Ker-Kb x TCR), Schönrich *et al.* generated a TCR transgenic model where anergy was induced against a tissue-restricted alloantigen (198). These mice express K^b in epithelial cells in the tongue, foot, hair as well as mTECs and the majority of thymocytes are clonotype$^+$. CD8$^+$ clonotype$^+$ thymocytes from these mice are unable to proliferate to anti-clonotype antibodies, even in the presence of exogenous IL-2 (198).

Clonal Deletion or Contraction

Interestingly, while clonal deletion is abrogated in the thymi of OT-I Bim$^{-/-}$ →RIPmOVA chimeras, in the majority of developmental models where tolerance was examined, clonal deletion was intact (186, 189) or thymocytes were partially deleted (145, 182, 186) **(Table 5)**. The one exception was in 0.8 Ker-Kb x TCR mice. CD8$^+$clonotype$^+$ thymocytes were not deleted in this model (198). In peripheral models, adoptively transferred T cells are initially activated and expand, before contraction of the populations leaves a small subset of anergic T cells (111-113) **(Table 5)**. There was one exception in which transfer of pigeon cytochrome *c* (PCC) specific CD4$^+$ T cells into lymphopenic hosts expressing membrane targeted PCC resulted in a large expansion and a limited contraction. The remaining T cells were functionally similar to other peripheral models (114). It is possible that the lack of clonal deletion in OT-I Bim$^{-/-}$→RIPmOVA chimeras may unmask more commonalities between these various forms of anergy that may be obscured by deletional or contraction mechanisms.

CD8 dulling and T cell Activation

Thymocytes and splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras exhibit CD8 dulling and induction of Nur77-GFP suggesting they have encountered antigen, however they remain CD44^{lo} **(Fig 3-7 and 3-11**), which suggests that they are not overtly activated **(Table 5)**. This is contrast to what is observed in *in vitro* and peripheral anergy models. In these models, T cell activation precedes the onset of the anergic state (110, 111, 113, 195, 196). The developmental models of anergy induction for the most part did not examine this aspect (152, 180, 182-186, 189). However, in a TCR transgenic TRA model where a TCR transgenic mouse was crossed to a mouse expressing the cognate Ag under control of the albumin promoter (TCR$_{GAG}$xAlb:GAG), T cells were partially deleted but were CD44^{hi} (145). This discrepancy may be a result of the abrogated clonal deletion in OT-I Bim$^{-/-}$→RIPmOVA chimeras, though it is not clear why CD44 expression would be lower.

Impaired proliferation of peripheral cells

One aspect that was universal among all anergy induction models as well as thymocytes and splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras was impaired proliferation upon *in vitro* stimulation with Ag and APCs (110, 113, 114, 181, 186, 189), stimulation via MLR (152, 180, 183-185), or after immunization (145) **(Table 5)**. While the proliferation of splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras could be rescued with stimulation by plate-bound αCD3/αCD28, there are conflicting reports in the literature about the response of anergic cells to antibody-mediated stimulation. In the SJL→ (B10.SxAKR)F1 model, Vβ6^{+} and Vβ17^{+} TCR bearing T cells, which are enriched for populations specific for Mls^{a} SA and I-E, are

unable to proliferate in MLR against host-type Ag. Monoclonal antibodies against Vβ6 and Vb11 are also unable to induce proliferation (180). Similarly, in a TCR transgenic model to UbA, anergic hemagglutinin specific T cells were unable to proliferate to TCR specific antibody stimulation (181). If anergy was induced by adoptive transfer of male antigen-specific HY T cells into athymic male mice, the resulting T cells did not proliferate to the HY TCR specific monoclonal antibody T3.70 (111). In contrast, in the HY model generated by Carlow *et al* using BM chimeras, anergic T cells from HY H-2D^{d} female BM→H-2D^{b} male chimeras were able to respond to T3.70 stimulation (186). It must be noted that these stimulations were done in the absence of αCD28 antibodies. Additionally, we did not stimulate with an OT-I TCR specific antibody; we used an αCD3 antibody, which would have stimulated all T cells in the assay. The difference in these stimulation conditions could account for the discrepancy in the response to antibody stimulation. Alternatively, this discrepancy could be a result of the manner in which anergy was induced. The only model that demonstrated a rescue of proliferation was a model where clonal deletion was impaired using transgenic TCR BM chimeras (186), which is similar to OT-I Bim$^{-/-}$→RIPmOVA chimeras. Interestingly, the TCR$_{GAG}$xAlb:GAG demonstrated a proliferation defect *in vivo* to GAG expressing *Listeria monocytogenes* (LM-GAG) (145). It may be of interest to see if OT-I Bim$^{-/-}$→RIPmOVA T cell would also be tolerant to LM-OVA challenge.

Impaired effector function

In addition to impaired proliferation, two of the most important characteristics of the non-responsiveness in OT-I Bim$^{-/-}$→RIPmOVA chimeras are

impaired effector function *in vivo* and impaired cytokine production *in vitro* **(Table 5)**. I address these separately as not all studies explicitly studied both aspects of T cell function. Effector function is clearly impaired in OT-I $Bim^{-/-}$→RIPmOVA chimeras, as they do not develop diabetes **(Table 4)** despite the large numbers of OVA specific T cells in the periphery and OVA expression on pancreatic β cells **(Fig 3-8)**. Additionally, transfer of OT-I $Bim^{-/-}$→RIPmOVA $CD8^+$ T cells into RIPmOVA chimeras did not induce diabetes in these mice **(Fig 4-5).** Similarly, SJL→(B10.S x AKR)F1 lymph node cells from the previously mentioned study (180) were unable to cause graft-versus-host disease **(GVHD)** after injection into irradiated (B10.SxAKR)F1 mice, while transfer of normal SJL cells resulted in the death of the recipient (180). While function was not directly examined in intact HY male mice and the HY H-$2D^d$ female BM→H-$2D^b$ male BM chimeras, these mice did not show any signs of autoimmunity, suggesting that effector functions of HY specific T cells in these models were also impaired *in vivo* (186, 189). Transfer of B6 T cells into irradiated mice expressing I-E as a TRA under the control of the elastase promoter in acinar cells of the pancreas (EL-I-E mice) resulted in the destruction of the exocrine pancreas (184). In contrast, transfer of anergic T cells or thymocytes from intact EL-I-E mice into irradiated EL-I-E mice did not result in destruction of the exocrine pancreas (184). This trend was also present in TCR_{GAG}xALb:GAG mice with no evidence of liver damage in these mice (145). Similarly, in contrast to non-transgenic littermates, 0.8 Ker-Kb single transgenic mice were tolerant to K^b skin grafts (198). A defect in T cell function was also detected in peripheral models where HY T cells were adoptively transferred into irradiated or athymic male mice (110-

112). These experiments clearly demonstrate a common *in vivo* impairment of effector function between our model and several developmental models of anergy, however it is not clear if this is achieved by the same mechanisms.

Impaired cytokine production

Splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras are unable to produce IFNγ and TNFα when restimulated with OVA peptide after an initial stimulation with OVA-pulsed splenocytes or plate-bound αCD3/αCD28 **(Fig. 4-4).** In the PCC peripheral model of anergy, IFN-γ, IL-3, IL-4 and IL-10 production in anergized CD4$^+$ PCC-specific T cells were greatly impaired after *in vitro* stimulation (114). Similarly, CD4$^+$ OVA specific DO11.10 T cells that had been transferred into a Balb/c host and anergized by injection of soluble OVA, showed a defect in IL-2, IL-6 and TNFα production (113). The TCR$_{GAG}$xAlb:GAG model also demonstrated a defect in IFNγ and TNFα production after antigen challenge. While these *in vivo* models shared cytokine production and effector function impairment with our OT-I Bim$^{-/-}$→RIPmOVA model, *in vitro* models of anergy did not **(Table 5)**. This suggests that the anergic state established *in vivo* and in our OT-I Bim$^{-/-}$→RIPmOVA model is more robust or complex than what occurs with *in vitro* anergy models.

Rescue of proliferation by exogenous IL-2

The proliferative impairment in T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras is not reversible by addition of exogenous IL-2. This is similar to other

developmental models of anergy in which exogenous IL-2 does not rescue proliferation (181, 182), or it does not completely rescue proliferation (152). This is in contrast to some $CD4^+$ and $CD8^+$ *in vitro* models of anergy in which the proliferative impairment can be completely reversed with the addition of exogenous IL-2 (41). This again points to *in vivo* anergy and the non-responsive state induced in OT-I $Bim^{-/-}$→RIPmOVA chimera being more complex and robust than *in vitro* anergy models.

Persistent Ag required to maintain anergy

Persistent Ag encounter was required for peripheral cells in OT-I $Bim^{-/-}$→RIPmOVA chimeras to remain non-responsive **(Fig. 5-9, 5-10, 5-11, 6-1)**. This aspect was not observed in *in vitro* anergy models (41), however it was identified in some *in vivo* anergy models **(Table 5)**. Anergic male-specific HY T cells from the adoptive transfer of female HY T cells into athymic male nude mice, regained the ability to proliferated to T3.70 antibody-mediated stimulation after transfer into a female nude host for two months and then retransfer into a nude male host (111). Similarly, after transfer of anergic male HY T cells into lethally irradiated hosts reconstituted with varying ratios of male and female BM, only hosts reconstituted with 90% male BM maintained the anergic phenotype (110). Transfer of anergic T cells from SJL→(B10.SxAKR)F1 (Mls^{axb}, $I\text{-}E^+$) chimeras into B10.S (Mls^b, $I\text{-}E^-$) mice resulted in anergic $V\beta6^+$ (Mls^a specific) and $V\beta17^+$ (I-E specific) T cells regaining the ability to proliferate to antibody-mediated stimulation (152). $CD4^+$ T cells from the DO11.10 and PCC models also demonstrated a reversal of anergy in the absence of Ag. If anergized $CD4^+$ PCC specific T cells were parked in a non-antigen bearing host,

they would proliferate to antigen challenge *in vitro* (114). In the DO11.10 model, where anergy was induced by immunization by soluble OVA peptide in Complete Freund's Adjuvant, mice needed continuous administration of OVA in Incomplete Freund's Adjuvant to maintain the anergic state of DO11.10 T cells. (113). However, in the TCR_{GAG}XAlb:GAG model and in *in vitro* models of anergy, persistent antigen is not required to maintain anergy (41, 145). The *in vitro* systems cannot completely replicate an *in vivo* system and this could explain the discrepancy in Ag requirement to maintain anergy. It is not clear why the TCR_{GAG}XAlb:GAG model differs from other *in vivo* models that examined requirement of persistent Ag to maintain anergy.

The anergic states induced *in vitro* and *in vivo* models share many overlapping characteristics that are also observed in OT-I $Bim^{-/-}$→RIPmOVA chimeras. It is possible that the lack of clonal deletion in OT-I $Bim^{-/-}$→RIPmOVA chimeras may unmask more commonalities between these various forms of anergy that may be obscured by deletion mechanisms.

Common Anergy Markers

I examined a wide variety of molecular markers that have generally been implicated in T cell activation or the impairment of T cell function (BTLA, CTLA4, Lag3, PD-1, Tim-3, Egr2, GITR, CD28, Helios) however few had expression patterns that were consistent with functional impairment of T cells **(Fig 4-7, 4-9, 5-2 and 5-4).** CTLA -4 and CD28 blockade have been implicated in several forms of anergy (41), however CTLA-4 extracellular and intracellular expression *ex vivo* in OT-I $Bim^{-/-}$→RIPmOVA chimeras does not suggest a role of CTLA-4 in the observed anergy **(Fig 4-7)**. Similarly, CD28 expression post-stimulation does not support a role for CD28

in this anergy. CD28 expression is lower in thymocytes, splenocytes and pancreatic lymphocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras compared to OT-I Bim$^{-/-}$→B6 chimera **(Fig. 4-9, 5-2, 5-4)**, which is consistent with impaired CD28 co-stimulation playing a role in anergy induction. However, stimulation with plate-bound αCD3/αCD28, which rescues proliferation in splenocytes from OT-I Bim$^{-/-}$→RIPmOVA chimera, does not rescue CD28 induction **(Fig. 4-9)** nor was CD28 expression rescued in splenocytes from OT-I Bim$^{-/-}$→RT chimeras **(Fig. 5-11).** As both proliferation and cytokine production can be rescued without CD28 rescue, it is likely that impaired CD28 co-stimulation does not play a causal role in the anergic state in OT-I Bim$^{-/-}$→RIPmOVA chimeras.

It should also be noted CD28 may not only function as a qualitative receptor, simply being required for T cell activation, but also as a quantitative receptor, able to amplify TCR signaling to reach the activation threshold (199). It is possible that APCs used for peptide stimulation did not express sufficient levels of CD28 ligands CD80 and CD86 to overcome any dampening of TCR signaling that may have occurred in OT-I Bim$^{-/-}$→RIPmOVA T cells. Stimulation with activated APCs or under inflammatory conditions such as with LM-OVA may result in sufficient CD28 signaling to overcome impaired TCR signaling that might be present in anergic OT-I Bim$^{-/-}$→RIPmOVA T cells. This could also shed light as to whether the functional impairment in OT-I Bim$^{-/-}$→RIPmOVA T cells results solely from TCR proximal events or it downstream mechanisms are also involved.

PD-1 has been associated with various forms of T cell dysfunction, PD-1 deficient mice develop autoimmune disorders (200-202) and blockade of PD-1 can

lead to autoimmune disorders (203-206). It has also been recently implicated in $CD8^+$ T cell anergy in transplant tolerance (168). Upregulation of PD-1 expression in $V\alpha2^+CD8^+$ SP thymocytes and pancreatic lymphocytes compared to splenocytes *ex vivo* combined with the more robust proliferative defect in thymocytes and pancreatic lymphocytes suggested that PD-1 may be an important inhibitory mechanism at play **(Fig. 4-7, 5-1, 5-2, 5-4).** However, PD-1 expression in OT-I $Bim^{-/-}$ →RIPmOVA splenocytes is higher in cells stimulated with plate-bound αCD3/αCD28 compared to OVA-pulsed splenocyte stimulation and does not change between splenocytes from OT-I $Bim^{-/-}$→RIPmOVA and OT-I $Bim^{-/-}$→RT chimeras **(Fig 5-11)**. If PD-1 were playing a role in the functional impairment of thymocytes and T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras, we would expected that PD-1 expression be lower in instances of functional rescue. That is not the case; in fact PD-1 expression seems to follow the opposite trends. This suggests that PD-1 is not playing a role in the observed functional impairment of T cells and thymocytes from OT-I $Bim^{-/-}$ →RIPmOVA chimeras at least in *in vitro* stimulations.

Egr2 is a negative regulator of T cell activation (207), and has been shown to be required for T cell anergy *in vitro* with T_h1 $CD4^+$ T cells and *in vivo* with a SA model (173). However Egr2 does not appear to be induced *ex vivo* in thymocytes and T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras **(Fig 4-7)**. This inconsistency may suggest the presence of different molecular mechanisms being at play in $CD4^+$ and $CD8^+$ T cell anergy. It may also stem from the model systems used as both Th1 models were peripherally or *in vitro* induced anergy.

Interestingly, while peripheral antigen encounter was required to maintain the anergic stated established in the thymi of OT-I Bim-/-→RIPmOVA and OT-I Bim-/-→RT chimeras **(Fig. 5-11 and 5-12)**, thymocytes and T cells from OT-I Bim-/-→RIPmOVA chimeras transferred in to lymphopenic C57BL/6 hosts did not expand up to seven days post transfer **(Fig. 4.10)**. As lymphopenia and homeostatic proliferation have been associated with the induction of autoimmunity (147-150) and the rescue of T cells from anergic states (151, 152), this was surprising. This may be the result of differences between anergic states established in CD8+ and CD4+ T cells or a consequence of differences in how anergy was induced in these models. It is possible that the discrepancy in LIP could be due to the length of time away from Ag. Anergic T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras may simply need more time away from antigen before rescue is observed.

Helios is upregulated in CD8SP thymocytes from OT-I $Bim^{-/-}$→RIPmOVA chimeras when compared to those from OT-I $Bim^{-/-}$→B6 chimeras. This increased expression is maintained in the spleen and in the pancreatic LN of OT-I $Bim^{-/-}$→RIPmOVA chimeras **(Fig. 4-7)**. In addition to being upregulated during negative selection and downregulated during positive selection (86, 87), Helios has been implicated in the function, stability and survival of $CD4^{+}Foxp3^{+}$ and $Ly49^{+}CD8^{+}$ T_{reg} cells under inflammatory conditions (93, 208, 209). There are some discrepancies in regards to the extent of the impairment of the suppressive capabilities of T_{reg} cells in two studies using $Helios^{fl/fl}$ x $Foxp3^{YFP}$-Cre mice. In these mice, Helios is conditionally knocked out in Foxp3 expressing cells (93, 209). One group reported only a partial impairment in suppressive capabilities *in vivo* of $CD4^{+}Foxp3^{+}$ T_{reg} cells (209).

Another group reported a more complete loss of *in vivo* suppressive capabilities of both $CD4^+Foxp3^+$ and $Ly49^+CD8^+$ T_{reg} cells (93). Both studies noted the slow onset of autoimmunity in these mice as well as the loss of T_{reg} cell stability under inflammatory conditions with T_{reg} cells converting into effector T cells. Helios may also be important in the survival and maintenance of the anergic state of T_{reg} cells. It was suggested that Helios upregulates STAT5, which can in turn improve IL-2 responsiveness in $CD4^+Foxp3^+$ T_{reg} cells, driving survival and Foxp3 expression. In $CD8^+$ T_{reg} cells, Helios may similarly improve survival through the IL-2 response and also impair terminal differentiation and exhaustion (93). However, despite upregulation of Helios *ex vivo* in CD8SP thymocytes and $CD8^+$ T cells from OT-I $Bim^{-/-}$ →RIPmOVA chimeras, the IL-2 response of CD8 T cells was impaired in these chimeras (**Fig 4-6 and 4-7)**. Additionally, unlike the proposed model (93), STAT5 activation appears to be impaired in anergic CD8SP and $CD8^+$ T cells from OT-I $Bim^{-/-}$ →RIPmOVA chimeras **(Fig. 4-10C).** However it must be noted that previous studies examined T_{reg} cells whereas I have not demonstrated explicitly the existence of a T_{reg} cell population in OT-I $Bim^{-/-}$→RIPmOVA chimeras. While it is clear some of the functional details previously described using conditional Helios-deficiency in T_{reg} cells may not apply to effector T cells from OT-I $Bim^{-/-}$→RIPmOVA chimeras, it is clear that Helios deficiency does affect the anergic state of T_{reg} cells (93, 209). It is not entirely clear how Helios enforces an anergic phenotype, however, it has been suggested that in T_{reg} cells, Helios can bind and epigenetically silence the *Il2* promoter (210). Additionally, in a CD4 model of negative selection against TRA and autoimmune gastritis, Helios upregulation was associated with antigen encounter by

TCR transgenic CD4$^+$ T cells in the absence of inflammation. Additionally, these Helios$^+$ T cells showed impaired function *in vitro* (86), though not to the same extent as seen in OT-I Bim$^{-/-}$→RIPmOVA chimeras. In this model, thymocytes undergoing negative selection also express higher levels of Helios, consistent with what is observed in OT-I Bim$^{-/-}$→RIPmOVA chimeras (86). It has also been suggested that in thymocytes, Helios may impair NF-κB activity or its targets, IL-2 and c-Myc (87). My data is consistent with this, as c-Myc induction is impaired after stimulation of OT-I Bim$^{-/-}$→RIPmOVA thymocytes **(Fig 5-8)**. The slight response to exogenous IL-2 by Vα2$^+$CD8$^+$ T cells from OT-I Bim$^{-/-}$→RIPmOVA but not OT-I Bim$^{-/-}$→B6 chimeras **(Fig 4-6)**, suggests that while T cells from OT-I Bim$^{-/-}$→B6 already produce sufficient IL-2 for optimal proliferation, T cells from OT-I Bim$^{-/-}$→RIPmOVA do not. As the proliferative rescue of T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras is not complete, these data also suggest that these T cells have an impaired IL-2 response.

Bim-independent negative selection in OT-I Bim$^{-/-}$→Act-mOVA chimeras

Negative selection is clearly intact in OT-I Bim$^{-/-}$→Act-mOVA chimeras with deletion of both DP and CD8SP thymocyte compartments **(Fig 3-2 and 3-3)**. While these data support earlier findings that Bim is not required for negative selection to UbA, the mechanisms driving Bim-independent negative selection remain unclear (99, 100). Potential mediators of Bim-independent negative selection include Fas, Nur77 and other BH3-only Bcl-2 family members.

While Fas can cooperate with Bim in controlling autoimmunity (211-213), there is little evidence pointing to a role for Fas or other death receptors in negative selection to UbA (40). Negative selection has been shown to occur independently of

Fas and other death receptors (214), however this does not preclude Fas and other death receptors from acting in the absence of Bim during negative selection to UbA. Ultimately, Fas drives apoptosis through the activation of effector caspases, with caspase 3 being the primary active caspase during clonal deletion (215). Contrary to what would be expected if Fas was involved in Bim-independent negative selection, little cleaved caspase 3 is detected in antigen specific thymocytes from OT-I $Bim^{-/-}$ →Act-mOVA chimeras **(Fig 3-4)**.

Nur77 has been shown to be induced during negative selection alongside Bim (77). Overexpression of Nur77 is sufficient to drive apoptosis in DP thymocytes (80) while inhibition of Nur77 with a dominant negative form or Nur77 deficiency can impair but not completely abrogate negative selection in some TCR transgenic models (80-82). In the physiological HY^{cd4} model, it was shown that Nur77 was not required for negative selection to UbA (83). Transgenic Nur77 expression was able to induce caspase 3 activation and apoptosis in thymocytes between the DN3 and DN4 stage in a polyclonal population. This effect was inhibited in TCR transgenic models of non-deletion and negative selection to UbA, suggesting that TCR signaling inhibits Nur77 pro-apoptotic activity (83). However, Nur77 may be able to modulate TCR signaling by increasing the TCR signaling threshold, thus altering the balance between positive and negative selection (83).

In the context of negative selection to TRA in a polyclonal model, single deficiency in other BH3-only Bcl-2 family members does not impair negative selection (142). However, compound deletion of Bim and family member Puma leads to the development of a far more extensive multi-organ autoimmunity than Bim

deficiency alone, with a similar severity to that seen in *Aire*-deficient mice (142). In TCR transgenic models against TRA, Puma and Bim double deficiency resulted in a more extensive impairment in clonal deletion, particularly in $CD69^{+}CD24^{hi}$ semi-mature thymocytes. However, like in OT-I $Bim^{-/-}$→RIPmOVA chimeras, the substantial increase in antigen-specific T cells was not accompanied by the induction of autoimmunity in Bim and Puma double knockout TCR transgenic models (142). While this study certainly identified a role for Puma in clonal deletion to TRA, it did not specifically examine a TCR transgenic model of negative selection to UbA. It remains possible that Puma or other proapoptotic BH-3 only Bcl-2 family members may play a role in negative selection to UbA.

Bim polymorphisms and disease

My data clearly demonstrates that in the absence of Bim, clonal deletion to TRA is abrogated, however, the surviving, self-reactive T cells are rendered anergic. For individuals with Bim polymorphisms that may impact the function of Bim, this suggests that while autoreactive T cells may escape into the periphery, there are robust tolerance mechanisms in place to maintain anergy, dependent upon persistent antigen encounter. However, in cases where peripheral tolerance is maintained through ignorance, whereby antigen is sequestered in an immune privileged site or antigen is present at too low a concentration to elicit a response, it might be possible for autoreactive T cells to persist in the periphery without encountering antigen. This may result in a loss of the anergic phenotype in these T cells. Should these T cells then encounter antigen, perhaps due to injury of the immune privilege site, they may be able to mediate disease.

It is important to note that we examined T cell function *in vivo* under non-inflammatory conditions. It is possible that under inflammatory conditions or in the presence of other PAMPs or danger signals, the anergic state observed in T cells that fail to undergo Bim-mediated clonal deletion, due to Bim polymorphisms or Bim deficiency, can be broken and result in the onset of autoimmunity. However, there exist Bim-independent mechanisms to eliminate activated T cells. For example, NK cells can mediate the deletion of activated T cells in chronic antiviral responses through the extrinsic arm of apoptosis by the recognition of TRAIL receptors (216-218) or FasL (219) on activated T cells. Similarly, recognition of NKG2D ligands on T cells by NKG2D$^+$ NK cells can signal NK cells to kill these T cells (220, 221). These killing mechanisms are caspase-dependent, however they are Bim-independent (222-224) and may be alternative methods of peripheral deletion for eliminating activated these autoreactive T cells.

Genome wide association studies have associated single nucleotide polymorphisms **(SNPs)** with loci including the Bim encoding Bcl2l11 locus in two autoimmune diseases: alopecia areata and primary sclerosing cholangistis (225, 226). Additionally, Bim lies within the NOD mouse autoimmune susceptibility locus *Idd13* and is associated with the impaired thymic deletion in NOD mice (94). Incidentally, the *Idd13* locus has also been associated with promoting T cell influx into pancreatic islets (227), however a recent study suggests this is mediated by the signal inhibitory receptor protein on myeloid cells (228). One caveat about these studies is that they do not determine if the association of Bim with disease is in target cells or T cells and it is difficult to draw any direct conclusions.

Bim polymorphisms can also affect the ability of target cells to undergo apoptosis. In nonsmall-cell lung cancer (NSCLC), Bim polymorphisms have been shown to protect tumor cells from apoptosis induced by epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) (229). Our understanding of anergic mechanisms in thymocytes and T cells failing to undergo Bim-mediated clonal deletion may complement existing and new treatment strategies for cancers by rescuing tumor specific T cells from anergy. While target cells may be protected from Bim-mediated apoptosis by Bim polymorphisms, they may still be susceptible to T cell mediated, Bim-independent apoptotic mechanisms. Additionally, identifying Bim-independent mechanisms of thymocyte and peripheral T cell deletion may offer alternative targets for cancer drugs that can function even if Bim polymorphisms protect tumor cells from intrinsic apoptosis.

Conclusion

I have demonstrated further support for the observation that clonal deletion to UbA can occur in a Bim-independent manner. It is not clear by what mechanism clonal deletion is occurring in the absence of Bim, but it is efficient and robust. In contrast, I have shown that clonal deletion to TRA requires Bim. Thymocytes that escape clonal deletion are anergized after high-affinity Ag encounter and are unable to proliferate *in vitro*. This anergic phenotype persists in peripheral cells and requires peripheral Ag encounter to be maintained. While proliferation of splenocytes can be rescued with plate-bound αCD3/αCD28 stimulation, cytokine production cannot. It is not clear how this anergic state is enforced, as the usual culprits (CD28, CTLA-4, Egr2 and PD-1) do not appear to be involved at least in the

context of *in vitro* stimulation with OVA-pulsed splenocytes. Despite this, the anergic state shares many characteristics with previously described models of anergy including: impaired proliferation that is not rescued by exogenous IL-2, or lymphopenia but is rescued by plate-bound αCD3/αCD28 stimulation; requirement for persistent Ag; impaired cytokine production; and CD8 dulling.

This is the first demonstration of differences in molecular requirements for negative selection to UbA versus TRA. While the exact nature and mechanism of this anergy unknown, it is clear that there exist multiple mechanisms of control in this model. Understanding the regulatory mechanisms at play may shed light on the progression of autoimmunity beyond impaired clonal deletion as a well as have implications in cancer immunotherapy.

Future Directions

The molecular mechanisms driving the induction of anergy in thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras were not identified with these experiments and further work will be needed to uncover them. While expression patterns suggest that PD-1 is not involved in the observed functional impairment, *in vitro* and *in vivo,* α-PD-1 antibody blocking experiments *in vitro* and *in vivo* can confirm whether PD-1 is involved in anergy induction or maintenance.

In *in vitro* anergy models, the activation of the calcium/NFAT pathway (108, 109) and inhibition of ERK and JNK pathways (230) have been implicated respectively in the induction and maintenance of anergy. Understanding how and what signaling pathways are involved in anergy induction will shed light on the molecular mechanisms involved in this anergic state. Additionally examining

functional molecules such as perforin, granzyme B and Fas/FasL will further expand our understanding the anergic state in OT-I Bim$^{-/-}$→RIPmOVA.

While I identified a Foxp3^{+}CD25^{-} population enriched for Vα2^{+}CD8^{+} thymocytes in the thymus and Vα2^{+}CD8^{+} T cells in the periphery, I was not able to determine if they demonstrated any regulatory function. Mixed *in vitro* stimulations of splenocytes or thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras and OT-I Bim$^{-/-}$→B6 chimeras could help to identify any regulatory functions in T cells or thymocytes from OT-I Bim$^{-/-}$→RIPmOVA chimeras. Likewise, co-transfer of T cells from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras into sub-lethally irradiated RIPmOVA recipients could also address this question *in vivo*. One limitation of these experiments would be that if there is a regulatory population in OT-I Bim$^{-/-}$→RIPmOVA chimeras there might not be sufficient numbers of to exert regulatory function in a mixed stimulation or co-transfer. Ideally, we would like to be able to sort out potential T_{reg} to examine suppressor function. However, CD8^{+}Foxp3^{+} T cells from OT-I Bim$^{-/-}$→RIPmOVA chimeras do not exclusively express markers associated with CD8^{+}Foxp3^{+} T_{reg} such as GITR, CTLA-4, CD103 (177), PD-1, and PD-L1 (178). While we do have the OT-I Bim$^{-/-}$ TCR transgenic mice crossed to Foxp3-GFP mice expressing the diphtheria toxin receptor **(DTR)**, the GFP expression is weak and the resolution is not suitable for sorting. With the DTR, it should be possible to selectively deplete Foxp3^{+} expressing T cells and thymocytes by treating OT-I Bim$^{-/-}$→RIPmOVA chimeras with diphtheria toxin. Chimeras would then be observed for the onset of diabetes in order to examine the importance of Foxp3^{+} cells in the non-responsive state observed in OT-I Bim$^{-/-}$→RIPmOVA chimeras.

We have performed microarrays on Vα2^{+}CD8^{+}CD24^{lo} thymocytes from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras, however the data from these arrays has not been fully analyzed yet. It might be of interest to also perform microarrays or RNA seq on stimulated and unstimulated thymocytes and splenocytes from OT-I Bim$^{-/-}$→RIPmOVA and OT-I Bim$^{-/-}$→B6 chimeras. This would allow us to examine what kind of mechanism are at work before stimulation and also identify differential transcriptional programs between OT-I Bim$^{-/-}$→RIPmOVA chimeras and OT-I Bim$^{-/-}$→B6 thymocytes and T cells. Additionally we could observe if there are any changes between thymocytes and peripheral T cells.

We have demonstrated that in the absence of clonal deletion, thymic mechanisms can efficiently anergize self-reactive thymocytes. This anergic state requires persistent antigen encounter to be maintained. A better understanding of how thymically induced states of anergy can be broken has important applications in cancer immunotherapy, especially in adoptive T cell immunotherapy. Tumor associated antigens are fundamentally self-antigens and T cells are likely to be selected against them. Breaking tolerance in tumor infiltrating, tumor-specific T cells in a targeted manner is important for the development of effective and safe cancer immunotherapies. Additionally, these same lessons could be applied to treating chronic infection in which T cells have long become exhausted or anergized. In contrast, this knowledge also has implications in how to re-establish tolerance after a break in tolerance or in preventing a break from occurring. These findings help outline and lay down the foundation for the development of safe and effective immunotherapies.

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