Investigating the role of PD-1 in T cell tolerance

by

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ABSTRACT

T cells are a vital part of the adaptive immune system that function to eliminate foreign threats while remaining tolerant to healthy-self. During T cell development in the thymus, T cells are generated with various T cell receptor (TCR) specificities, such that they can respond to a near endless variety of threats. However, some T cells deleteriously generate a TCR specific for self and thus, have the potential to induce autoimmune disease. To prevent this, self-reactive thymocytes undergo negative selection after receiving a high affinity signal in the thymus. T cells are negatively selected against ubiquitous antigen (UbA) in the thymic cortex or tissue restricted antigen (TRA) in the thymic medulla. Various molecular factors are differentially important for ensuring self-tolerance to either UbA or TRA; one such protein is programmed cell death protein 1 (PD-1).

Given that our group has previously investigated the role of PD-1 in T cell tolerance to UbA, we used the OT-I TCR transgenic mouse model to investigate the role of PD-1 in T cell tolerance to TRA. We determined that $CD8^+$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA bone marrow chimeras were intrinsically impaired but were not functionally rescued by blocking PD-1 signalling *in vitro*. Subsequently, we generated OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras and found that 100% of OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras develop autoimmunity, compared to 0% of control OT-I WT > RIP-mOVA controls. We then isolated OT-I $PD\text{-}1^{-/-}$ T cells from the thymus of OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras and found that clonal deletion was intact, suggesting that PD-1 signals impact T

cell tolerance outside of deletional mechanisms. In the periphery, we found a greater number of $CD8^+$ T cells from OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls, and these cells exhibited differences in proliferation capacity, trafficking potential and cytokine production. Collectively, this data suggests that PD-1 plays a significant role in establishing T cell tolerance to TRA via non-deletional tolerance mechanisms.

PREFACE

This thesis is composed of original work by Rees Kelly. Throughout, it contains content co-authored by collaborators and republished with permission:

- Kelly RG, Golec DP, Henao-Caviedes LM, Baldwin TA. 2018. Microenvironment Regulation of Thymic Progenitors: How Form Follows Function. Encyclopedia of Tissue Engineering and Regenerative Medicine 1st Edition. Elsevier Publishing Group.

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Understanding the cellular and molecular requirements underlying negative selection in the thymus and breeding colony", Protocol No. AUP 220.

DEDICATION

I'd like to dedicate this thesis to my father Bruno, who passed away over the course of this degree.

It is my sincere hope that this research will be used to improve immunotherapeutics such that others will have more time with their loved ones before they pass away.

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I would first like to thank my supervisor Dr. Troy Baldwin for giving me the opportunity to pursue graduate studies in his lab. Dr. Baldwin not only taught me the tools needed to become a strong, self-sufficient scientist, but he also served as an exemplary role-model for professional and personal conduct. I am grateful for his unwavering support over the years, which has enabled me to produce research that I am immensely proud of.

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I would like to especially thank the two most important women in my life – my mother Wendy and my girlfriend Laura. I would not have finished this degree without their unconditional love, support, and encouragement.

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LIST OF RECURRING ABBREVIATIONS

APC – antigen presenting cell

Ab – antibody

Ag – antigen

B6 – C57BL/6 wild type mice

Bim – Bcl-2-interacting mediator of death

BM – bone marrow

BMC – bone marrow chimeras

CMJ – corticomedullary junction

cTEC – cortical thymic epithelial cell

DC – dendritic cell

DN – double negative ($CD4^-CD8^-$)

DP – double positive ($CD4^+CD8^+$)

ERK – extracellular signal-regulated kinase

HSC – hematopoietic stem cell

ITAM – immunoreceptor tyrosine-based activation motif

ITIM – immunoreceptor tyrosine-based inhibitory motif

ITSM – immunoreceptor tyrosine-based switch motif

MFI – mean or median fluorescent intensity

MHC – major histocompatibility complex

NOD – non-obese diabetic mouse

NK – natural killer cell

OVA – chicken ovalbumin

PD-1 – programmed cell death protein 1

pLn – pancreatic lymph node

RAG – recombination-activating gene

SCZ – subcapsular zone

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

TCR – T cell receptor

Tg - transgenic

TRA – tissue restricted antigen

Treg – T regulatory cell

UbA – ubiquitous antigen

WT – wild type

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CHAPTER 1: INTRODUCTION

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The Immune System

We live in an inherently dangerous world, where we are constantly under assault from a gamut of evolutionarily diverse pathogens. Given these ever-present threats, we have evolved simple and complex protection mechanisms that work synergistically such that we are able to limit serious illness. There are three major branches of defence: epithelial and mucosal barriers which provide physical separation and chemical protection; the innate immune system which provides a rapid cellular yet non-antigen specific wave of defence; the adaptive immune system which takes a significant period of time to engage, but is antigen specific, powerful and can provide long-lasting fortification. The adaptive immune system is primarily mediated by B cells, which produce antibodies (Ab), and T cells that help coordinate host immune cells and kill pathogenic cells directly. The T cell compartment is highly heterogeneous in nature; it is probable that there are functionally distinct T cell subsets that have not yet been elucidated. The major T cell lineages are: $CD8^+$ T cells, $CD4^+$ Th1 cells, $CD4^+$ Th2 cells, $CD4^+$ T Regulatory cells (Treg), and $CD4^+$ Th17 cells. Both $CD8^+$ and $CD4^+$ T cells recognize peptide antigen in the context of major histocompatibility complex (MHC), however, $CD8^+$ T cells are restricted to MHC Class I and $CD4^+$ T cells to MHC Class II. The interplay between T cell subsets, in conjunction with other host immune cells, provides us with a prodigious amount of protection against deleterious threats. However, some T cells that are generated have the potential to cause disease by inappropriately becoming active against self; this is the basic premise behind autoimmunity. Central to this idea, the dysregulation of T cell development in the thymus often underlies the development of autoimmune disease.

T Cell Development

Progenitor Development and Migration to the Thymus

The most immature progenitors harbouring T cell potential are hematopoietic stem cells (HSC) found in the bone marrow. HSCs mature into multipotent progenitor cells (MPPs) at which point they undergo the first steps of lineage commitment [1,2]. The earliest lymphoid committed population are lymphoid-primed multipotent progenitors (LMPPs), which have lost their erythroid and megakaryocyte lineage potential. Both LMPPs and the further differentiated common lymphoid progenitors (CLPs) have the potential to seed the thymus [3]. Thymus settling progenitors (TSPs) are reliant on a number of chemokines and adhesion molecules for importation from the blood to the thymus. Chemokine receptors CXCR4, CCR7, and CCR9 have been shown to be crucial in homing TSPs to the thymus, while corresponding chemokine ligands CXCL12, CCL21, and CCL25 are expressed by both vascular endothelial cells and thymic stromal cells, with some overlap [4–7]. Additionally, expression of the adhesion molecule P-selectin glycoprotein ligand-1 (PSGL-1) on TSPs was shown to be important in ensuring efficient extravasation through P-selectin expressing thymic vascular endothelium [8]. Once TSPs have arrived inside the thymus at the CMJ, the Notch signalling pathway has proven to drive the transition of TSPs into early thymic progenitors (ETPs), which comprise the most immature population of T cell progenitors that are readily detectable within the thymus under homeostatic conditions [9,10]. Ultimately, productive Notch signalling in ETPs facilitates the development of immature double negative (DN) thymocytes, named as such due to a lack of CD4 and CD8 co-receptor expression.

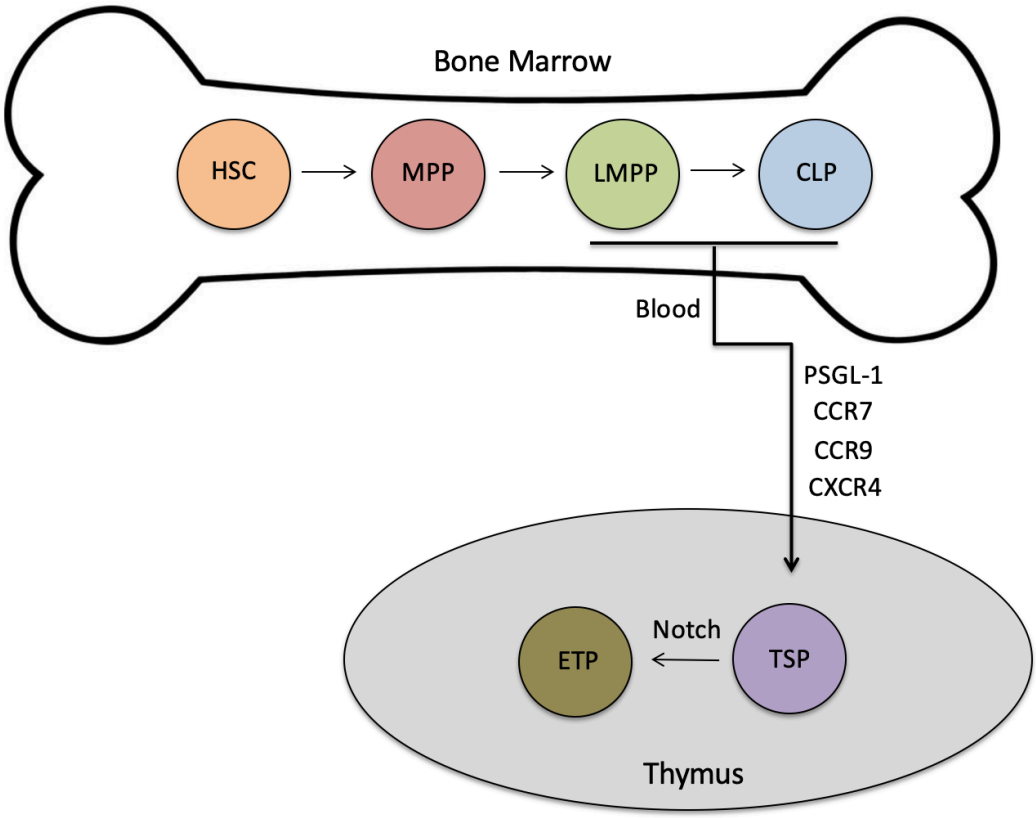


Figure 1-1. **Overview of Bone Marrow Progenitor Development and Migration**. Hematopoietic stem cells (HSC) are found in the bone marrow and mature into multipotent progenitor cells (MPPs). MPPs differentiate into lymphoid-primed multipotent progenitors (LMPPs), which have lost their erythroid and megakaryocyte lineage potential. Both LMPPs and the further differentiated common lymphoid progenitors (CLPs) have the potential to seed the thymus. Importation of TSPs is reliant on a number of chemokines and adhesion molecules. Once TSPs have arrived inside the thymus at the cortico-medullary junction (CMJ), the Notch signalling pathway drives the transition of TSPs into early thymic progenitors (ETPs), which comprise the most immature population of T cell progenitors that are readily detectable within the thymus under homeostatic conditions.

DN Thymocyte Development

There are four major stages of DN thymocyte development: DN1-4, and they are generally marked by differential expression of CD25, CD44 and CD117. DN1 thymocytes ($CD25^{-}CD44^{+}CD117^{hi}$) are located in the thymic corticomedullary junction (CMJ). Temporally, DN1 thymocytes reside in the CMJ for roughly a week and a half, proliferate extensively, and begin to become more restricted in various lineage potentials [11]. Strong Notch1 signalling mediated by transcription factor RBP-Jκ has been shown to be critical in restricting fate potential at this stage of development [12,13]. Subsequently, DN2 thymocytes ($CD25^{+}CD44^{+}CD117^{hi}$) leave the CMJ and travel towards the subcapsular zone (SCZ). This migration is facilitated by chemokine gradients corresponding with CCR7, CCR9 and CXCR4 expression on DN2 thymocytes [14,15]. Early and late DN2 thymocytes can be separated according to CD117 expression, which is more pronounced with increasing proximity to the SCZ [16]. DN2 thymocytes are also subjected to signals from cortical thymic epithelial cells (cTECs) and fibroblasts as they migrate to the SCZ, which combine to facilitate the loss of dendritic cell (DC) and natural killer (NK) lineage potential [17,18]. V(D)J recombination, a unique and powerful mechanism of genetic recombination, begins at the DN2 stage of development at the T cell receptor (TCR) β, γ, and δ loci. The bifurcation of DN thymocytes into α/β or γ/δ T cells is dependent on which loci produce a productive rearrangement first. Should a successful rearrangement at the β locus occur before rearrangements at the γ and δ loci, γ/δ rearrangement is halted and the DN thymocyte is committed in a Notch-dependant manner to the α/β lineage [19]. Conversely, if productive rearrangements at the γ and δ loci occur before a successful rearrangement at the β locus, β rearrangement is terminated

and the DN thymocyte is committed to the γ/δ lineage, provided there is adequate IL-7 in the cortical microenvironment [20]. Both the Notch signalling pathway and the Wnt/B-catenin pathway are essential for productive lineage commitment during V(D)J recombination [21,22]. DN3 thymocytes ($CD25^{+}CD44^{-}$) are primarily located in the SCZ and undergo a major T cell development checkpoint, β-selection. DN3 thymocytes now express a TCR β-chain, which thereafter pairs with a pre-Tα chain, culminating in the formation of the pre-TCR. If DN3 thymocytes with a pre-TCR are able to signal through the pre-TCR, in other words, have a functional TCR rearrangement, they in turn receive survival, proliferation, and lineage commitment signals. Conversely, DN3 thymocytes that are unable to signal through the pre-TCR are eliminated by apoptosis. Signalling pathways that have been implicated in this process are the Notch and phosphoinositide 3-kinase (PI3K) pathways, with CXCR4 associated signalling playing an important role as well [23,24]. Presuming successful β-selection, DN4 thymocytes ($CD25^{-}CD44^{-}$) begin to undergo recombination at the TCR α-locus, begin to migrate towards the medulla, and upregulate CD4/CD8 co-receptors, which marks a major transition from the DN to DP stage of development. Temporally, it has been experimentally determined that it takes roughly 19 days for an individual T cell progenitor, since its initial arrival in the thymus, to develop into a DP thymocyte [11].

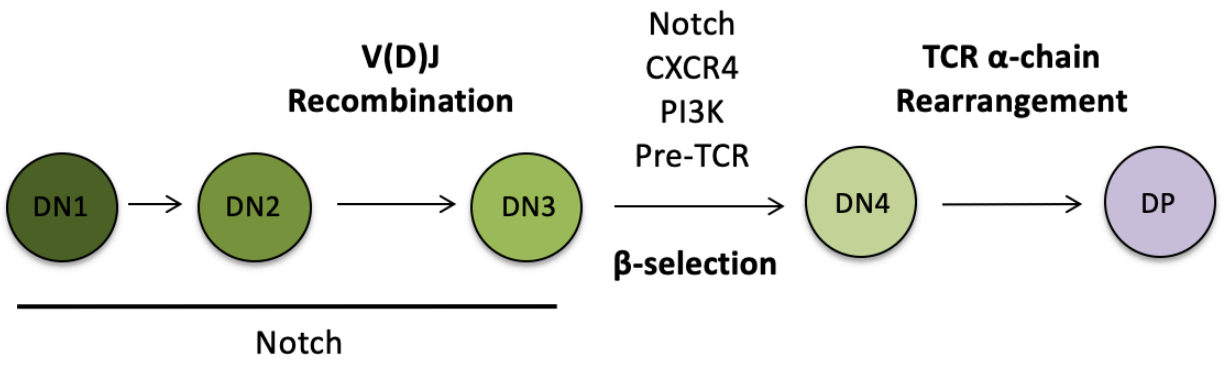


Figure 1-2. **Overview of DN Thymocyte Development**

There are four major stages of DN thymocyte development: DN1-4. Early DN1 thymocytes are considered to be TSPs, and they undergo proliferation and the first steps of lineage commitment. DN1 thymocytes develop into DN2 thymocytes which migrate towards the SCZ and start to undergo V(D)J recombination. After successful rearrangement at the TCR β-locus given adequate Notch signalling, DN3 thymocytes undergo β-selection, leading to expression of the pre-TCR. Presuming successful β-selection, DN4 thymocytes undergo recombination at the TCR α-locus, migrate towards the medulla, upregulate CD4/CD8 co-receptors, and now express a fully functional TCR as DP thymocytes.

DP Thymocyte Development

DP thymocytes that express an α/β TCR, undergo a number of different developmental fates depending on the ability of their TCR to recognize and bind to peptide presented in the context of major histocompatibility complex (pMHC). Most DP thymocytes (80-90%) do not express a TCR that is able to recognize pMHC, thus they die by neglect. A relatively small portion (5-10%) of DP thymocytes bind to self-pMHC with low to moderate affinity and are positively selected; an equally small portion (5-10%) bind to self-pMHC with strong affinity and are eliminated via apoptosis (negative selection to ubiquitous antigen - UbA). These selection events in the cortex are mediated by cTECs and DCs, which work together to ensure that T cells expressing a useful TCR are positively selected, while T cells with potentially self-reactive TCRs are deleted. As a whole, cTECs are the most important mediators of positive selection, due to their expression of specific pMHC complexes. cTECs express a specific proteasomal subunit, β5t, that has been shown to interact with the proteasome to form a so-called thymoproteasome, capable of generating a pMHC repertoire that can positively select DP thymocytes in the context of MHC CI [25,26]. This effect is well known as studies investigating β5t knockout mice have shown defects in positive selection and functionality of $CD8^+$ SP T cells [27,28]. cTECs also express the lysosomal protease Cathepsin L, which can generate a pMHC repertoire restricted to MHC CII; deficiencies in Cathepsin L lead to a marked reduction of $CD4^+$ SP thymocytes [29].

DP thymocytes become either $CD8^+$ or $CD4^+$ SP thymocytes depending on the ability of their TCR to bind peptide presented on MHC CI or MHC CII, respectively. There are three major models that have been proposed to explain the mechanism behind CD4/CD8 lineage commitment: the instructive model, the stochastic model, and the kinetic signalling model. The kinetic signalling model has harmonized historical evidence with modern experimental data, and it now stands as the most widely accepted model explaining lineage commitment [30]. It postulates that DP thymocytes commit to a particular lineage by assessing the CD8 dependence of their TCR. Firstly, CD8 co-receptor is downregulated and CD8 gene expression is halted after positive selection. If these DP thymocytes receive continued signals through TCR-pMHC interaction despite a loss of CD8, these DP thymocytes commit to the CD4 lineage. Conversely, if this signal is interrupted by the absence of CD8, CD4 is downregulated and the DP thymocyte will upregulate CD8 and commit to the CD8 lineage. Certain transcription factors are also critical for lineage commitment mechanisms. Many studies have elucidated ThPOK and GATA3 as the key transcription factors that are seminal for the development of $CD4^+$ T cells, whereas Runx3 is essential for $CD8^+$ T cell generation [31–34].

SP Thymocyte Development

After initial commitment to either the CD4 or CD8 lineage, SP thymocytes upregulate chemokine receptor CCR7 which binds to medullary thymic epithelial cells (mTECs) secreted cytokines CCL19 and CCL21 that direct them to the medulla, where they undergo another round of negative selection, mediated by mTECs and DCs [35]. It's

important to keep in mind that there are significant differences in negative selection that occurs in the cortex and medulla, not pertaining to the stage of development. In the cortex, thymocytes are negatively selected to ubiquitous antigen (UbA), which are antigens that are present throughout the body, such as actin. This is different from the medulla, which negatively selects thymocytes that are able to bind strongly to tissue-restricted antigens (TRA), of which a couple of examples are insulin and myelin basic protein (MBP).

One of the most important features of mTECs is their ability to express a wide variety of self-peptides. Freshly migrated SP thymocytes from the cortex do not undergo proliferation after strong pMHC-TCR signalling to TRA; instead most undergo apoptosis, which underlies the mechanism behind negative selection in the thymic medulla [36]. Given random TCR rearrangement during T cell development, some thymocytes may have TCRs specific for self-peptides present in various locations throughout the body. Traditionally, the ability of the immune system to prevent the development of T cells whose cognate antigen was located outside the thymus was a mystery. However, we now know that a large number of gene transcripts, controlled primarily by the autoimmune regulator (AIRE) gene, are ectopically produced by mTECs, marking a unique capability compared to the related cTECs located in the cortex [37–39]. Additionally, the transcription factor Fezf2 has also been shown to induce the expression of genes important for tolerance to TRA [40]. Presentation of TRAs to T cells is important; mutations in the *AIRE* and *Fezf2* genes are associated with autoimmune polyendocrine syndrome type 1 (APS-1) and other autoimmune diseases [41–43].

Dendritic cells are also vital in the negative selection of self-specific thymocytes. While DCs themselves do not have the inherent ability to express TRAs, they are able to cross-present TRA from adjacent mTECs. Studies where DCs are ablated genetically or are deficient in MHC CII expression have illustrated a clear impairment of negative selection to $CD4^+$ thymocytes, surprisingly, in a non-redundant role compared to mTEC mediated deletion [44,45]. Temporally, mTECs associate with DCs in large part due to the production of chemokine XCL1 that attracts medullary DCs and subsequently co-localizes them with mTECs [46]. Together, mTECs and DCs ensure that the majority of SP thymocytes which express a TCR specific for self are negatively selected in the thymus and thus are not able to seed the periphery and induce autoimmune disease.

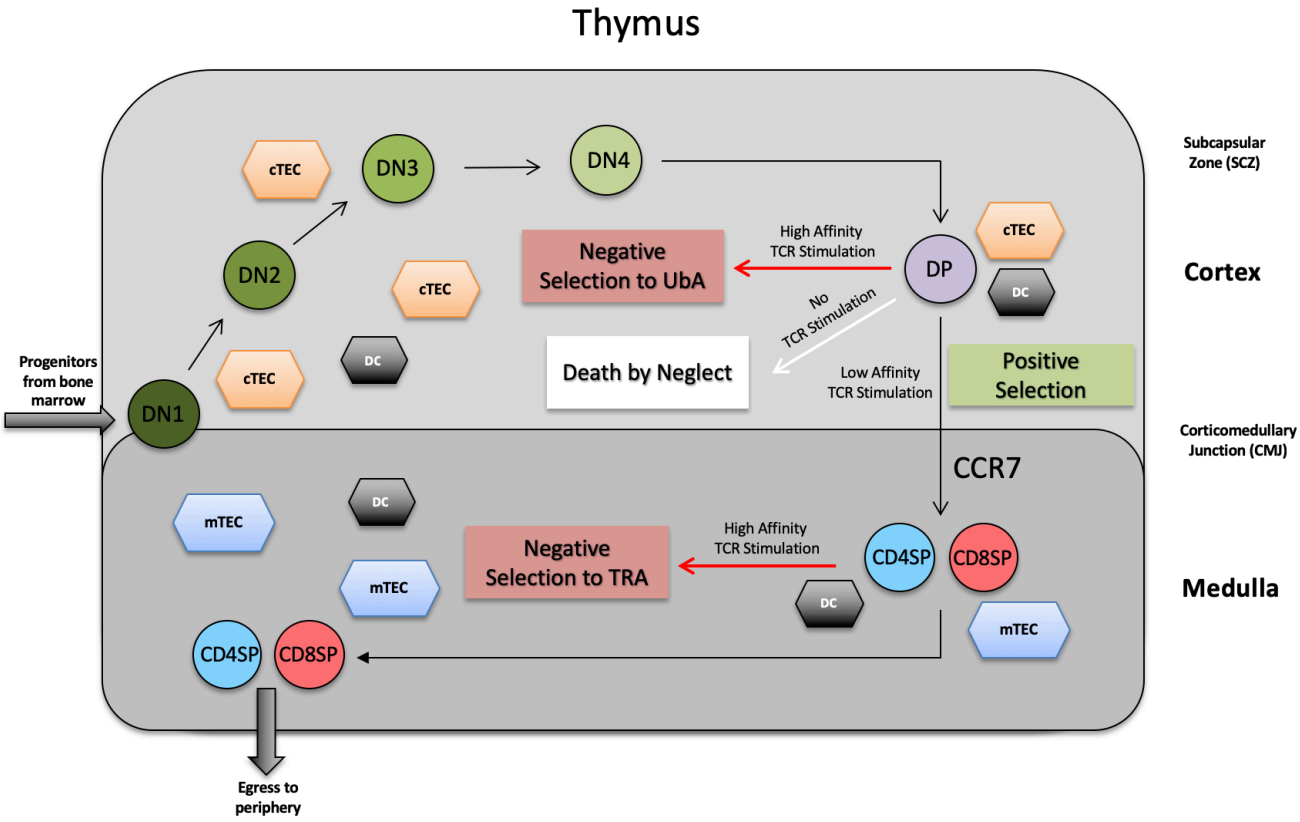


Figure 1-3. **Overview of DP Thymocyte and SP Thymocyte Development**

DP thymocytes that express a fully functional α/β TCR, undergo a number of different developmental fates depending on their ability to recognize and bind to pMHC. Most DP thymocytes (80-90%) do not express a TCR that is able to recognize pMHC, thus they die by neglect. A relatively small portion (5-10%) of DP thymocytes bind with low to moderate affinity and are positively selected; an equally small portion (5-10%) bind with strong affinity and are eliminated via apoptosis (negative selection to UbA). These selection events in the cortex are mediated by cTECs and DCs. After positive selection, SP thymocytes upregulate chemokine receptor CCR7 which binds to mTEC secreted cytokines CCL19 and CCL21 that direct them to the medulla, where they undergo another round of negative selection to TRA, mediated by mTECs and DCs.

TCR Signalling During Positive and Negative Selection

Interactions between the TCR and pMHC initiates a number of distinct proximal and distal TCR signalling events that drive positive and negative selection. In general, strong intermittent signalling leads to negative selection, in contrast to weak/medium yet sustained signalling, which is conducive to positive selection. Positive and negative selection signals are quantitatively and kinetically distinct; mechanisms used to convert changes in TCR-pMHC affinity to fate decisions are evident in proximal and distal TCR signalling events. To begin, TCR-pMHC engagement facilitates phosphorylation of lymphocyte-specific protein tyrosine kinase (Lck) and CD3 subunits on their immunoreceptor tyrosine-based activation motifs (ITAMs), which promotes localization of zeta-chain-associated protein kinase 70 (ZAP-70) to the TCR complex. Both positive and negative selection requires ZAP-70 localization to the intracellular ζ-chains of CD3. Recruitment of ZAP-70 after high affinity TCR-pMHC engagement (negative selection) is strong and transient, in contrast to low affinity TCR engagement (positive selection), which elicits reduced and delayed recruitment of ZAP-70 [47]. After recruitment and activation of ZAP-70 by Lck, it is able to phosphorylate a key adapter protein, linker for activation of T cells (LAT). Low-to-moderate affinity pMHC-TCR interactions induce sustained and weak phosphorylation of LAT compared to negative selection, where there is a strong early peak of phosphorylation which fades over time [48]. LAT functions as an essential docking point for two distinct signalosomes: Grb2/Themis/Shp1 and Itk/SLP76/PLCγ. The non-classical Grb2/Themis/Shp1 signalosome functions primarily to dampen TCR signaling through phosphatase Shp1 and THEMIS, and to activate the

Ras-ERK signalling pathway through Grb2 mediated recruitment of Sos1 [49,50]. The classical LAT signalosome functions as a scaffold for PLCγ phosphorylation by Itk bound to SLP76 that upon PLCγ activation is able to catalyze the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG, in tandem with Ca2+, mediates the activation of protein kinase C (PKC), leading to the phosphorylation of I-kB and subsequent liberation of physiologically active NF-kB dimers [51,52]. DAG also has the capacity to bind RasGRP, which in turn activates the Ras-ERK signalling pathway, leading to ERK1/2 activation and its nuclear translocation. In the nucleus, active ERK1/2 has been shown to regulate the expression of transcription factor heterodimer AP-1, particularly through c-FOS subunit stabilization [53]. A few steps upstream from ERK, and in addition to DAG, IP3 is cleaved from PIP2, which subsequently binds to the IP3 receptor on the ER and facilitates the release of intracellular Ca2+, and later, store-operated Ca2+. Groups have shown that the release of Ca2+ has widespread cellular effects including the activation of the transcription factor NFAT, which translocates to the nucleus and regulates the expression of a variety of target genes; a large release of Ca2+ is associated with negative selection but modest and sustained Ca2+ release is associated with positive selection [54,55]. Perhaps the most traditional example of differences in intracellular signalling corresponding with positive or negative selection is evident in differential ERK1/2 signalling. Positive selection induces weak, but sustained ERK signals dependant on RasGRP1, culminating in the upregulation of survival factors such as Bcl-2, Mcl-1, and IL-7R; conversely, negative selection leads to strong but transient ERK signalling, accompanied by JNK and p38 mediated initiation of Bim-mediated apoptosis [56–61].

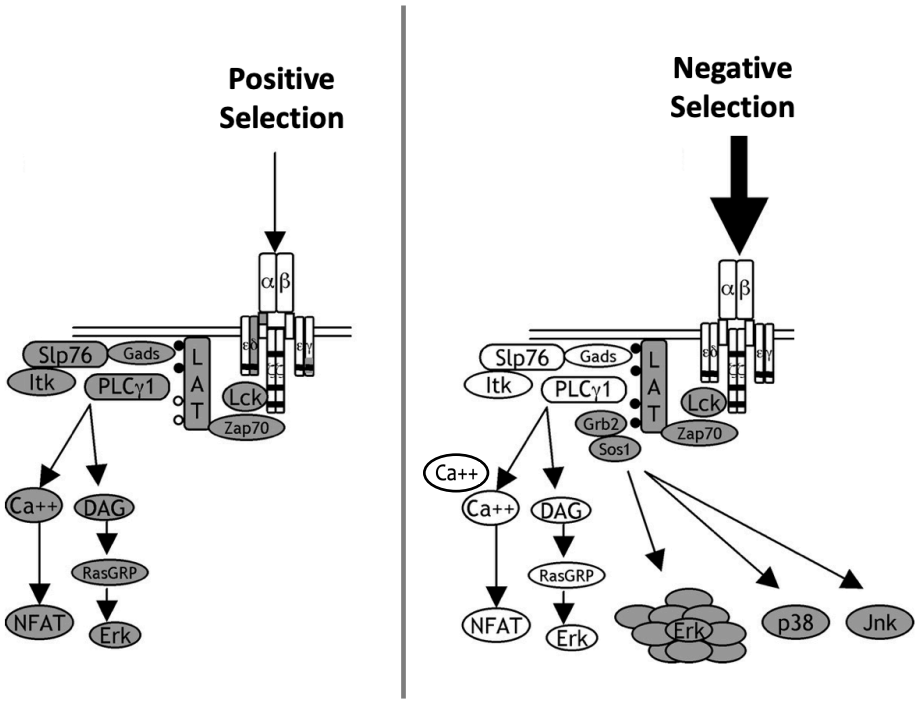


Figure 1-4. **Signaling Events After TCR Stimulation**

TCR-pMHC initiates a number of TCR signalling events: TCR-pMHC engagement facilitates phosphorylation of Lck and CD3, which then promotes localization of ZAP-70. ZAP-70 is then phosphorylated by Lck and is subsequently able to phosphorylate LAT, which functions as an important docking point for other proteins (signalosomes). Downstream of LAT, strong intermittent ERK signalling and a large calcium release is associated with negative selection; whereas, weak sustained ERK signals and modest calcium release is associated with positive selection. Figure adapted from Hogquist et al, Annual Review of Immunology, 2003.

T Cell Tolerance

The "mission statement" of T cell development is to ensure the generation of T cells with TCRs that are able to effectively respond against a wide variety of threats, without causing autoimmunity. This balance between immunocompetency and inappropriate self-reactivity is not trivial. Given V(D)J recombination, there are between 10^{15} to 10^{20} unique TCR structures that may be generated, theoretically providing host immunity for all pathogens but also virtually guaranteeing the production of a significant heterogenous population of T cells that may induce autoimmune disease [62].

Central Tolerance

The processes involved with preventing the generation and propagation of these autoreactive T cells during development in the thymus is called central tolerance. Central tolerance is primarily enforced by clonal deletion of thymocytes with strong affinity for either UbA or TRA, as mentioned previously. However, there is also strong evidence that non-deletional tolerance mechanisms such as anergy induction and clonal diversion are significant enforcers of central tolerance [63]. Anergy is a blanket term used to describe various cell-intrinsic functional impairments that render lymphocytes unable to mount an immune response; classical anergy induction occurs when a naïve T cell encounters its cognate antigen but does not receive co-stimulatory signals through CD28 [64]. Clonal diversion, also known as agonist selection, refers to thymocytes with strong affinity for thymic pMHC that are not clonally deleted and are instead diverted into an alternative lineage, such as into natural T regulatory $CD4^+$ cells. While central tolerance mechanisms

are robust, they are not perfect; even in healthy organisms, a fraction of self-reactive thymocytes manage to escape the thymus, however they may not cause disease [65,66].

Peripheral Tolerance

The mechanisms in place to prevent potentially autoreactive T cells from causing disease after emigrating from the thymus are considered to be peripheral tolerance mechanisms. Similar to central tolerance, peripheral tolerance is mediated by a number of distinct mechanisms: potentially autoreactive T cells may not respond to low levels of cognate antigen (ignorance), as evidenced from studies using TCR-GP double transgenic mice and RIP-OVA^{lo} transgenic mice; T cells may be rendered anergic from a lack of co-stimulatory signals, as shown initially by stimulating naïve CD4$^+$ T cells with immobilized anti-CD3 without CD28 *in vitro*; T cells may undergo peripheral deletion owing to cell intrinsic (Bim mediated) and cell extrinsic (Fas mediated) mechanisms that may act to prevent autoimmunity; T cells may also be cell-extrinsically suppressed by T regulatory cells or tolerogenic dendritic cells, as shown *in vitro* and *in vivo* by many groups using different model systems [67–73,79]. Furthermore, a lack of CD4$^+$ T cell "help" for Ag-specific CD8$^+$ T cells can lead to inactivation/tolerance of CD8$^+$ T cells and dynamic regulation of certain proteins such as CD5 can "tune" T cell sensitivity to antigen thus leading to tolerance [74–76]. Globally, central and peripheral tolerance mechanisms have been shown to effectively prevent the induction of autoimmune disease. However, less is known regarding individual molecular factors that are important for T cell tolerance.

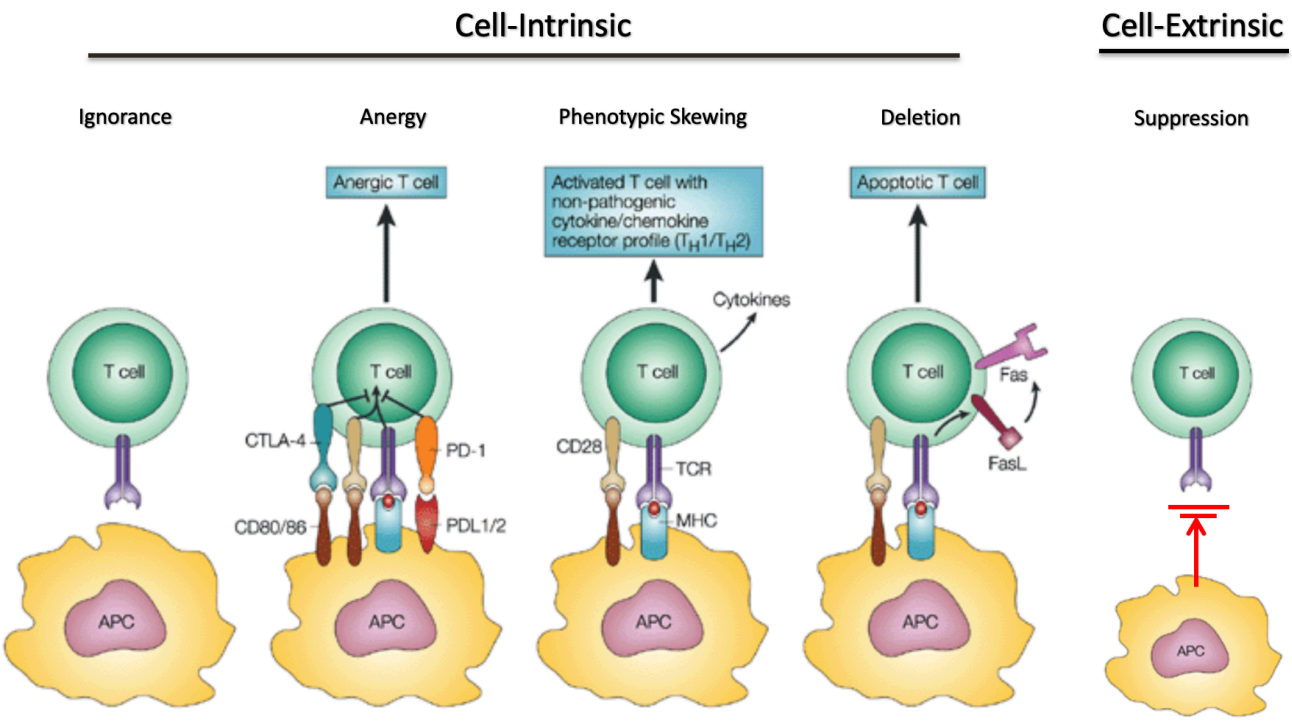


Figure 1-5. **Overview of Peripheral Tolerance Mechanisms**

A fraction of self-reactive T cells escape central tolerance mechanisms (clonal deletion, anergy) and enter the periphery where they are subjected to peripheral tolerance mechanisms which can be grouped into T cell extrinsic and T cell intrinsic mechanisms. T cell extrinsic mechanisms include ignorance, anergy, phenotypic skewing and deletion; T cell intrinsic mechanisms include suppression from tolerogenic dendritic cells and regulatory T cells. Figure adapted from Abbas and Walker, Nature Reviews Immunology, 2002.

Animal Models Used to Investigate Tolerance

Research groups use an assortment of different animal models to investigate the role of various molecular factors that impact T cell tolerance. Broadly speaking, they can be classified into distinct categories: polyclonal models which generate T cells with a variety of different specificities for pMHC, and transgenic monoclonal models that generate T cells with identical TCRs specific for the same pMHC. Polyclonal model systems have been valuable tools to investigate the inner workings of complex immune systems, as they model heterogenous T cell development. However, most immune cells generated in this way do not have strong affinity for any particular pMHC, nor is there a large frequency of T cells specific for the same pMHC. These factors limit the ability of researchers to effectively investigate potentially autoreactive T cells. Therein, TCR transgenic model systems engineered to generate T cells specific for a particular high-affinity antigen are a more powerful tool used to investigate the role of molecular factors of T cell tolerance to high affinity antigen, given their predominant frequency.

HY^{cd4} Model – Negative Selection to UbA

One model system often used to investigate the role of molecular factors important in tolerance to UbA is the HY^{cd4} model, engineered using a Cre/lox based conditional strategy to express the TCR at the DP stage of thymocyte development [77]. The HY TCR is specific for an epitope from the male-specific smcy gene (KCSRNRQYL) presented in the context of $H\text{-}2D^{b}$. In this way, the HY^{cd4} female mouse

is a model for positive selection, whereas the HY^{cd4} male mouse is a model for negative selection; male antigen is first encountered by DP thymocytes in the cortex and thus, it models negative selection to UbA. Important to keep in mind, is that molecular factors that may be important in T cell tolerance for UbA may not be important for TRA, or vice-versa; for example, previous work from our group using this model system suggests that PD-1 is not important for T cell tolerance to UbA [78].

OT-I Model - T Cell Tolerance to TRA

To investigate molecular factors of T cell tolerance to TRA, the OT-I transgenic model in conjunction with RIP-mOVA recipients have been well studied [79–82]. The OT-I TCR is MHC Class I restricted and specific for OVA-peptide (SIINFEKL) in the context of $H\text{-}2K^b$. RIP-mOVA mice express a membrane bound form of OVA under the control of the rat insulin promotor. RIP-mOVA mice express OVA predominantly on mTECs and islet β-cells but also on kidney tubule proximal cells and in testes. Bone marrow chimeras generated using OT-I donor bone marrow and RIP-mOVA recipients are an optimal strategy for studying T cell tolerance to TRA, as OT-I thymocytes encounter OVA in both the thymus during development and in the periphery. Should a breakdown of tolerance occur in OT-I > RIP-mOVA chimeras, they develop autoimmune diabetes as a tangible readout for autoimmunity.

Adoptive transfer of OT-I $CD8^+$ T cells can induce autoimmunity in RIP-mOVA recipients without OT-II $CD4^+$ help; tolerance induction is mediated by interactions with

antigen presenting DCs, as peripheral deletion was shown to be sufficient for tolerance contingent on the number of OT-I $CD8^+$ T cells transferred [80,83]. The islet infiltrating OT-I $CD8^+$ T cells also produce inflammatory cytokines such as IFNγ, though its precise role in autoimmune diabetes pathogenesis is still unclear. Furthermore, while certain trafficking molecules such as CXCR3 have been shown to be important for T cell trafficking to inflammatory tissues in other model systems, these findings have yet to be recapitulated in the OT-I → RIP-mOVA adoptive transfer model.

NOD Model – Comparison for OT-I Mediated Diabetes

Most animal models of diabetes aim to recapitulate Type I Diabetes, of which the NOD model system is most widely used. In Type I Diabetes as well as in NOD mice, pancreatic β-cells undergo destruction mediated by infiltrating $CD4^+$ and $CD8^+$ T cells that are specific for a variety of auto-antigens. Additionally, other immune cells such as macrophages and DCs play a significant role in diabetes induction [84,85]. There are sex specific differences between NOD mice, as female NOD mice develop diabetes at an enhanced frequency, though the precise rate is dependent on exposure to bacterial antigen or infection of the colony, as neonatal immunostimulation can prevent diabetes induction [86]. The basic disease pathogenesis in the NOD model system is as follows: potentially autoreactive $CD4^+$ and $CD8^+$ T cells escape central tolerance mechanisms and enter the periphery; naïve $CD4^+$ T cells interact with auto-antigen expressing DCs in the pLn leading to development into mature Tfh and Th1 cell types; Tfh cells elicit auto-antibody production by B cells, while Th1 cells can help activate naïve CD8 T cells in the pLn;

Th1 cells traffic to the pancreas and produce cytokines that stimulate M1 macrophages and DCs; cytokines such as IFNγ produced by M1 macrophages, Batf3 expressing DCs and Th1 $CD4^+$ T cells directly induce β-cell death; damaged islets secrete CXCL10 and CXCL9 which recruit $CXCR3^+$ $CD8^+$ T cells; islet infiltrating $CD8^+$ T cells secrete further pro-inflammatory cytokines and they directly kill β-cells via perforin and granzyme-mediated processes [87]. One major drawback to using NOD mice for investigating molecular factors important for T cells tolerance is that it does not generate large numbers of T cells specific for high affinity antigen. This limitation can be overcome by using TCR transgenic (Tg) NOD mice such as the BDC2.5 Tg mouse which generates monoclonal $CD4^+$ T cells specific for an islet β-cell islet protein [88,89]. In contrast to non-Tg NOD mice, virtually every T cell from OT-I > RIP-mOVA bone marrow chimeras are specific for the same high affinity antigen.

It is ultimately well understood that $CD8^+$ T cells are required for disease pathogenesis in NOD mice, though the initial progression of diabetes pathogenesis seems to be mediated by $CD4^+$ T cells [90]. We use the NOD model of diabetes pathogenesis to compare with diabetes induction in RIP-mOVA recipients, though we do not actually study NOD mice – we use its identified characteristics as a reference for our OT-I model system.

The Role of PD-1 in T Cell Tolerance

Our group has published data on the role of various molecular factors important for T cell tolerance using the OT-I model system, such as the pro-apoptotic Bcl-2 family member Bim [91]. Bim has been shown to be important for the intrinsic pathway of apoptosis and is induced during negative selection in the thymus; experiments using OT-I $Bim^{-/-}$ > RIP-mOVA chimeras and OT-I $Bim^{-/-}$ > Act-mOVA chimeras demonstrate that Bim is required for clonal deletion to TRA, though not UbA. Surprisingly, the OT-I $Bim^{-/-}$ thymocytes that escape clonal deletion after cognate antigen encounter do not go on to cause autoimmunity, and instead are rendered functionally impaired. While Bim is not important for T cell tolerance to TRA, PD-1 signals may be critical for tolerance, as PD-1 is expressed highly on $V\alpha2^{+}$ $CD8^{+}$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras.

For this project, we used the OT-I model system to investigate the role of PD-1 signalling in tolerance to TRA, as it was previously shown to not be required for tolerance to UbA using the HY^{cd4} model system. PD-1 is a co-inhibitory receptor that can be expressed on T cells, B cells, NK cells, natural killer T cells (NKT), innate lymphoid cells (ILCs), and on some myeloid and antigen presenting cell (APC) populations. PD-1 signalling serves to counter positive signals received through the TCR and CD28 through ligation of PD-L1 and PD-L2. The role of PD-1 signalling is most often studied in the context of T cells. PD-1 has two ligands: PD-L1 and PD-L2, which differ significantly in their expression patterns. PD-L1 is expressed on both hematopoietic lineages such as T cells, B cells, macrophages and DCs and on non-hematopoietic lineages such as cortical

thymic epithelial cells, vascular endothelial cells and pancreatic islets. Most of these cells constitutively express PD-L1, though pro-inflammatory signals increase the amount of PD-L1 expression. Conversely, PD-L2 expression is largely restricted to DCs, macrophages and B cells, and its expression is typically low at steady-state, though it can be induced by inflammatory signalling. PD-L1 and PD-L2 each have an additional binding partner: PD-L1 can bind to CD80 and PD-L2 to repulsive guidance molecule B (RGMB), though their precise roles in immune tolerance are unclear [92]. While there are no conserved signalling motifs within the intracellular tails of PD-L1 and PD-L2, there exists some evidence to suggest signals can be transduced "backwards" into PD-L1 or PD-L2 expressing cells [93]. PD-1 contains two canonical signalling motifs, an ITIM and an ITSM sequence. Currently, there is little evidence to support a functional role for the ITIM motif; experiments mutating the tyrosine residue of the ITSM but not the ITIM impaired the ability of PD-1 to exert its co-inhibitory effects [94]. The phosphatase SHP-2 recognizes the phosphorylated ITSM residue after engagement of the TCR and is subsequently able to promiscuously dephosphorylate TCR-proximal signalling proteins such as ZAP-70, Ras, PI3K and the intracellular domain of CD28. Collectively, this results in a weakened signal transduced through the TCR after engagement, thereby attenuating signals to transcription factors such as AP-1, NFAT and NF-κB that are important for functional outcomes such as activation, proliferation, effector function and survival.

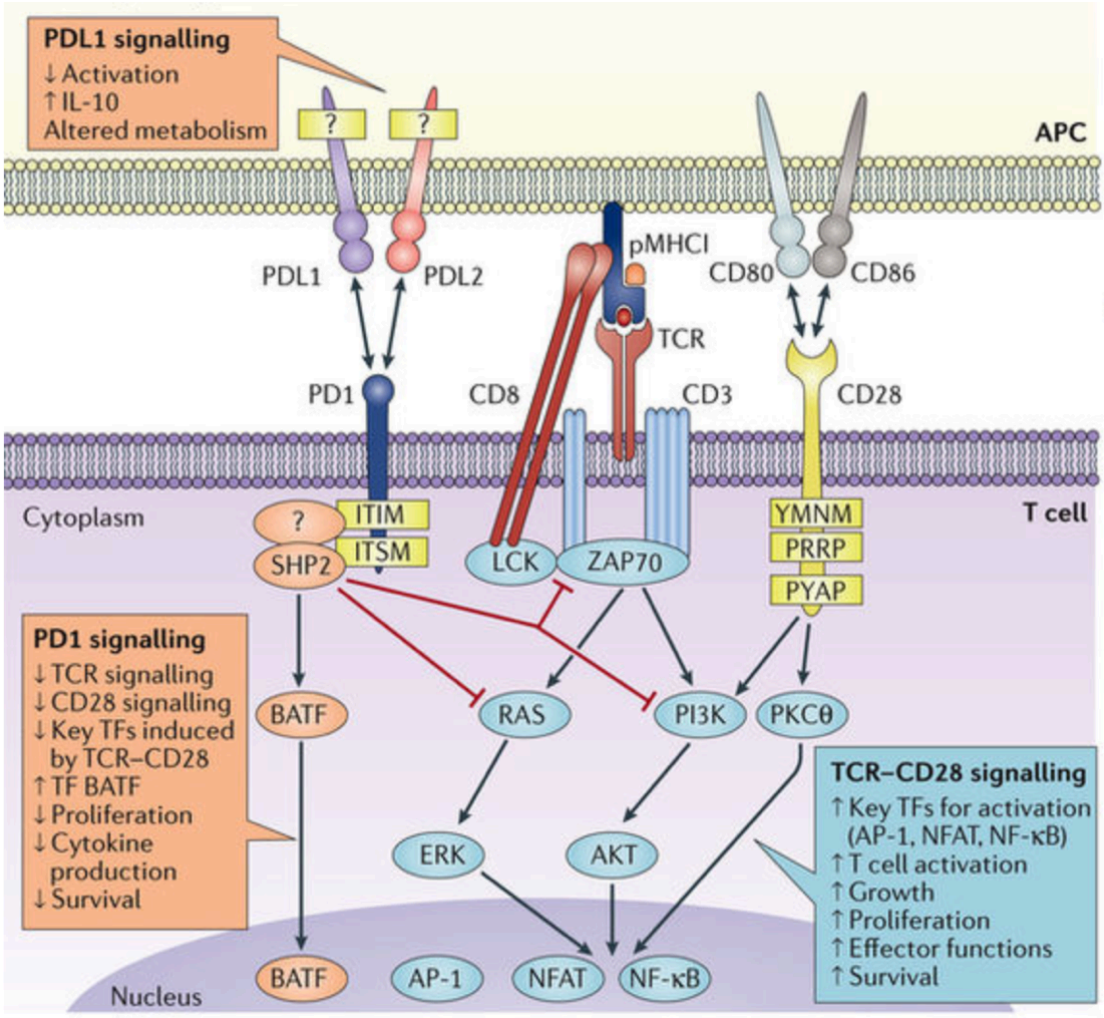


Figure 1-6. **Overview of PD-1 Signaling**

PD-1 contains two canonical signalling motifs, an ITIM and ITSM sequence. The phosphatase SHP-2 recognizes the phosphorylated ITSM residue after engagement of the TCR and is subsequently able to promiscuously dephosphorylate TCR-proximal signalling proteins such as ZAP-70, Ras, PI3K and the intracellular domain of CD28. Collectively, this results in a weakened signal transduced through the TCR after engagement, thereby attenuating signals to transcription factors such as AP-1, NFAT and NF-κB that are important for functional outcomes such as activation, proliferation, effector function and survival. Figure adapted from Abbas and Walker, Nature Reviews Immunology, 2002.

Role in Non-Self Immune Response

Kinetically, PD-1 is expressed on T cells during development in the thymus as early as the DP stage, where the expression of PD-1 is correlated with the strength of the signal received through the TCR [95,96]. After thymic egress, PD-1 is upregulated when naïve T cells come into contact with antigen in the periphery. In this context, PD-1 is not expressed on all naïve T cells, but it is expressed on most T cells during initial antigen encounter. Functionally, PD-1 is expressed on T cells involved with clearing acute infections as well as during chronic viral infections and during certain long-term disease states such as cancer. While the expression of PD-1 is useful to temper over-activation and protect tissues form immunopathology during acute infections, severe PD-1 upregulation leads to an impaired immune response due to T cell exhaustion [97–100]. Should a chronic infection remain unimpaired, PD-1 expression remains high; conversely, pathogen clearance permits PD-1 expression to decrease on responding T cells. Practically, some cancers gain "adaptive resistance" by upregulating PD-L1 in the tumour milieu, thereby inhibiting PD-1^+ T effector cells [101].

Role in Autoimmunity

PD-1 signalling has been shown to play a role in a number of distinct mechanisms that regulate autoimmunity; PD-1 signals during thymocyte development, during the priming and differentiation of naïve T cells in the periphery, and on T effector functions in target tissues combine to significantly control autoimmunity. In the thymus, a lack of

PD-1 signals alters thymic development in some model systems and leads to the development of a greater population of double negative (DN) thymocytes [102]. PD-1 signals can also prevent the overactivation and proliferation of Tregs, which would ordinarily lead to apoptosis [103]. In secondary lymphoid organs (SLOs) PD-1 signals fine-tune memory and effector cell differentiation and functionality: peripheral PD-1$^{-/-}$ cells are skewed towards a central memory phenotype and exhibit increased proliferation in response to secondary challenge [104]. Globally, PD-1 has been shown to be important for restraining autoreactive T cells in various animal models as PD-1 deficient mice develop strain-specific spontaneous autoimmune disease [105]. Similarly, humans with particular PD-1 single nucleotide polymorphisms (SNPs) have been shown to be more susceptible to certain autoimmune diseases such as Type I diabetes and systemic lupus erythromatosis [106,107].

Rationale

Though PD-1 has been broadly shown to be important for T cell tolerance, it is not clear when precisely PD-1 signals are important for conferring tolerance. Indeed, there is mixed evidence supporting the role for PD-1 in establishing or maintaining T cell tolerance [108–110]; this may be because many of the model systems used to investigate this question often do not account for potentially tolerizing PD-1 signals that may occur in the thymus. Our model system using OT-I > RIP-mOVA bone marrow chimeras allows us to investigate the importance of PD-1 signals during development in the thymus and in the periphery. We used previously described OT-I Bim$^{-/-}$ > RIP-mOVA

chimeras to investigate if PD-1 signals are required to maintain tolerance *in vitro* as OT-I $Bim^{-/-}$ $CD8^{+}$ T cells are functionally impaired and highly express PD-1. Additionally, we will use OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras to investigate the role of PD-1 signals in establishing tolerance *in vivo* and *ex vivo*.

Hypotheses

1) We hypothesize that: PD-1 signals contribute to the functional impairment observed with OT-I $Bim^{-/-}$ > RIP-mOVA $CD8^{+}$ T cells and that *in vitro* PD-1 blockade will restore the functionality of these cells as measured by activation, proliferation and effector function.

2) We hypothesize that PD-1 signals are important for the establishment of tolerance to TRA such that OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA but not OT-I WT > RIP-mOVA chimeras will develop autoimmune diabetes.

CHAPTER 2: MATERIALS AND METHODS

Mice

C57BL/6 mice, C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA) mice and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were originally purchased from The Jackson Laboratory, but were mostly bred in-house. PD-$1^{-/-}$ mice were originally provided by Dr. Tasuku Honjo (Kyoto University, Kyoto, Japan). OT-I $Bim^{-/-}$ mice were provided by Dr. Maureen McGargill (St. Jude Children's Research Hospital, Memphis, TN). OT-I PD-$1^{-/-}$ were generated from intercrossing OT-I and PD-$1^{-/-}$ mice in animal facilities at the University of Alberta. All mice 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 Chimera Generation

Bone marrow chimera donor mice were injected intraperitoneally with 100 μg of purified anti-Thy1.2 Ab (clone 30H12 from BioXCell) 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 70 μm nylon cell strainers (Fisherbrand). Recipient mice were irradiated twice at 4.5-4.8 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. At Day +1 or +2 post bone marrow chimera generation, recipient mice were injected intraperitoneally with 100 ug of purified anti-Thy1.2 Ab (clone 30H12 from BioXCell). For competitive bone marrow chimera experiments, OT-I WT ($CD45.1^+$) and OT-I PD-$1^{-/-}$ or PD-$1^{-/-}$ ($CD45.1.2^+$) BM cells were

mixed 1:1 and at least 5 x10^6 cells were transplanted into lethally irradiated (9.0-9.6 Gy) into RIP-mOVA recipients (CD45.2$^+$) by tail vein injection. Mice were provided with novotrimel for 4 weeks post injection in order to protect chimeras from infection and were allowed to reconstitute for at least 8 weeks or until diabetes induction.

Flow Cytometry and Antibodies

Antibodies and flow cytometry antibodies were purchased from ThermoFisher Scientific, BD Biosciences and BioLegend. Cells were stained with antibody 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 intracellular cytokines as per manufacturer's protocol. Cells were treated with the FoxP3 staining kit (eBiosciences) for intracellular staining of Ki67 as per manufacturer's protocol. Cell events were collected on a BD Fortessa SORP (BD Biosciences) and analysed by FlowJo software (Tree Star).

PD-1 Blockade

For *in* vitro blockade experiments, OT-I Bim$^{-/-}$ > RIP-mOVA/B6 splenocytes and thymocytes were treated with 5ug of αPD-1 (clones: J43 or RMP1-14 from BioXCell) per mL of media on Day 0. For *in vivo* PD-1 blockade, chimeras were treated with 250 ug of RMP1-14 every second day for a minimum of four weeks (4 milligrams total) to 16 weeks (16 milligrams total).

Tissue Collection

Mice were anesthetized with isoflurane and subsequently euthanized via CO2 asphyxiation. Thymus, spleen, and lymph nodes were harvested and gently ground into wire mesh screens inside of petri dishes and subsequently filled with Hank's Balanced Salt Solution (HBSS) to achieve single cell suspensions. If samples were needed for cell culture, cells would be collected in sterile RP10 (RPMI 1640 with 2.05 mM L-glutamine from HyClone, 10% fetal calf serum (FCS), 5 mM HEPES, 50 mM 2- mercaptoethanol, 50 mg/mL penicillin/streptomycin, 50 mg/mL gentamycin sulfate) instead. Cellularity was determined by Trypan Blue exclusion in a hemocytometer (Fisher Scientific) under a light microscope (Zeiss).

Pancreatic T Cell Isolation

Pancreatic digestion buffer is prepared and placed in 37°C water bath: 9 mL of RP10 + 1 mL of 10% BSA in PBS + 10 mg (1 mg/mL) of Collagenase Type 4 (Sigma C5138) + 50 uL of DNAse I (2000U/mL). Pancreas was removed and sliced into 1 mm pieces using a scalpel in 1 mL of PBS in a petri dish. Pieces were placed into digestion buffer in 37°C water bath with slight shake for 20 minutes. Once the digest was complete, pancreas slices were passed through a cell-trainer and resuspended in RP-10.

Blood Glucose Monitoring

OT-I chimeras were monitored weekly for blood glucose levels using a One-Touch UltraMini system with OneTouch Ultra Test Strips starting from 3 weeks and up to 16 weeks after bone marrow reconstitution or diabetes induction. Blood samples were obtained by tail vein bleeding. If blood glucose exceeded 15 mmol/L, another reading was taken 24 hours later. Mice were considered diabetic upon two consecutive readings of over 15 mmol/L.

Proliferation Assay and Stimulation Protocol

Stimulator splenocytes were harvested from WT B6 mice, washed and resuspended at 20 x 10^6 cells/mL 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) and pulsed with 100 nM of SIINFEKL peptide at 37°C for 1 hour with gentle shaking every 15 – 20 minutes. Stimulators were washed with three times with RP10 before being resuspended in RP10 1 x 10^7 cells/mL. Effector cells were harvested from indicated mice, washed and resuspended in sterile PBS at 1 x 10^7 (for Carboxyfluorescein succinimidyl ester staining) or 5 x 10^6 (for cell trace violet 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 4x the volume of RP10 and cells were then 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 4x the volume of RP10. Cells were washed once in RP10 then resuspended in RP10 to 1 x 10^7 cells/mL. Effector cells were mixed with stimulators at a ratio of 4:1 and incubated at 37°C for the indicated time points. Division and proliferation indices were calculated by Flowjo software (Tree Star) version 7.6.5. 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 Production 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 fresh RP10. On Day 5, effectors were re-stimulated with 100 nM of 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.

Statistics

Four different statistical analyses were used to determine *p* values depending on the data: two-sided unpaired student's *t* test, two-sided paired student's *t* test, ordinary one-way ANOVA, or using a Mantel-Cox logrank test. *P* values were calculated using Prism (GraphPad Software).

CHAPTER 3: RESULTS – PD-1 is Necessary for Tolerance to TRA

Vα2$^+$ CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras are functionally impaired and upregulate PD-1 *ex vivo*

Previous published works from our lab have characterized a functional impairment in Vα2$^+$ CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA bone marrow chimeras (BMC) compared to OT-I Bim$^{-/-}$ > B6 controls, as measured by markers of activation (CD69, CD25), proliferation (CFSE/CTV labelling) and effector function (TNFα, IFNγ) [91]. Additionally, further unpublished works from our group have illustrated an increase in PD-1 transcript on Vα2$^+$ CD8$^+$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras compared to OT-I Bim$^{-/-}$ > B6 controls. We hypothesized that the observed increase in PD-1 transcript on Vα2$^+$ CD8$^+$ splenocytes will positively correlate with a translational increase in PD-1 protein expression. To test this hypothesis, we generated bone marrow chimeras using OT-I Bim$^{-/-}$ BM and lethally irradiated wild-type (B6) or RIP-mOVA recipients (**Fig. 3-1)**. Eight weeks or more post-transplant, we harvested the spleen, thymus and pancreatic lymph nodes (pLn) and performed an *ex vivo* analysis of both OT-I Bim$^{-/-}$ > RIP-mOVA and OT-I Bim$^{-/-}$ > B6 chimeras. We found that Vα2$^+$ CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras express more PD-1 compared to OT-I Bim$^{-/-}$ > B6 controls **(Fig. 3-2)**. Therein, we hypothesized that PD-1 interactions may establish and/or maintain the functional impairment evident in Vα2$^+$ CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras in a cell-intrinsic manner.

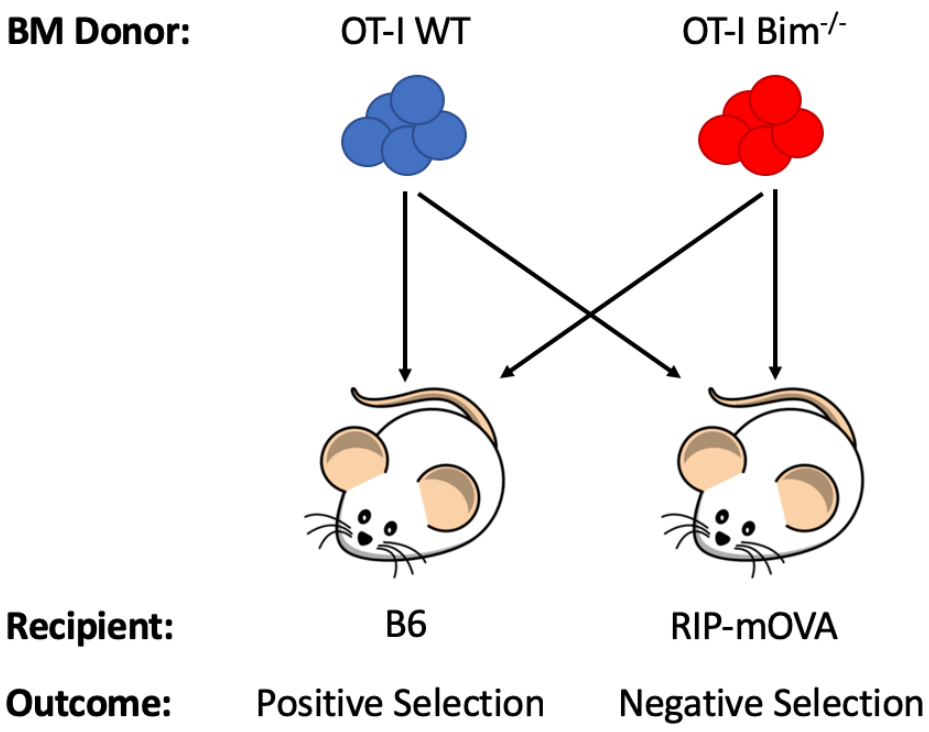


Figure 3-1. **Schematic of OT-I Bim$^{-/-}$ bone marrow chimera setup**

Bone marrow donors were T cell depleted using 100 ug of anti-Thy1.2 (clone: 30H12) administered by intraperitoneal injection on days -2 and -1. Recipients were irradiated twice at 4.5-4.8 Gy and subsequently 1e6 to 1e7 BM cells were injected into lethally irradiated recipients via tail vein injection. Recipient mice received 100 ug anti-Thy1.2 on day +1. Chimeric mice were placed on antibiotic water for four weeks post-transfer and cells were examined eight weeks post-transplant or later.

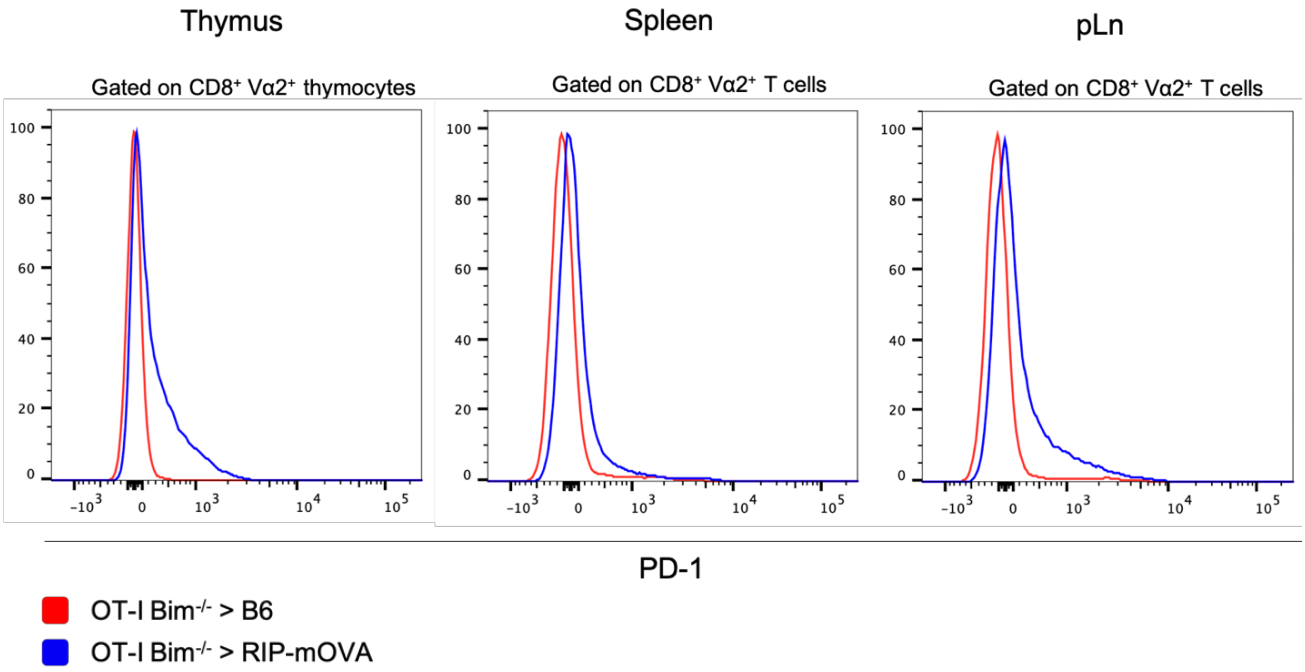


Figure 3-2. ***Ex vivo*** **analysis of PD-1 expression on thymocytes or T cells from OT-I Bim$^{-/-}$ chimeras**

Cells from the thymus, spleen and pLn of OT-I Bim$^{-/-}$ > RIP-mOVA or OT-I Bim$^{-/-}$ > B6 chimeras were harvested and analyzed by flow cytometry. Vα2^{+} CD8^{+} thymocytes or T cells were electronically gated and examined for PD-1 expression. OT-I Bim$^{-/-}$ > RIP-mOVA (n=10), OT-I Bim$^{-/-}$ > B6 (n=9). Data was collected over six separate cohorts.

In vitro stimulation assays reveal no population of dominant cell-extrinsic suppressors in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras

While there are no previously characterized populations of T-cell suppressors in the OT-I transgenic model system, we wanted to examine the possibility that such suppressors in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras were generated. If present, cells with a suppressive capacity may contribute to the observed functional impairment in a cell-extrinsic manner. We hypothesized that if a population of dominant suppressors existed in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras, they would be able to suppress the activation and proliferation of OT-I WT splenocytes. CFSE-labelled splenocytes from CD45.1^{+} OT-I WT mice were mixed with splenocytes from CD45.2^{+} OT-I Bim$^{-/-}$ > RIP-mOVA chimeras at a 1:1 ratio and stimulated with OVA peptide-pulsed splenocytes *in vitro*. On Day 2 after stimulation, we found that the expression of CD69 on splenocytes from OT-I WT CD45.1^{+} mice was not significantly lower in mixed culture compared to unmixed OT-I WT splenocytes **(Fig. 3-3, A-B)**. On Day 3, the presence of OT-I Bim$^{-/-}$ > RIP-mOVA splenocytes in mixed culture did not negatively impact the proliferation of mixed OT-I WT splenocytes, as measured by the division index (DI) and the proliferation index (PI) **(Fig. 3-3, C-E)**. Surprisingly, the PI of mixed culture OT-I splenocytes was significantly higher than that of unmixed OT-I splenocytes. These data suggest that a population of Ag-specific suppressors are not generated in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras and that the functional impairment of OT-I Bim$^{-/-}$ T cells from cognate antigen encounter in RIP-mOVA recipients is cell-intrinsic.

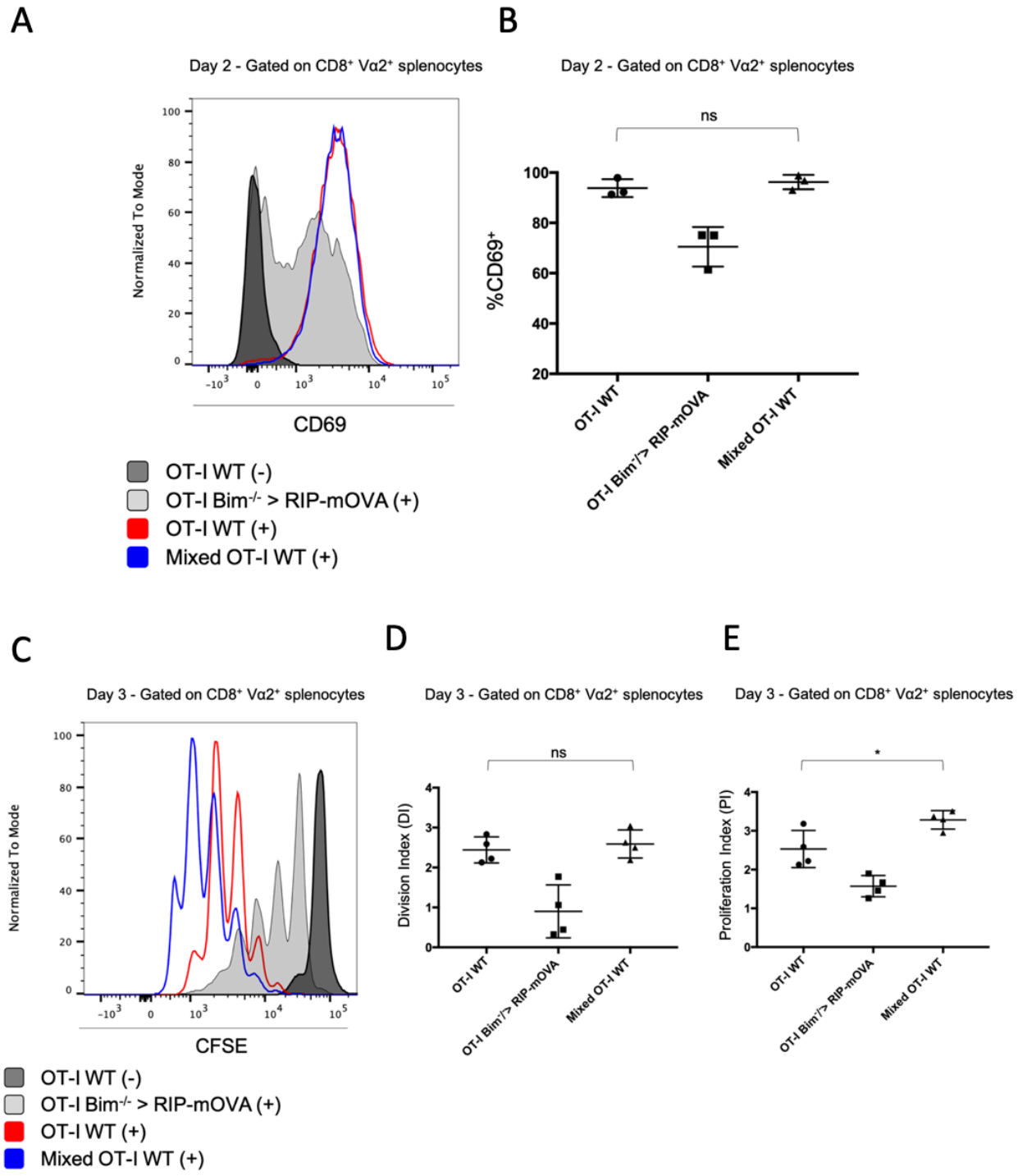

A
Day 2 - Gated on CD8⁺ Vα2⁺ splenocytes
Normalized To Mode
CD69
OT-I WT (-)
OT-I Bim-/- > RIP-mOVA (+)
OT-I WT (+)
Mixed OT-I WT (+)
B
Day 2 - Gated on CD8⁺ Vα2⁺ splenocytes
ns
%CD69⁺
OT-I WT
OT-I Bim-/-> RIP-mOVA
Mixed OT-I WT
C
Day 3 - Gated on CD8⁺ Vα2⁺ splenocytes
Normalized To Mode
CFSE
OT-I WT (-)
OT-I Bim-/- > RIP-mOVA (+)
OT-I WT (+)
Mixed OT-I WT (+)
D
Day 3 - Gated on CD8⁺ Vα2⁺ splenocytes
ns
Division Index (DI)
OT-I WT
OT-I Bim-/-> RIP-mOVA
Mixed OT-I WT
E
Day 3 - Gated on CD8⁺ Vα2⁺ splenocytes
*
Proliferation Index (PI)
OT-I WT
OT-I Bim-/-> RIP-mOVA
Mixed OT-I WT

Figure 3-3. **Stimulation assays reveal no population of T cell suppressors in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras**

Cells from the spleen of OT-I Bim$^{-/-}$ > RIP-mOVA chimeras (CD45.2^{+}) and/or OT-I WT mice (CD45.1^{+}) were harvested and stimulated *in vitro* using WT splenocytes pulsed with 100 nm of SIINFEKL (OVA-peptide). Splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras and OT-I WT mice were mixed at a 1:1 ratio in culture totaling 2e6 splenocytes. Unmixed samples also contained a total of 2e6 cells. Cells were electronically gated using CD45 congenics and analyzed for expression of CD69 on Day 2 and proliferation on Day 3. The division index (DI) represents the average number of division that all cells within the population undergo. The proliferation index (PI) is the average number of divisions that only proliferating cells undergo. The (+) represents stimulation and the (-) symbol represents the unstimulated control(s). **A.** Representative FACS plot of CD69 profile of OT-I splenocytes. OT-I WT (n=3), OT-I Bim$^{-/-}$ > RIP-mOVA (n=3), Mixed 1:1 OT-I WT and OT-I Bim$^{-/-}$ > RIP-mOVA (n=3) **B.** Fraction of OT-I CD8^{+} CD69^{+} splenocytes. OT-I WT (n=3), OT-I Bim$^{-/-}$ > RIP-mOVA (n=3), Mixed 1:1 OT-I WT and OT-I Bim$^{-/-}$ > RIP-mOVA (n=3). **C.** Representative FACS plot of proliferation labelling of OT-I splenocytes. OT-I WT (n=4), OT-I Bim$^{-/-}$ > RIP-mOVA (n=4), Mixed 1:1 OT-I WT and OT-I Bim$^{-/-}$ > RIP-mOVA (n=4). **D.** Division Index and Proliferation Index of OT-I splenocytes. OT-I WT (n=4), OT-I Bim$^{-/-}$ > RIP-mOVA (n=4), Mixed 1:1 OT-I WT and OT-I Bim$^{-/-}$ > RIP-mOVA (n=4). Data was collected over three separate cohorts. P values were calculated using a two-tailed unpaired *t* test.

PD-1 blockade does not ameliorate the functional impairment of Vα2$^+$ CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras *in vitro*

Given that Vα2$^+$ CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras express PD-1 and are ostensibly impaired in a cell-intrinsic manner, we next hypothesized that blocking PD-1 interactions with αPD-1 antibodies would restore the impaired functionality of these cells. To address this question, we stimulated splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras with OVA-peptide pulsed splenocytes *in vitro*, treated with monoclonal PD-1 blocking antibodies (clones: J43 or RMP1-14) and assessed functionality as measured by activation, proliferation and cytokine production. We compared the results to untreated Vα2$^+$ CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras and OT-I Bim$^{-/-}$ > B6 controls. Furthermore, we investigated if PD-1 blockade of OT-I Bim$^{-/-}$ > RIP-mOVA chimeras was sufficient to induce autoimmune diabetes *in vivo*.

Activation

We stimulated Vα2$^+$ CD8$^+$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA and OT-I Bim$^{-/-}$ > B6 chimeras with OVA-peptide and compared the samples with and without PD-1 blockade *in vitro*. On Day 2, we found that PD-1 blockade did not increase the fraction of Vα2$^+$ CD8$^+$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras that induced co-expression of CD25 and CD69 compared to untreated controls; treated and untreated OT-I Bim$^{-/-}$ > RIP-mOVA splenocytes exhibited significantly fewer Vα2$^+$ CD8$^+$ T cells that co-expressed CD25 and CD69 compared to OT-I Bim$^{-/-}$ > B6 controls **(Fig. 3-4, A-B)**.

Given that *pdcd1*, the genetic locus for PD-1 transcript, has been shown to undergo epigenetic regulation on T cells that chronically engage with cognate antigen, we hypothesized that thymocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras may be more susceptible to PD-1 blockade as they have not yet been in contact with cognate antigen in the periphery [111]. On Day 2, we found that PD-1 blockade did not increase the fraction of $V\alpha2^{+}$ $CD8^{+}$ thymocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras that induced co-expression of CD25 and CD69 compared to untreated controls; treated and untreated OT-I $Bim^{-/-}$ > RIP-mOVA thymocytes exhibited significantly fewer $V\alpha2^{+}$ $CD8^{+}$ T cells that co-expressed CD25 and CD69 compared to OT-I $Bim^{-/-}$ > B6 controls (**Fig. 3-4, C-D)**.

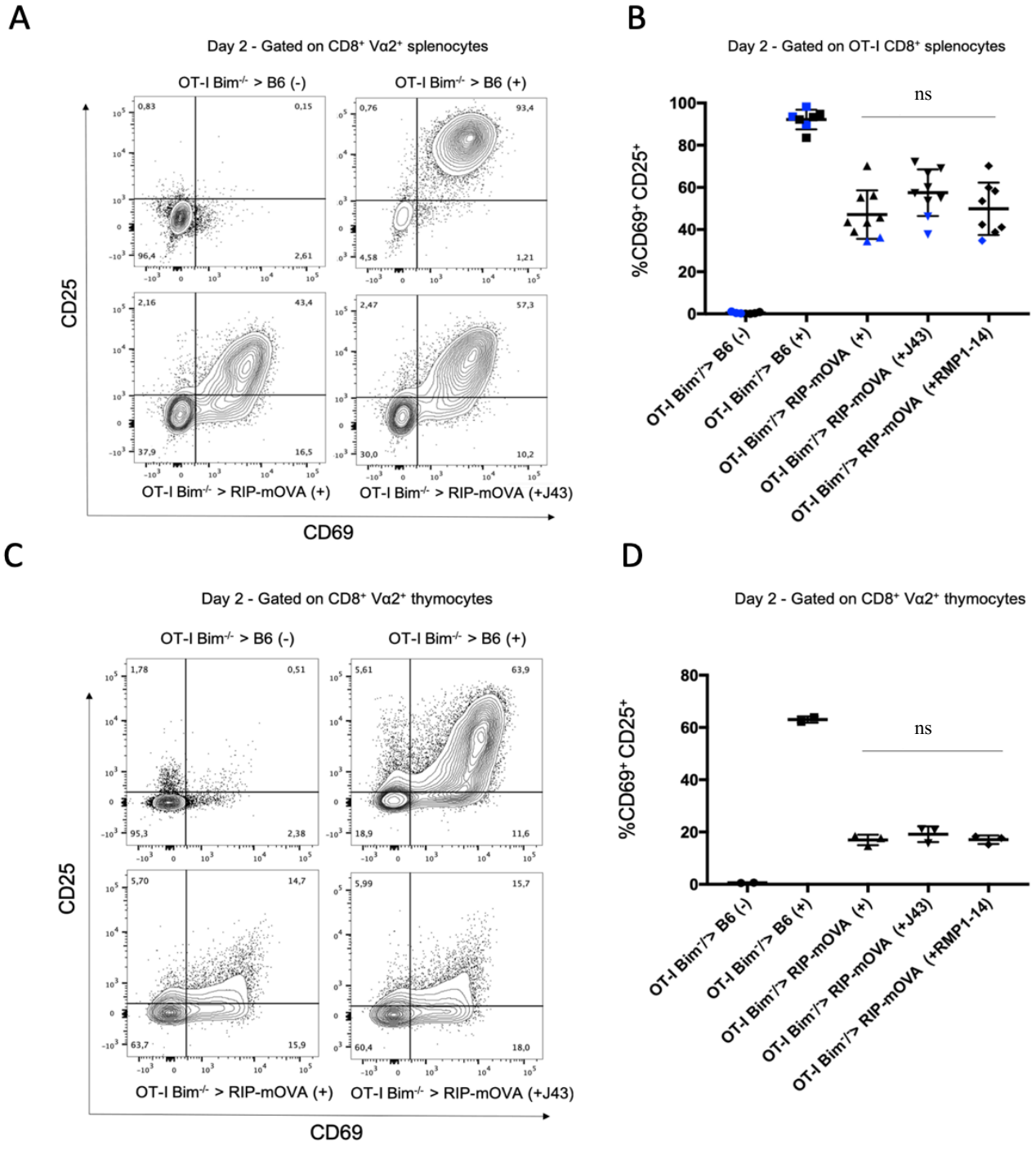

A
Day 2 - Gated on CD8⁺ Vα2⁺ splenocytes
OT-I Bim-/- > B6 (-)
OT-I Bim-/- > B6 (+)
0,83
0,15
96,4
2,61
0,76
93,4
4,58
1,21
2,16
43,4
37,9
16,5
2,47
57,3
30,0
10,2
CD25
CD69
OT-I Bim-/- > RIP-mOVA (+)
OT-I Bim-/- > RIP-mOVA (+J43)
B
Day 2 - Gated on OT-I CD8⁺ splenocytes
%CD69⁺ CD25⁺
ns
OT-I Bim-/- > B6 (-)
OT-I Bim-/- > B6 (+)
OT-I Bim-/- > RIP-mOVA (+)
OT-I Bim-/- > RIP-mOVA (+J43)
OT-I Bim-/- > RIP-mOVA (+RMP1-14)
C
Day 2 - Gated on CD8⁺ Vα2⁺ thymocytes
OT-I Bim-/- > B6 (-)
OT-I Bim-/- > B6 (+)
1,78
0,51
95,3
2,38
5,61
63,9
18,9
11,6
5,70
14,7
63,7
15,9
5,99
15,7
60,4
18,0
CD25
CD69
OT-I Bim-/- > RIP-mOVA (+)
OT-I Bim-/- > RIP-mOVA (+J43)
D
Day 2 - Gated on CD8⁺ Vα2⁺ thymocytes
%CD69⁺ CD25⁺
ns
OT-I Bim-/- > B6 (-)
OT-I Bim-/- > B6 (+)
OT-I Bim-/- > RIP-mOVA (+)
OT-I Bim-/- > RIP-mOVA (+J43)
OT-I Bim-/- > RIP-mOVA (+RMP1-14)

Figure 3-4. **PD-1 blockade does not increase the fraction of OT-I CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras that co-express CD25 and CD69**

Cells from the spleen and thymus of OT-I Bim$^{-/-}$ > RIP-mOVA and OT-I Bim$^{-/-}$ > RIP-mOVA chimeras were harvested and stimulated *in vitro* using WT splenocytes pulsed with 100 nm of SIINFEKL (OVA-peptide) with or without 5 ug of αPD-1 (clone: J43 or RMP1-14 as specified). The (+) represents stimulation and the (-) symbol represents the unstimulated control(s). Two different strategies were used to detect OT-I CD8$^+$ T cells: black dots represent data points collected using antibodies specific for Vα2 and CD45.2, blue dots represent data points collected using an OVA (257-264) peptide H-2K$_b$ tetramer. **A.** Representative FACS plot of CD25 by CD69 profile of OT-I splenocytes. OT-I Bim$^{-/-}$ > B6 (n=9), OT-I Bim$^{-/-}$ > RIP-mOVA (n=9) **B.** Fraction of OT-I CD8$^+$ splenocytes that co-express CD25 and CD69. OT-I Bim$^{-/-}$ > RIP-mOVA (n=10), OT-I Bim$^{-/-}$ > B6 (n=9). Data was collected over six separate cohorts. **C.** Representative FACS plot of CD25 by CD69 profile of OT-I thymocytes. OT-I Bim$^{-/-}$ > B6 (n=2), OT-I Bim$^{-/-}$ > RIP-mOVA (n=3). **D.** Fraction of OT-I CD8$^+$ thymocytes that co-express CD25 and CD69. OT-I Bim$^{-/-}$ > B6 (n=2), OT-I Bim$^{-/-}$ > RIP-mOVA (n=3). Data was collected over two separate cohorts. P values were calculated using an ordinary one-way ANOVA test on OT-I Bim$^{-/-}$ > RIP-mOVA samples.

Proliferation

Next, we investigated the proliferation capacity of $V\alpha2^+$ $CD8^+$ splenocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras as measured by division index (DI) and proliferation index (PI). On Day 3, we observed that PD-1 blockade did not increase the division index or proliferation index of $V\alpha2^+$ $CD8^+$ splenocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras as both treated and non-treated splenocytes exhibited similar proliferation defects when compared to OT-I $Bim^{-/-}$ > B6 controls **(Fig. 3-5, A-C)**.

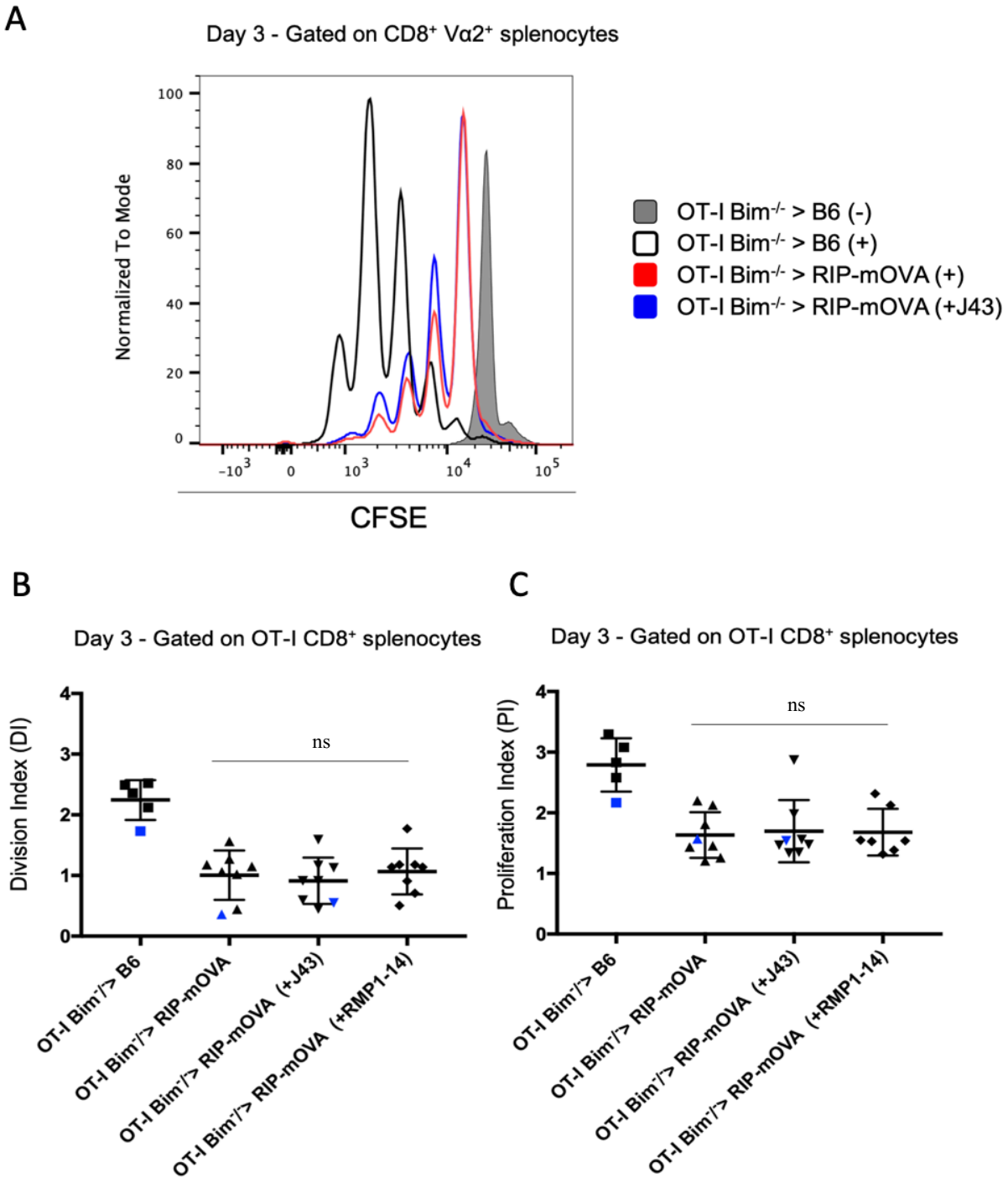

A
Day 3 - Gated on CD8+ Vα2+ splenocytes
Normalized To Mode
CFSE
OT-I Bim-/- > B6 (-)
OT-I Bim-/- > B6 (+)
OT-I Bim-/- > RIP-mOVA (+)
OT-I Bim-/- > RIP-mOVA (+J43)
B
Day 3 - Gated on OT-I CD8+ splenocytes
Division Index (DI)
ns
OT-I Bim-/-> B6
OT-I Bim-/-> RIP-mOVA
OT-I Bim-/-> RIP-mOVA (+J43)
OT-I Bim-/-> RIP-mOVA (+RMP1-14)
C
Day 3 - Gated on OT-I CD8+ splenocytes
Proliferation Index (PI)
ns
OT-I Bim-/-> B6
OT-I Bim-/-> RIP-mOVA
OT-I Bim-/-> RIP-mOVA (+J43)
OT-I Bim-/-> RIP-mOVA (+RMP1-14)

Figure 3-5. **PD-1 blockade does not increase the proliferation capacity of OT-I CD8$^+$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras**

Cells from the spleen of OT-I Bim$^{-/-}$ > RIP-mOVA and OT-I Bim$^{-/-}$ > RIP-mOVA chimeras were harvested and stimulated *in vitro* using WT splenocytes pulsed with 100 nm of SIINFEKL (OVA-peptide) with or without 5 ug of αPD-1 (clone: J43 or RMP1-14 as specified). The (+) represents stimulation and the (-) symbol represents the unstimulated control(s). Two different strategies were used to detect OT-I CD8$^+$ T cells: black dots represent data points collected using antibodies specific for Vα2 and CD45.2, blue dots represent data points collected using an OVA (257-264) peptide H-2K$_b$ tetramer. A. Representative FACS plot of CFSE labelling of OT-I splenocytes. OT-I Bim$^{-/-}$ > B6 (n=5), OT-I Bim$^{-/-}$ > RIP-mOVA (n=8) B. Division index of OT-I CD8$^+$ splenocytes. OT-I Bim$^{-/-}$ > RIP-mOVA (n=9), OT-I Bim$^{-/-}$ > B6 (n=5). C. Proliferation Index of OT-I CD8$^+$ splenocytes. OT-I Bim$^{-/-}$ > RIP-mOVA (n=9), OT-I Bim$^{-/-}$ > B6 (n=5). Data was collected over six separate cohorts. P values were calculated using an ordinary one-way ANOVA test on OT-I Bim$^{-/-}$ > RIP-mOVA samples.

Cytokine Production

PD-1 blockade has been shown to enhance the cytokine production of $CD8^+$ T cells in OT-I → RIP-mOVA adoptive transfer models and other animal models such as in NOD mice [112,113]. Herein, we assessed if PD-1 blockade would increase the fraction of OT-I $CD8^+$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras that were able to produce TNFα and IFNγ after restimulation following initial stimulation *in vitro*. On Day 5, we observed that PD-1 blockade of OT-I $CD8^+$ splenocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras did not restore the ability of these cells to produce TNFα or IFNγ compared to untreated OT-I $Bim^{-/-}$ > RIP-mOVA controls **(Fig. 3-6, A-B)**. Furthermore, both treated and untreated splenocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras were impaired in their ability to produce cytokines compared to OT-I $Bim^{-/-}$ > B6 controls.

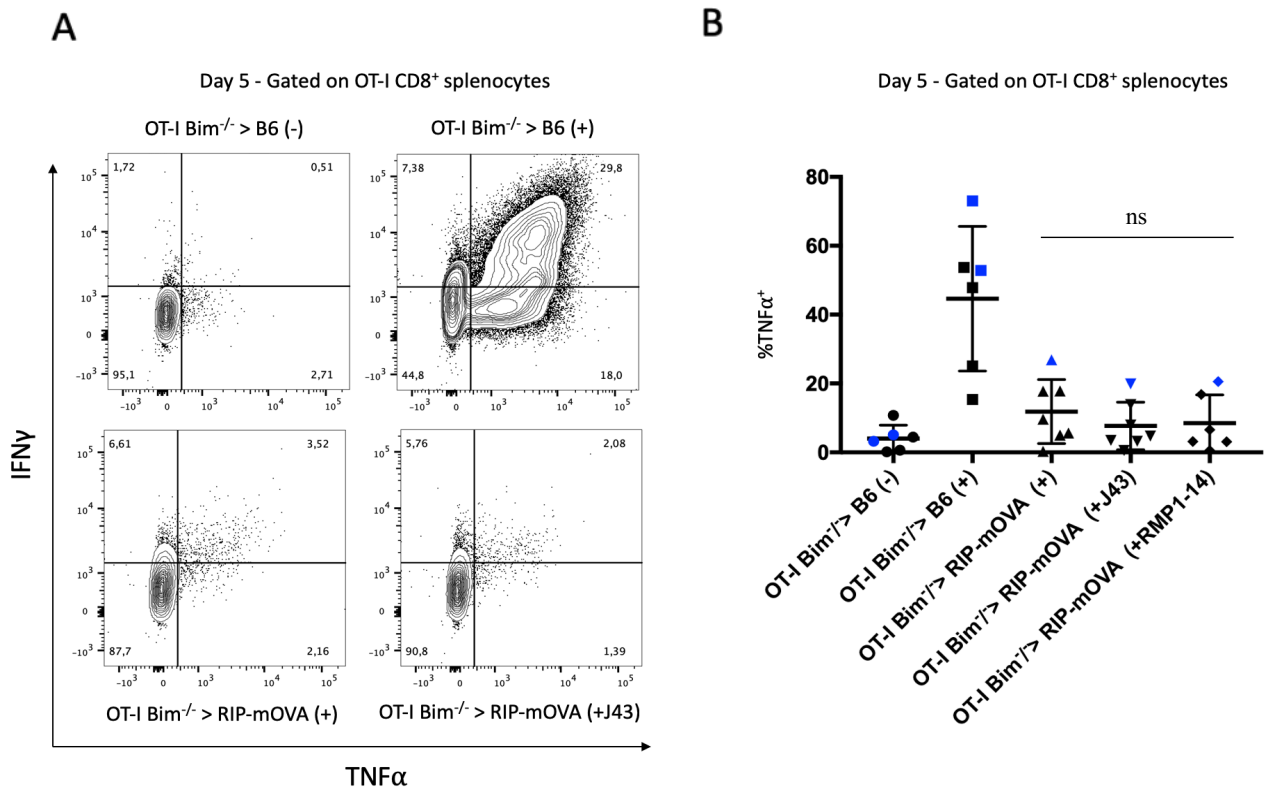


Figure 3-6. **PD-1 blockade does not enhance the ability of OT-I CD8$^+$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras to produce cytokines**

Cells from the spleen of OT-I Bim$^{-/-}$ > RIP-mOVA and OT-I Bim$^{-/-}$ > RIP-mOVA chimeras were harvested and stimulated *in vitro* using WT splenocytes pulsed with 100 nM of SIINFEKL (OVA-peptide) with or without 5 ug of αPD-1 (clone: J43 or RMP1-14 as specified). Cells were resuspended in fresh RP-10 media on Day 3 and allowed to rest until Day 5. Cells on Day 5 were restimulated with 100 nM of SIINFEKL (OVA-peptide) and 3 ug of Brefeldin A. The (+) represents stimulation and the (-) symbol represents the unstimulated control(s). Two different strategies were used to detect OT-I CD8$^+$ T cells: black dots represent data points collected using antibodies specific for Vα2 and CD45.2, blue dots represent data points collected using an OVA (257-264) peptide H-2K^b tetramer. **A.** Representative FACS plot of cytokine production from OT-I splenocytes. OT-I Bim$^{-/-}$ > B6 (n=6), OT-I Bim$^{-/-}$ > RIP-mOVA (n=7) **B.** Fraction of TNFα$^+$ OT-I CD8$^+$ splenocytes. OT-I Bim$^{-/-}$ > B6 (n=6), OT-I Bim$^{-/-}$ > RIP-mOVA (n=7). Data was collected over three separate cohorts. P values were calculated using an ordinary one-way ANOVA test on OT-I Bim$^{-/-}$ > RIP-mOVA samples.

In vivo PD-1 blockade

While *in vitro* PD-1 blockade was largely ineffective, we hypothesized that *in vivo* PD-1 blockade of OT-I Bim$^{-/-}$ > RIP-mOVA chimeras may induce autoimmune diabetes. We treated OT-I Bim$^{-/-}$ > RIP-mOVA and OT-I Bim$^{-/-}$ > B6 chimeras with 250 ug of αPD-1 antibody (clone: RMP1-14) every second day for four weeks and observed that both αPD-1 treated and isotype treated OT-I Bim$^{-/-}$ > RIP-mOVA chimeras did not develop diabetes, similar to OT-I Bim$^{-/-}$ > B6 controls **(Table. 3-1)**.

We determined that *in vitro* PD-1 blockade did not improve the functionality of Vα2^{+} CD8^{+} splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras compared to untreated controls and *in vivo* PD-1 blockade was not sufficient to induce autoimmune diabetes in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras. These data suggest that early PD-1 signals may have long-lasting effects and be sufficient for the establishment of tolerance to TRA.

Chimeras:	OT-I Bim$^{-/-}$ > B6	OT-I Bim$^{-/-}$ > RIP-mOVA	OT-I Bim$^{-/-}$ > RIP-mOVA (αPD-1)
Diabetes Incidence:	0/12	0/16	0/2

Table 3-1. ***In vivo*** **PD-1 blockade does not induce autoimmune diabetes in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras**

Table depicting autoimmune diabetes incidence in OT-I Bim$^{-/-}$ chimeras treated with or without αPD-1 antibodies. Bone marrow chimeras were maintained for a variable length of time from four weeks to 16 weeks, glucose measurements were taken every week and immediately preceding euthanization. Diabetes incidence was defined as having a reading of blood glucose at or above 15 mM on two consecutive measurements. Anti-PD-1 treated chimeras were administered 250 ug of anti-PD-1 (clone: RMP1-14) every second day for four weeks, administered by intraperitoneal injection. One anti-PD-1 treated OT-I Bim$^{-/-}$ > RIP-mOVA chimera died shortly after the last injection, the other was maintained for an additional four weeks until euthanization (eight weeks since first injection). One OT-I Bim$^{-/-}$ > RIP-mOVA chimera was treated with 250 ug of an isotype control antibody every second day for four weeks, administered by intraperitoneal injection. This chimera was maintained for four weeks past the last injection and then euthanized (eight weeks since first injection). OT-I Bim$^{-/-}$ > B6 (n=12), OT-I Bim$^{-/-}$ > RIP-mOVA (n=16), OT-I Bim$^{-/-}$ > RIP-mOVA +αPD-1 (n=2). Data was collected over seven different cohorts.

PD-1 is necessary for T cell tolerance to TRA

Given that we were not able to improve functionality of $V\alpha2^+$ $CD8^+$ splenocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras *in vitro* or induce autoimmunity with PD-1 blockade *in vivo*, we intercrossed OT-I and PD-$1^{-/-}$ mice to generate a colony of OT-I PD-$1^{-/-}$ mice so as to investigate the effects of a complete genetic loss of PD-1 on tolerance to TRA. We generated bone marrow chimeras using OT-I PD-$1^{-/-}$ BM and either wild-type (B6) or RIP-mOVA recipients and hypothesized that OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras would develop autoimmune diabetes due to a lack of PD-1 signals **(Fig. 3-7)**. Strikingly, we observed a 100% incidence of diabetes in OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras, compared to 0% incidence in OT-I WT > RIP-mOVA controls **(Fig. 3-8A)**, even when bone marrow chimerism was less than 60% as measured by donor $CD45.1^+$ congenics or by fraction of $V\alpha2^+$ $CD8^+$ splenocytes **(Fig. 3-8B)**. Next, we investigated if autoimmunity induction in OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras was dependent on all donor $CD8^+$ T cells being PD-$1^{-/-}$. To address this question, we generated mixed bone marrow chimeras with a 1:1 ratio of OT-I WT to OT-I PD-$1^{-/-}$ BM and similarly found that 100% of mixed OT-I WT/PD-$1^{-/-}$ > RIP-mOVA chimeras develop diabetes. These data suggest that only a fraction of the overall pool of OT-I T cells need be PD-$1^{-/-}$ to induce autoimmunity. Furthermore, this finding gives additional evidence that there are no dominant suppressors generated from OT-I BM. To ensure that antigen specific OT-I PD-$1^{-/-}$ T cells are seminal to the induction of diabetes in RIP-mOVA recipients, we generated 1:1 mixed non-OT-I PD-$1^{-/-}$: OT-I WT > RIP-mOVA chimeras, and these chimeras did not develop autoimmune diabetes. Lastly, given that a total genetic loss of PD-1 led to a

100% incidence of autoimmunity in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras, we treated OT-I WT > RIP-mOVA chimeras with PD-1 blockade (clone: RMP1-14) *in vivo* to re-capitulate diabetogenic OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras. While we hypothesized that all treated OT-I WT > RIP-mOVA chimeras would develop autoimmune diabetes, neither αPD-1 treated, isotype treated or untreated OT-I WT > RIP-mOVA chimeras developed autoimmunity **(Fig. 3-8A)**.

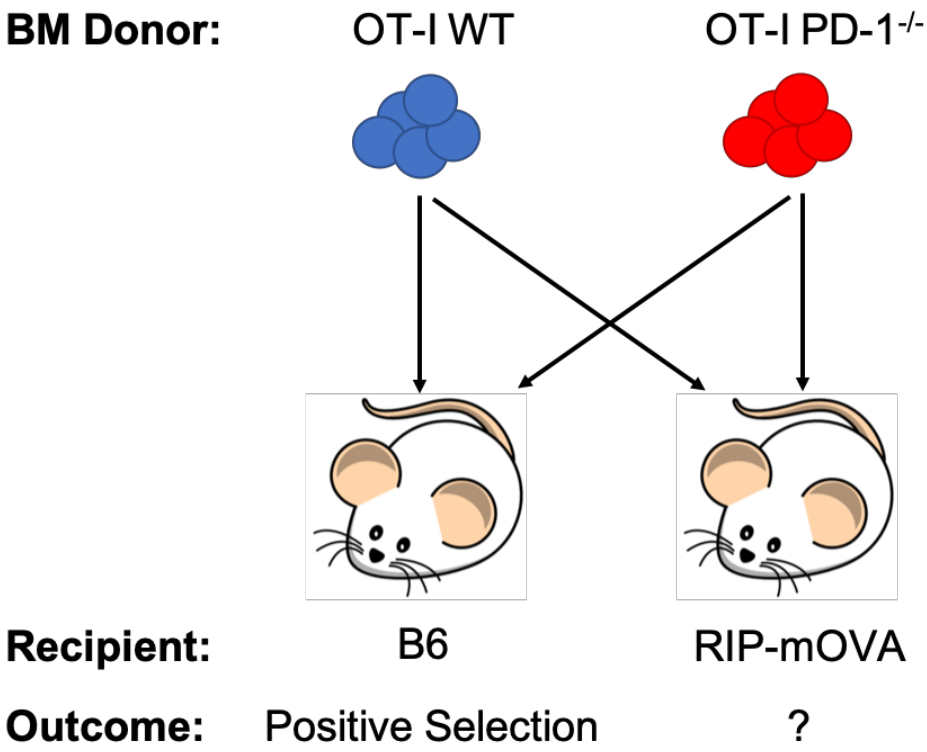
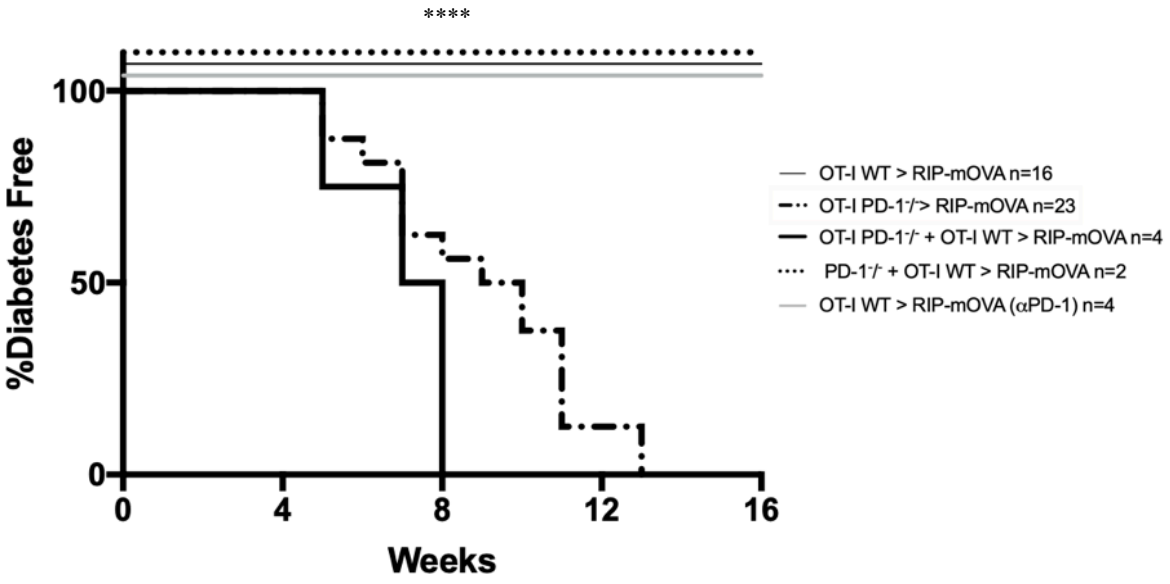


Figure 3-7. **Schematic of OT-I PD-1$^{-/-}$ bone marrow chimera setup**

Bone marrow donors were T cell depleted using 100 ug of anti-Thy1.2 (clone: 30H12) administered by intraperitoneal injection on days -2 and -1. Recipients were irradiated twice at ~4.8 Gy and subsequently 1e6 to 1e7 BM cells were injected into lethally irradiated recipients via tail vein injection. Recipient mice received 100 ug anti-Thy1.2 on day +1. Chimeric mice were placed on antibiotic water for four weeks post-transfer and cells were examined eight weeks post-transplant or later.

A


****
100
50
0
%Diabetes Free
0
4
8
12
16
Weeks
OT-I WT > RIP-mOVA n=16
OT-I PD-1-/-> RIP-mOVA n=23
OT-I PD-1-/- + OT-I WT > RIP-mOVA n=4
PD-1-/- + OT-I WT > RIP-mOVA n=2
OT-I WT > RIP-mOVA (αPD-1) n=4

B

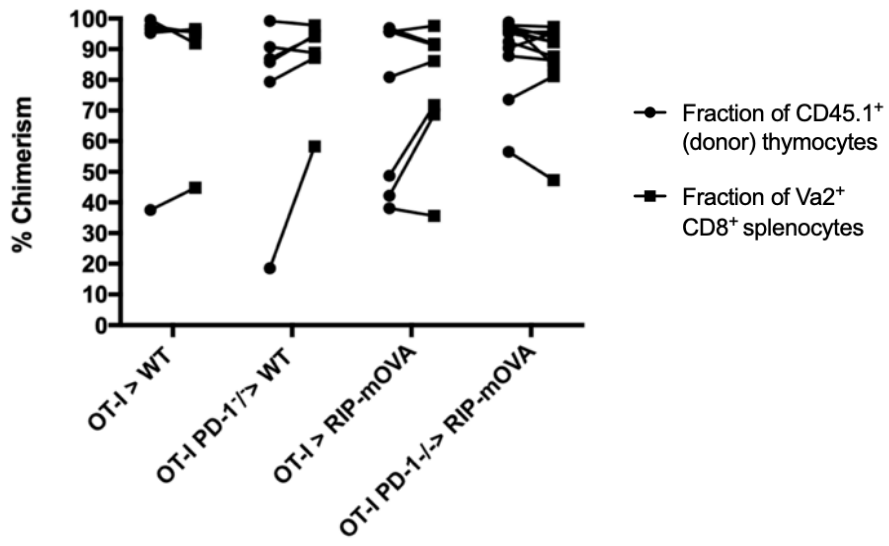

% Chimerism
100
90
80
70
60
50
40
30
20
10
0
OT-I > WT
OT-I PD-1-/-> WT
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
Fraction of CD45.1+ (donor) thymocytes
Fraction of Va2+ CD8+ splenocytes

Figure 3-8. **PD-1 expression on antigen specific T cells is necessary for T cell tolerance to TRA**

A. Survival curve depicting autoimmune diabetes incidence of OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA bone marrow chimeras. OT-I WT > RIP-mOVA (n=16), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=23), OT-I PD-1$^{-/-}$ + OT-I WT > RIP-mOVA (n=4), PD-1$^{-/-}$ + OT-I WT > RIP-mOVA (n=2), OT-I WT > RIP-mOVA +αPD-1 (n=4). Data obtained from six separate cohorts. P values were obtained using a Mantel-Cox logrank test. OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras tested positive for diabetes at a median age of 9.5 weeks. OT-I PD-1$^{-/-}$: OT-I WT > RIP-mOVA competitive chimeras tested positive for diabetes at a median age of 7.5 weeks. Bone marrow chimeras were maintained for a variable length of time from four weeks to 32 weeks, glucose measurements were taken every week and immediately preceding euthanization. Four OT-I WT > RIP-mOVA chimeras were administered 250 ug of anti-PD-1 (clone: RMP1-14) every second day for two weeks by intraperitoneal injection. One OT-I WT > RIP-mOVA chimera was administered 250 ug of anti-PD-1 (clone: RMP1-14) every second day for twelve weeks by intraperitoneal injection. This chimera was maintained for eight weeks past the last injection and then euthanized (twelve weeks since first injection) Four OT-I WT > RIP-mOVA chimeras were treated with 250 ug of an isotype control antibody every second day for two weeks, administered by intraperitoneal injection.. **B.** Bone marrow chimerism as measured by congenic donor CD45.1^{+} status of total thymocytes paired with fraction of Vα2^{+} CD8 splenocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=6), OT-I WT > RIP-mOVA (n=7), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=11). Data was collected over six separate cohorts.

PD-1 does not regulate clonal deletion in the thymus of OT-I WT > RIP-mOVA chimeras

As 100% of OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras develop autoimmune diabetes, we next investigated by what mechanism a lack of PD-1 signals abrogates tolerance to TRA. We hypothesized that PD-1 signals may be involved in clonal deletion of OT-I thymocytes after high-affinity antigen encounter in the thymus of RIP-mOVA recipients. Should clonal deletion fail due to a lack of PD-1 signals, we would hypothesize that OT-I PD-1$^{-/-}$ T cells would enter the periphery, recognize and eventually destroy the beta cells in the pancreatic islets leading to diabetes induction. We examined the CD4 by CD8 profile of the thymus in OT-I WT > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras and observed a modest reduction in the fraction of CD8SP thymocytes and a similar CD8 dulling in this compartment compared to OT-I WT/PD-1$^{-/-}$ > B6 controls **(Fig. 3-9A)**. Furthermore, we observed an equally significant reduction in the fraction and total number of mature (Vα2^{+} CD24^{lo}) CD8SP thymocytes in both OT-I WT and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT/PD-1$^{-/-}$ > B6 controls, signifying that clonal deletion is intact in both RIP-mOVA chimeras **(Fig. 3-9, B-C)**. Overall, these data suggest two-fold: that while the vast majority of OT-I thymocytes encounter cognate antigen and undergo clonal deletion in RIP-mOVA recipients, the few OT-I thymocytes that are unable to undergo PD-1 signaling and that manage to escape deletion ultimately end up inducing autoimmune diabetes; that PD-1 doesn't appear to regulate T cell tolerance by deletional mechanisms.

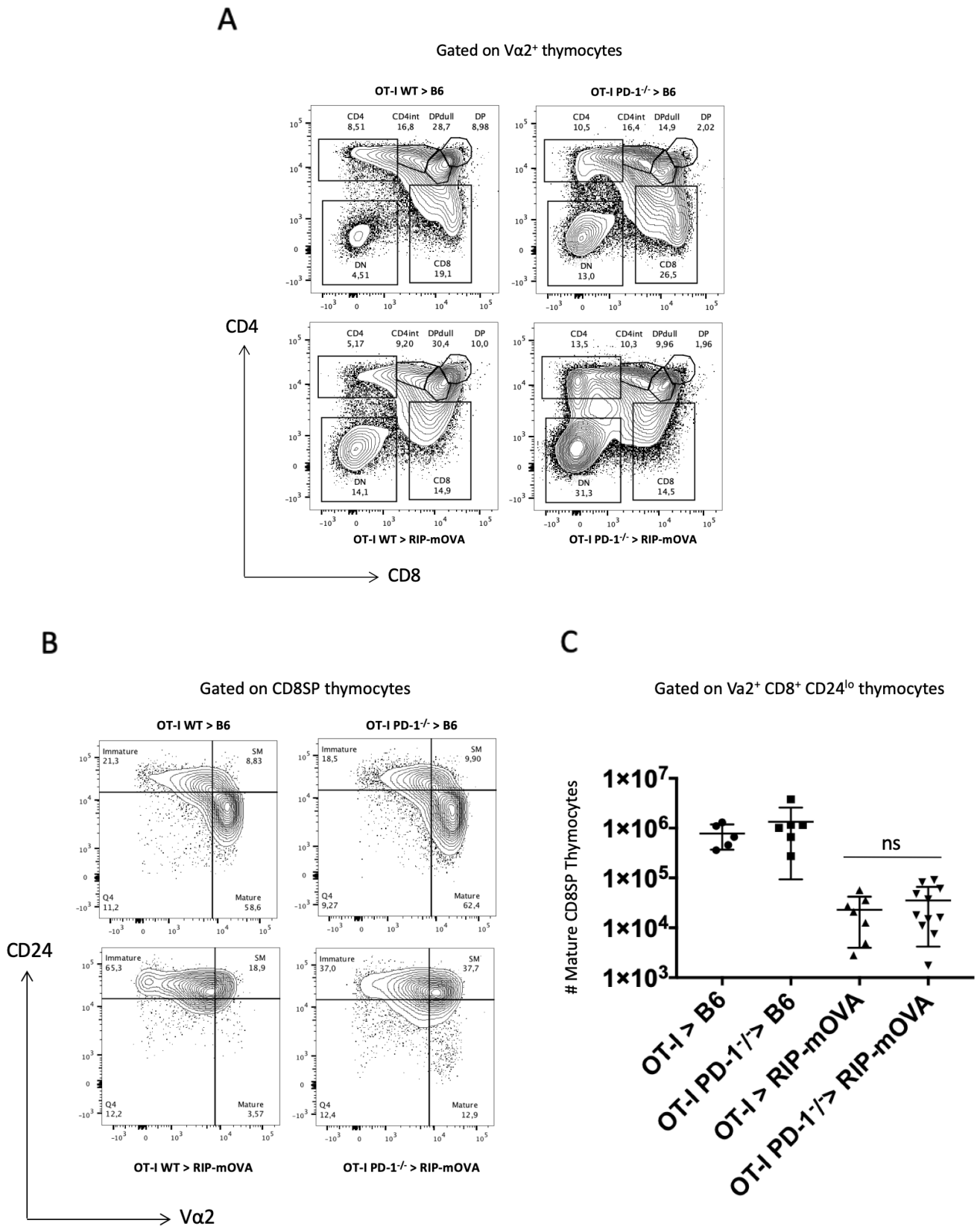

A
Gated on Vα2+ thymocytes
OT-I WT > B6
OT-I PD-1-/- > B6
CD4 8,51
CD4int 16,8
DPdull 28,7
DP 8,98
DN 4,51
CD8 19,1
CD4 10,5
CD4int 16,4
DPdull 14,9
DP 2,02
DN 13,0
CD8 26,5
CD4 5,17
CD4int 9,20
DPdull 30,4
DP 10,0
DN 14,1
CD8 14,9
CD4 13,5
CD4int 10,3
DPdull 9,96
DP 1,96
DN 31,3
CD8 14,5
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
CD4
CD8
B
Gated on CD8SP thymocytes
OT-I WT > B6
OT-I PD-1-/- > B6
Immature 21,3
SM 8,83
Q4 11,2
Mature 58,6
Immature 18,5
SM 9,90
Q4 9,27
Mature 62,4
Immature 65,3
SM 18,9
Q4 12,2
Mature 3,57
Immature 37,0
SM 37,7
Q4 12,4
Mature 12,9
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
CD24
Vα2
C
Gated on Va2+ CD8+ CD24lo thymocytes
Mature CD8SP Thymocytes
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
ns
OT-I > B6
OT-I PD-1-/- > B6
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA

Figure 3-9. **OT-I PD-1$^{-/-}$ > RIP-mOVA thymocytes undergo clonal deletion**

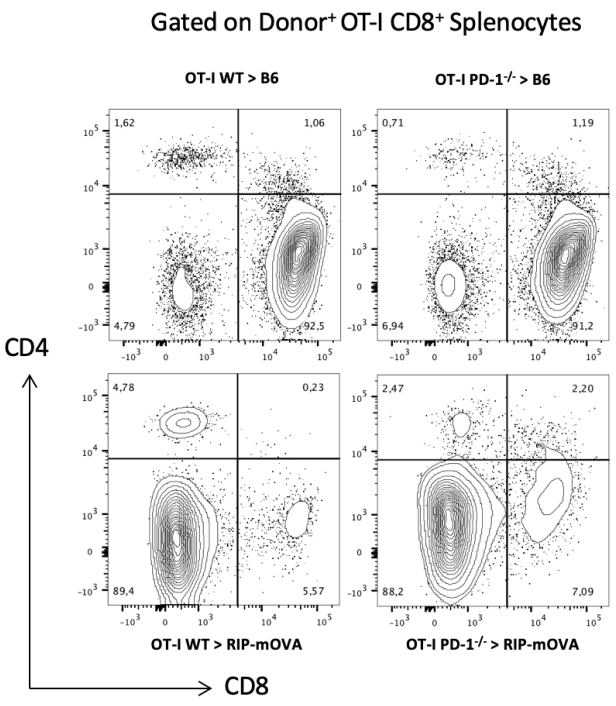
A. Representative FACS plot of CD4 by CD8 profiles of OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA chimeras. "CD4int" population is indicative of CD4 intermediate thymocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=6), OT-I WT > RIP-mOVA (n=7), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=11). **B.** Representative FACS plot of CD24 by Vα2 profiles of OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA chimeras. "Immature" population is CD24^{hi} Vα2^{lo}, "SM" population is CD24^{hi} Vα2^{hi}, "Mature" population is CD24^{lo} Vα2^{hi}. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=6), OT-I WT > RIP-mOVA (n=7), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=11). **C.** Compilation of number of "Mature" CD8SP thymocytes in OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA chimeras. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=6), OT-I WT > RIP-mOVA (n=7), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=11). Data was obtained from six separate cohorts. P values were obtained using a two-tailed unpaired *t* test.

Diabetic OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras exhibit increased numbers of OT-I T cells in the periphery

As there was no statistical difference in the number of OT-I T cells in the thymus of OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls, we next sought to determine if PD-1 deficiency had an impact on the number of peripheral OT-I CD8^{+} T cells in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls. We first examined the number of Vα2^{+} CD8^{+} T cells in the spleen and found a significant overall reduction in the number of Vα2^{+} CD8^{+} T cells in RIP-mOVA chimeras compared to B6 controls**;** Vα2^{+} CD8^{+} splenocytes were the dominant cell type in OT-I WT/PD-1$^{-/-}$ > B6 chimeras in contrast to Vα2^{+} DN splenocytes in OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras, likely due to CD8 co-receptor downregulation after cognate antigen encounter **(Fig. 3-10, A-B)**. There was also a significant increase in the number of Vα2^{+} CD8^{+} splenocytes from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls, suggesting that OT-I T cells unable to undergo PD-1 signals may have functional advantages such as increased proliferative capacity and/or resistance to peripheral deletion. We observed similar trends when we investigated the number of Vα2^{+} CD8^{+} T cells in the pLn; there were very few Vα2^{+} CD8^{+} T cells in RIP-mOVA chimeras compared to B6 controls, however there was a significant increase in the number of Vα2^{+} CD8^{+} T cells in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls (**Fig. 3-10, C-D)**. Lastly, we hypothesized we would be able to detect ostensibly diabetogenic Vα2^{+} CD8^{+} T cells in the pancreas of OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras but not in the pancreas of OT-I WT > RIP-mOVA chimeras. We

isolated a population of Vα2^{+} CD8^{+} T cells from the pancreas of diabetic OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras which was not found in the pancreas of non-diabetic OT-I WT > RIP-mOVA chimeras (**Fig. 3-10, E-F)**. Overall, we found a reduction in the number of Vα2^{+} CD8^{+} T cells from OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT/PD-1$^{-/-}$ > B6 controls, reflecting intact clonal deletion in RIP-mOVA recipients and positive selection in B6 recipients. The increase in Vα2^{+} CD8^{+} T cells from diabetic OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA chimeras suggests that OT-I PD-1$^{-/-}$ T cells may have altered functionality owing to a lack of inhibitory PD-1 signaling in the thymus and periphery.

A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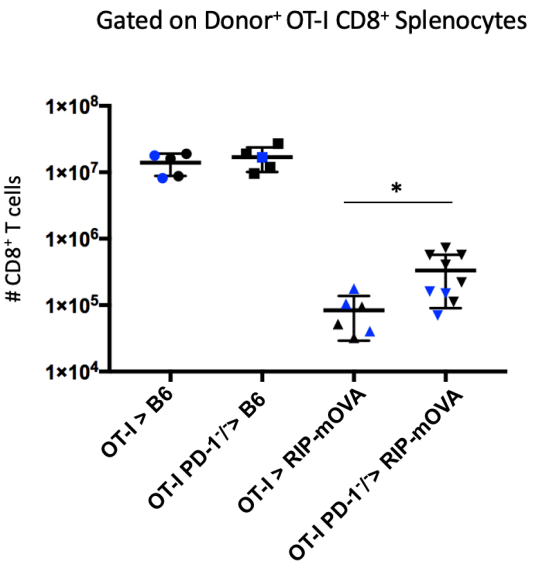
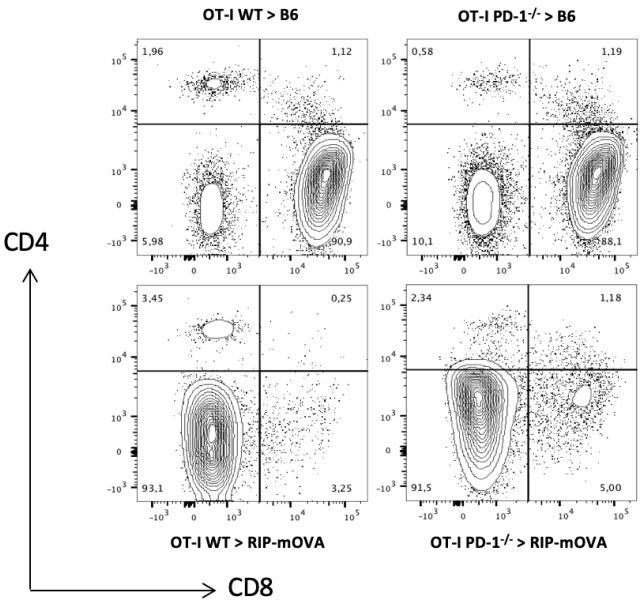
OT-I WT > B6
OT-I PD-1-/- > B6
1,62
1,06
4,79
92,5
0,71
1,19
6,94
91,2
4,78
0,23
89,4
5,57
2,47
2,20
88,2
7,09
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
CD4
CD8

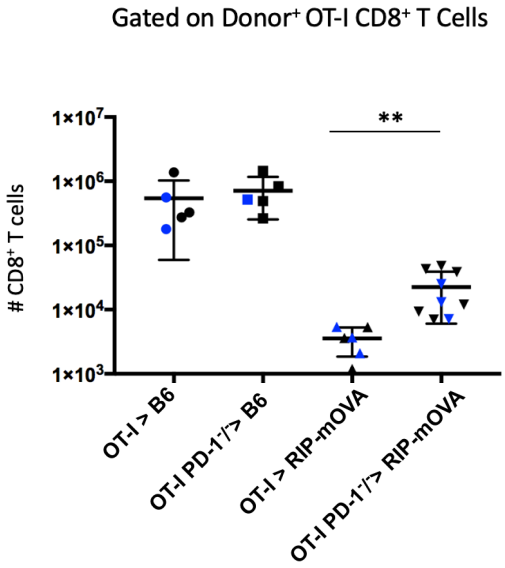
B


Gated on Donor+ OT-I CD8+ Splenocytes
CD8+ T cells
1×10^8
1×10^7
1×10^6
1×10^5
1×10^4
*
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA

C


Gated on Donor+ OT-I CD8+ T cells
OT-I WT > B6
OT-I PD-1-/- > B6
1,96
1,12
5,98
90,9
0,58
1,19
10,1
88,1
3,45
0,25
93,1
3,25
2,34
1,18
91,5
5,00
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
CD4
CD8

D


Gated on Donor+ OT-I CD8+ T Cells
CD8+ T cells
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
**
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA

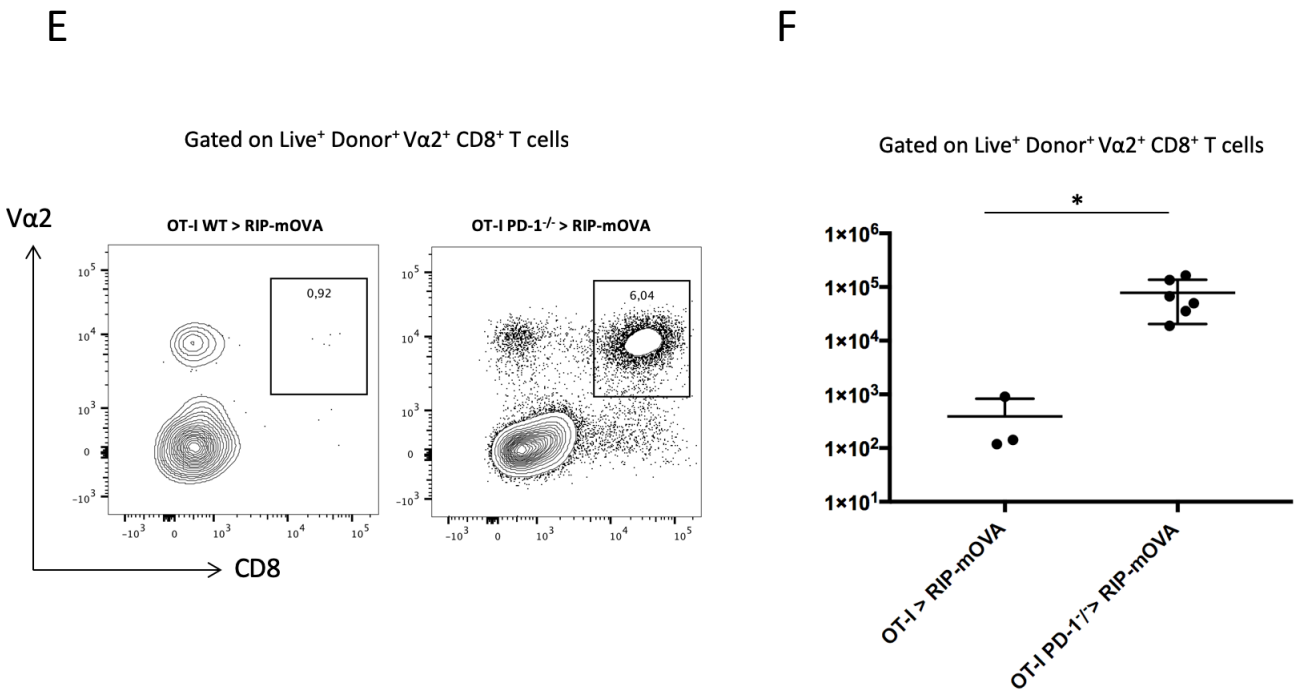


Figure 3-10. **OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras have more peripheral OT-I CD8^{+} T cells compared to OT-I WT > RIP-mOVA chimeras**

Two different strategies were used to detect OT-I CD8^{+} T cells: black dots represent data points collected using antibodies specific for Vα2 and CD45.2, blue dots represent data points collected using an OVA (257-264) peptide H-2K^{b} tetramer. A. Representative FACS plot of CD4 by CD8 profiles of OT-I splenocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9) B. Number of OT-I CD8^{+} splenocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9) C. Representative FACS plot of CD4 by CD8 profiles of OT-I T cells from the pLn. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9) D. Number of OT-I CD8^{+} T cells from the pLn. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9). E. Representative FACS plot of CD4 by CD8 profiles of pancreatic OT-I splenocytes. OT-I WT > RIP-mOVA (n=3), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=6) F. Number of OT-I CD8^{+} splenocytes. OT-I WT > RIP-mOVA (n=3), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=6). Data was collected over six separate cohorts. P values were calculated using two-tailed unpaired *t* test.

Peripheral T cells from OT-I WT > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras display evidence of antigen encounter and proliferation capacity

Given that there are an increased number of Vα2^{+} CD8^{+} T cells in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls, we next investigated what the functional differences were between the two populations in the spleen, pLn and pancreas *in vivo*. We hypothesized that OT-I T cells from OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras would show evidence of activation and proliferation by upregulating CD69 and expressing CD44 and Ki67 *ex vivo* compared to OT-I WT/PD-1$^{-/-}$ > B6 chimeras, and that diabetogenic OT-I PD-1$^{-/-}$ >RIP-mOVA CD8^{+} T cells would be phenotypically different than OT-I WT > RIP-mOVA CD8^{+} T cells.

Activation (CD69 expression)

To examine if OT-I PD-1$^{-/-}$ > RIP-mOVA CD8^{+} T cells were activated after cognate antigen encounter in any way different from OT-I WT > RIP-mOVA CD8^{+} T cells, we investigated the expression of CD69, which is typically upregulated shortly after antigen encounter. We observed that Vα2^{+} CD8^{+} splenocytes from OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras upregulated CD69 to a greater extent than Vα2^{+} CD8^{+} splenocytes from OT-I WT/PD-1$^{-/-}$ > B6 chimeras; however, we did not find a significant difference between OT-I WT > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA T chimeras **(Fig. 3-11, A-B)**. Interestingly, the Vα2^{+} CD8^{+} splenocytes from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras typically had a slightly lower CD69 MFI compared to OT-I WT > RIP-mOVA controls. We observed similar trends in the pLn, though there was a greater difference in

the CD69 MFI between OT-I PD-1$^{-/-}$ > RIP-mOVA and OT-I WT > RIP-mOVA chimeras **(Fig. 3-11, C-D)**. Lastly, we isolated Vα2^{+} CD8^{+} T cells from the pancreas of diabetic OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras and found that a larger fraction of Vα2^{+} CD8^{+} T cells upregulate CD69 cells compared to T cells from the pLn; the MFI of CD69^{+} Vα2^{+} CD8^{+} pancreatic T cells was typically much higher than T cells from the pLn or spleen. This data suggests that the presence of OVA-peptide is necessary for CD69 upregulation, and that the increased fraction of Vα2^{+} CD8^{+} CD69^{+} T cells from RIP-mOVA recipients' pLn and pancreas is likely due to the active antigen dissemination in these immune sites compared to the spleen. Furthermore, it is not clear why the MFI of CD69 of Vα2^{+} CD8^{+} CD69^{+} pLn T cells is lower in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls, though it may simply result from the migration of the most highly CD69 expressing Vα2^{+} CD8^{+} T cells having trafficked to the pancreas in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras.

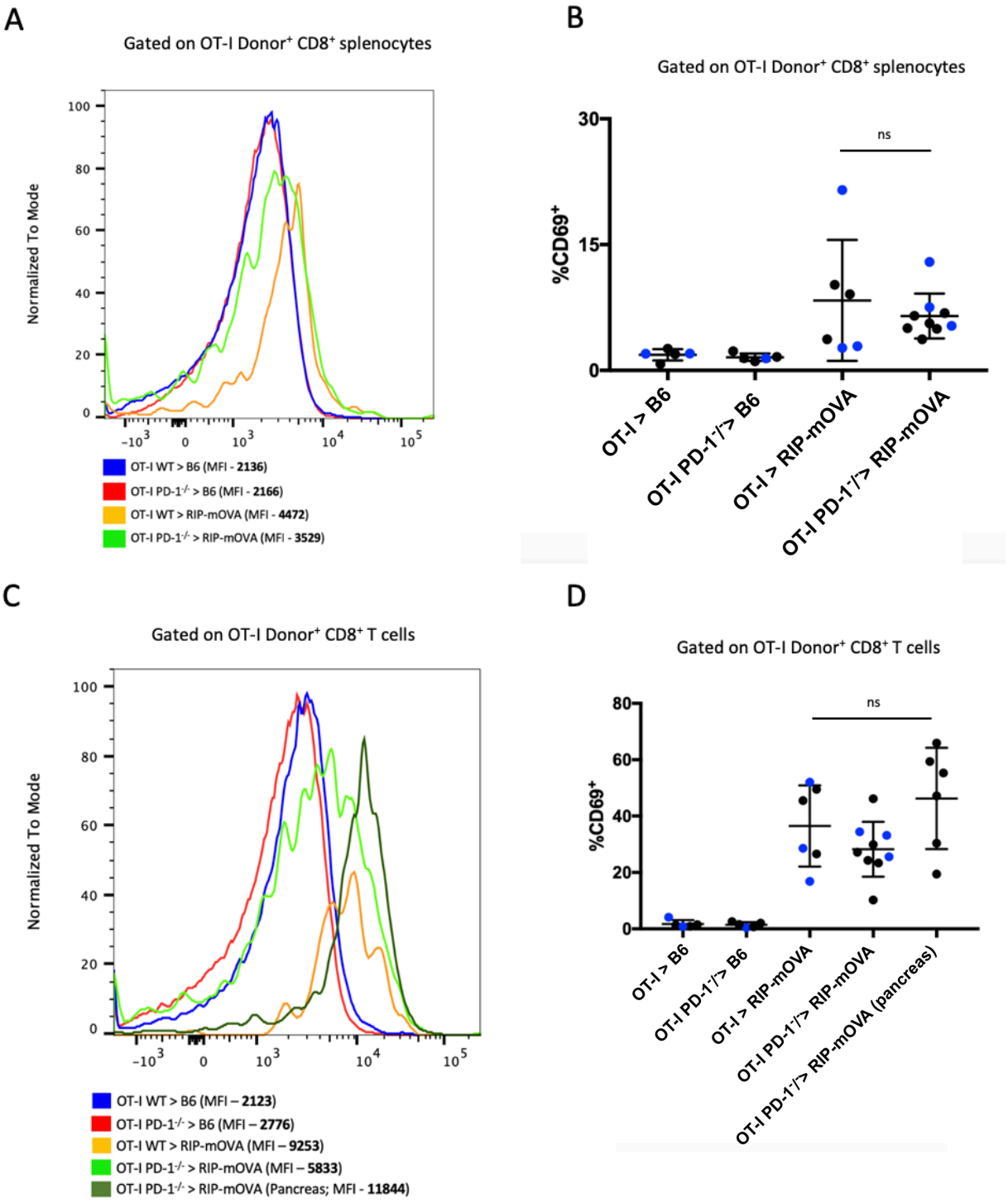

A
Gated on OT-I Donor+ CD8+ splenocytes
Normalized To Mode
OT-I WT > B6 (MFI - 2136)
OT-I PD-1-/- > B6 (MFI - 2166)
OT-I WT > RIP-mOVA (MFI - 4472)
OT-I PD-1-/- > RIP-mOVA (MFI - 3529)
B
Gated on OT-I Donor+ CD8+ splenocytes
%CD69+
ns
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
C
Gated on OT-I Donor+ CD8+ T cells
Normalized To Mode
OT-I WT > B6 (MFI – 2123)
OT-I PD-1-/- > B6 (MFI – 2776)
OT-I WT > RIP-mOVA (MFI – 9253)
OT-I PD-1-/- > RIP-mOVA (MFI – 5833)
OT-I PD-1-/- > RIP-mOVA (Pancreas; MFI - 11844)
D
Gated on OT-I Donor+ CD8+ T cells
%CD69+
ns
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
OT-I PD-1-/-> RIP-mOVA (pancreas)

Figure 3-11. **Peripheral T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA and OT-I WT > RIP-mOVA chimeras upregulate CD69 after antigen encounter**

Two different strategies were used to detect OT-I CD8^{+} T cells: black dots represent data points collected using antibodies specific for Vα2 and CD45.2, blue dots represent data points collected using an OVA (257-264) peptide H-2K^{b} tetramer. **A.** Representative histogram of CD69 expression of OT-I splenocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9) **B.** Fraction of OT-I CD8^{+} CD69^{+} splenocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9) **C.** Representative histogram of CD69 expression of OT-I T cells from pLn and pancreas. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=6) **D.** Fraction of OT-I CD8^{+} CD69^{+} T cells from pLn and pancreas. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=6). Data was collected over six separate cohorts. P values were calculated using two-tailed unpaired *t* test (**Fig. 3-11B**) and an ordinary one-way ANOVA test on OT-I WT/PD-1$^{-/-}$ > RIP-mOVA samples (**Fig. 3-11D**).

Activation (CD44 expression)

As OT-I T cells inevitably encounter OVA peptide in the periphery of RIP-mOVA recipients, we hypothesized that the majority of peripheral T cells in both OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras and OT-I WT > RIP-mOVA chimeras would express CD44 in contrast to OT-I WT/PD-$1^{-/-}$ > B6 controls. In this way, we used CD44 expression as another marker of antigen encounter/activation, though CD44 may also be expressed on T cells in lymphopenic environments [114]. We observed few $CD44^+$ splenocytes in OT-I WT/PD-$1^{-/-}$ > B6 chimeras, while there was a large and significant population of $CD44^+$ splenocytes in OT-I WT > RIP-mOVA and OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras, indicating antigen encounter and T cell maturation from naïve to effector/memory phenotype cells **(Fig. 3-12, A-B)**. Surprisingly, there was a greater frequency of $CD44^+$ splenocytes in OT-I WT > RIP-mOVA chimeras compared to OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras, which may due to migration of $CD44^+$ T cells to the pancreas in OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras. In the pLn, we identified few $CD44^+$ T cells in OT-I WT/PD-$1^{-/-}$ > B6 chimeras, but a significant population of $CD44^+$ T cells in the pLn from OT-I WT > RIP-mOVA and OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras **(Fig. 3-12, C-D)**. Compared to the spleen, there was a greater overall fraction of $CD44^+$ T cells in the pLn in OT-I WT/PD-$1^{-/-}$ > RIP-mOVA chimeras, though there was no significant difference between the fraction of $V\alpha2^+$ $CD8^+$ $CD44^+$ T cells OT-I WT > RIP-mOVA chimeras compared to OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras. In the pancreas of OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras, the majority of the $V\alpha2^+$ $CD8^+$ T cells were CD44 intermediate and $CD62^{lo}$ **(Fig. 3-12, C-D)**. This downregulation of CD62L likely contributes to the retention of diabetogenic T cells in the pancreas, as CD62L downregulation has been shown to be

important for preventing T cell egress from target tissues [115]. Lastly, it is unclear why CD44 expression is dulled in pancreatic T cells compared to $V\alpha2^{+}$ $CD8^{+}$ $CD44^{+}$ T cells in the pLn and spleen, though it may be an indication of a difference in trafficking potential that is unique to $V\alpha2^{+}$ $CD8^{+}$ $CD44^{+}$ T cells from OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras.

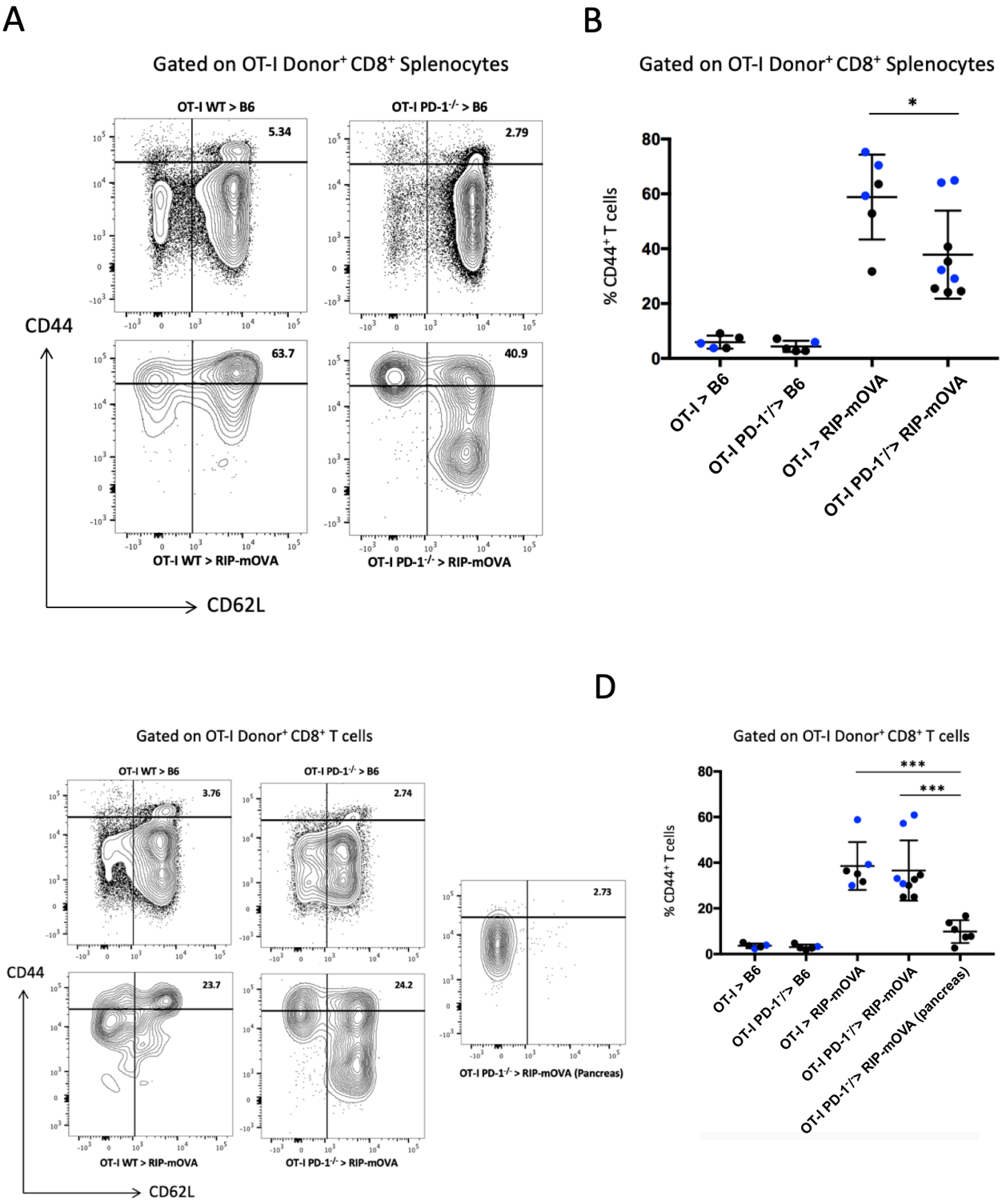

A
Gated on OT-I Donor+ CD8+ Splenocytes
OT-I WT > B6
OT-I PD-1-/- > B6
5.34
2.79
63.7
40.9
CD44
CD62L
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
B
Gated on OT-I Donor+ CD8+ Splenocytes
% CD44+ T cells
*
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
C
Gated on OT-I Donor+ CD8+ T cells
OT-I WT > B6
OT-I PD-1-/- > B6
3.76
2.74
23.7
24.2
2.73
CD44
CD62L
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA (Pancreas)
D
Gated on OT-I Donor+ CD8+ T cells
% CD44+ T cells
***
***
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
OT-I PD-1-/-> RIP-mOVA (pancreas)

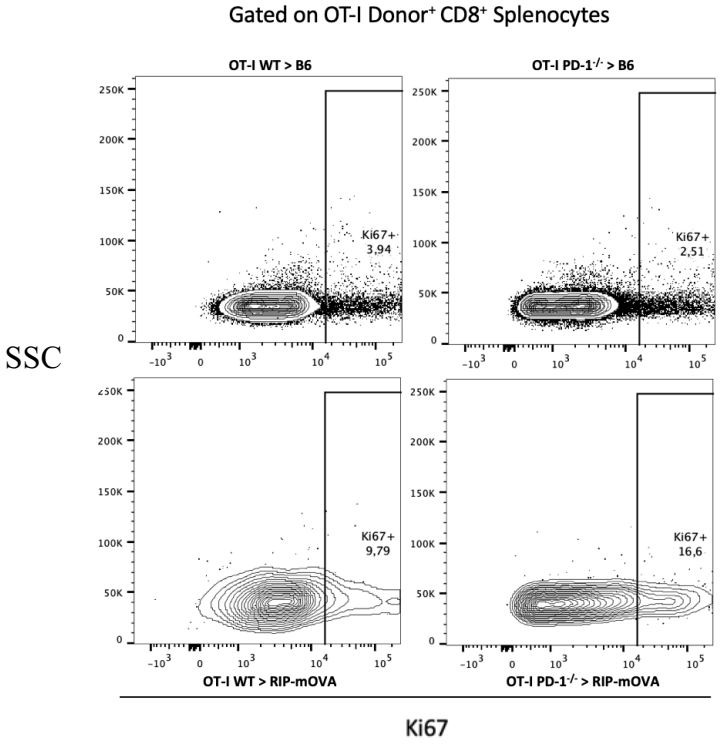
Figure 3-12. **Peripheral T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA and OT-I WT > RIP-mOVA chimeras upregulate CD44 after antigen encounter but pancreatic T cells downregulate CD62L**

Two different strategies were used to detect OT-I CD8^{+} T cells: black dots represent data points collected using antibodies specific for Vα2 and CD45.2, blue dots represent data points collected using an OVA (257-264) peptide H-2K^{b} tetramer. Numerical values on FACS plots represent the total fraction of CD44^{+} cells from both upper quadrants. **A.** Representative FACS plot of CD44 expression of OT-I splenocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9) **B.** Fraction of OT-I CD8^{+} CD44^{+} splenocytes. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9) **C.** Representative FACS plot of CD69 expression of OT-I T cells from pLn and pancreas. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=6) **D.** Fraction of OT-I CD8^{+} CD44^{+} T cells from pLn and pancreas. OT-I WT > B6 (n=5), OT-I PD-1$^{-/-}$ > B6 (n=5), OT-I WT > RIP-mOVA (n=6), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=9), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=6). Data was collected over six separate cohorts. P values were calculated using two-tailed unpaired *t* test (**Fig. 3-12B**) and an ordinary one-way ANOVA test on OT-I WT/PD-1$^{-/-}$ > RIP-mOVA samples (**Fig. 3-12D**).

Proliferation

One possible explanation for the increased number of peripheral Vα2$^+$ CD8$^+$ T cells in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA chimeras is that OT-I PD-1$^{-/-}$ T cells have enhanced proliferative capacity due to a lack of PD-1 signaling. To address this idea, we examined the expression of Ki67 on Vα2$^+$ CD8$^+$ T cells from the spleen, pLn and pancreas from OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA chimeras. Our preliminary experiments indicated that a fraction of Vα2$^+$ CD8$^+$ T cells from the spleen of OT-I WT > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras expressed Ki67, indicating that they had been in cell cycle in contrast to OT-I WT/PD-1$^{-/-}$ > B6 controls **(Fig. 3-13, A-B)**. Given our limited replicates, we could not determine if there were any significant differences in the Ki67$^+$ fraction of Vα2$^+$ CD8$^+$ splenocytes from OT-I WT > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras. We observed similar trends in the pLn and pancreas, as there was a fraction of Vα2$^+$ CD8$^+$ T cells from OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras that expressed Ki67 **(Fig. 3-13, C-D)**. The fraction of Ki67 expressing cells seemed to be higher in OT-I PD-1$^{-/-}$ > RIP-mOVA T cells in all three compartments; however, given our limited replicates we cannot definitely report if there are any significant differences in proliferation capacity of these cells when PD-1 signals are absent.

A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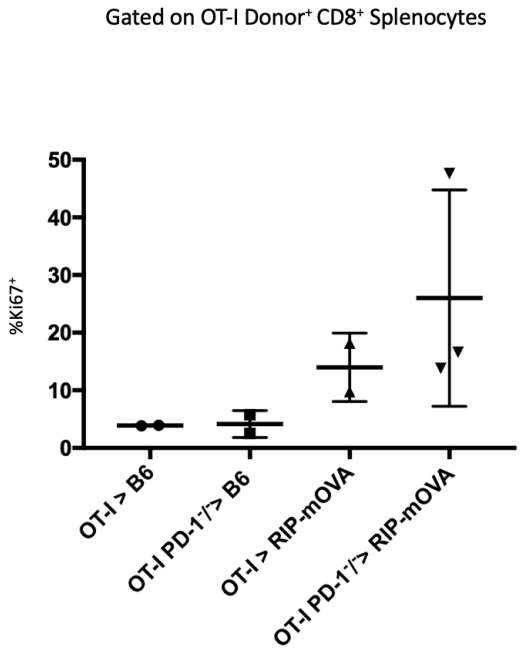
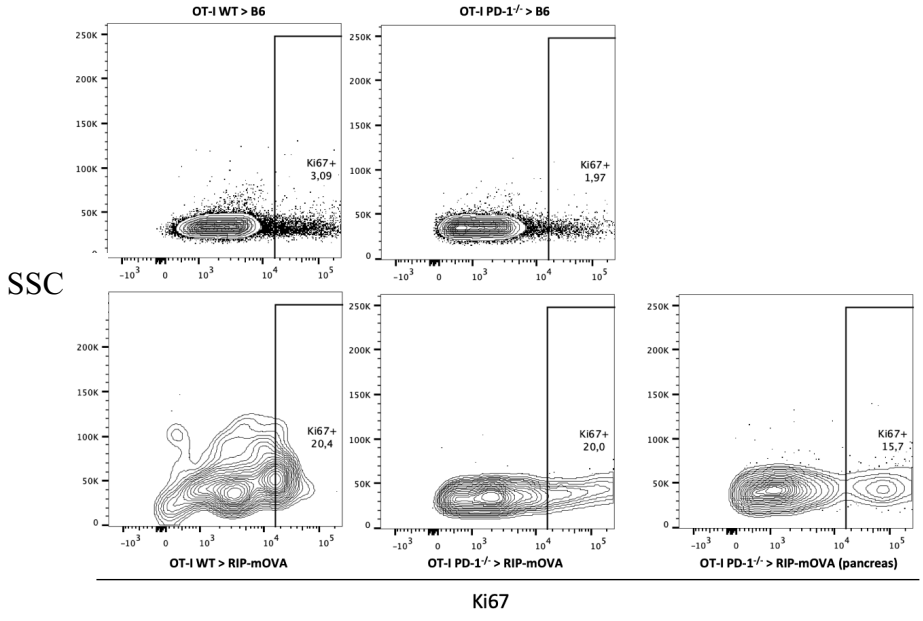
OT-I WT > B6
OT-I PD-1-/- > B6
Ki67+ 3,94
Ki67+ 2,51
SSC
Ki67+ 9,79
Ki67+ 16,6
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
Ki67

B


Gated on OT-I Donor+ CD8+ Splenocytes
%Ki67+
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA

C


Gated on OT-I Donor+ CD8+ T cells
OT-I WT > B6
OT-I PD-1-/- > B6
Ki67+ 3,09
Ki67+ 1,97
SSC
Ki67+ 20,4
Ki67+ 20,0
Ki67+ 15,7
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA (pancreas)
Ki67

D

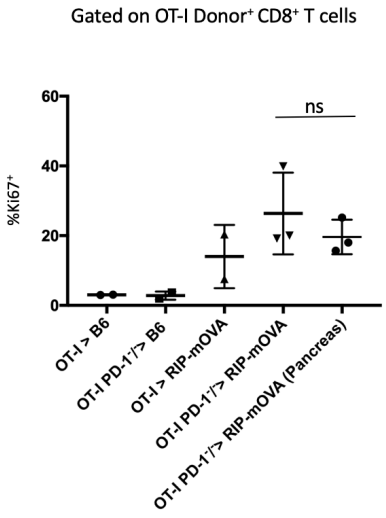

Gated on OT-I Donor+ CD8+ T cells
ns
%Ki67+
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
OT-I PD-1-/-> RIP-mOVA (Pancreas)

Figure 3-13. **Peripheral T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA and OT-I WT > RIP-mOVA chimeras upregulate Ki67**

Black dots represent data points collected using antibodies specific for Vα2 and CD45.2. **A.** Representative FACS plot of Ki67 expression from OT-I splenocytes. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3) **B.** Fraction of OT-I Ki67^{+} splenocytes. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3) **C.** Representative FACS plot of Ki67 expression from OT-I T cells from pLn and pancreas. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3). **D.** Fraction of OT-I Ki67^{+} T cells from pLn and pancreas. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3). Data was collected over two separate cohorts. P values were calculated using two-tailed unpaired *t* test.

Vα2$^+$ CD8$^+$ T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras may have altered trafficking potential compared to OT-T WT > RIP-mOVA chimeras

We hypothesized that differential trafficking potential of Vα2$^+$ CD8$^+$ T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras may play a seminal role in autoimmune diabetes induction. One chemokine receptor that has been shown to be important for trafficking to inflammatory sites, such as the diabetic pancreas, is CXCR3 [116,117] . As such, we next investigated CXCR3 expression on Vα2$^+$ CD8$^+$ T cells from the spleen, pLn and pancreas of OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA chimeras. In both RIP-mOVA and B6 recipient chimeras, we observed a decrease in the fraction of OT-I PD-1$^{-/-}$ T cells that expressed CXCR3 **(Fig. 3-14, A-B)**. Furthermore, we found that the fraction of CXCR3$^+$ CD8$^+$ T cells decreased from the spleen to the pLn to the pancreas. Similar to our CD62L expression data, pancreatic Vα2$^+$ CD8$^+$ T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras completely downregulate CXCR3, potentially contributing to the retention of diabetogenic T cells in the pancreas **(Fig. 3-14C)**. These data suggest that CXCR3 expression is different when PD-1 signaling is not available, even without the presence of cognate antigen, and that this difference in trafficking potential may be seminal to diabetes pathogenesis in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras.

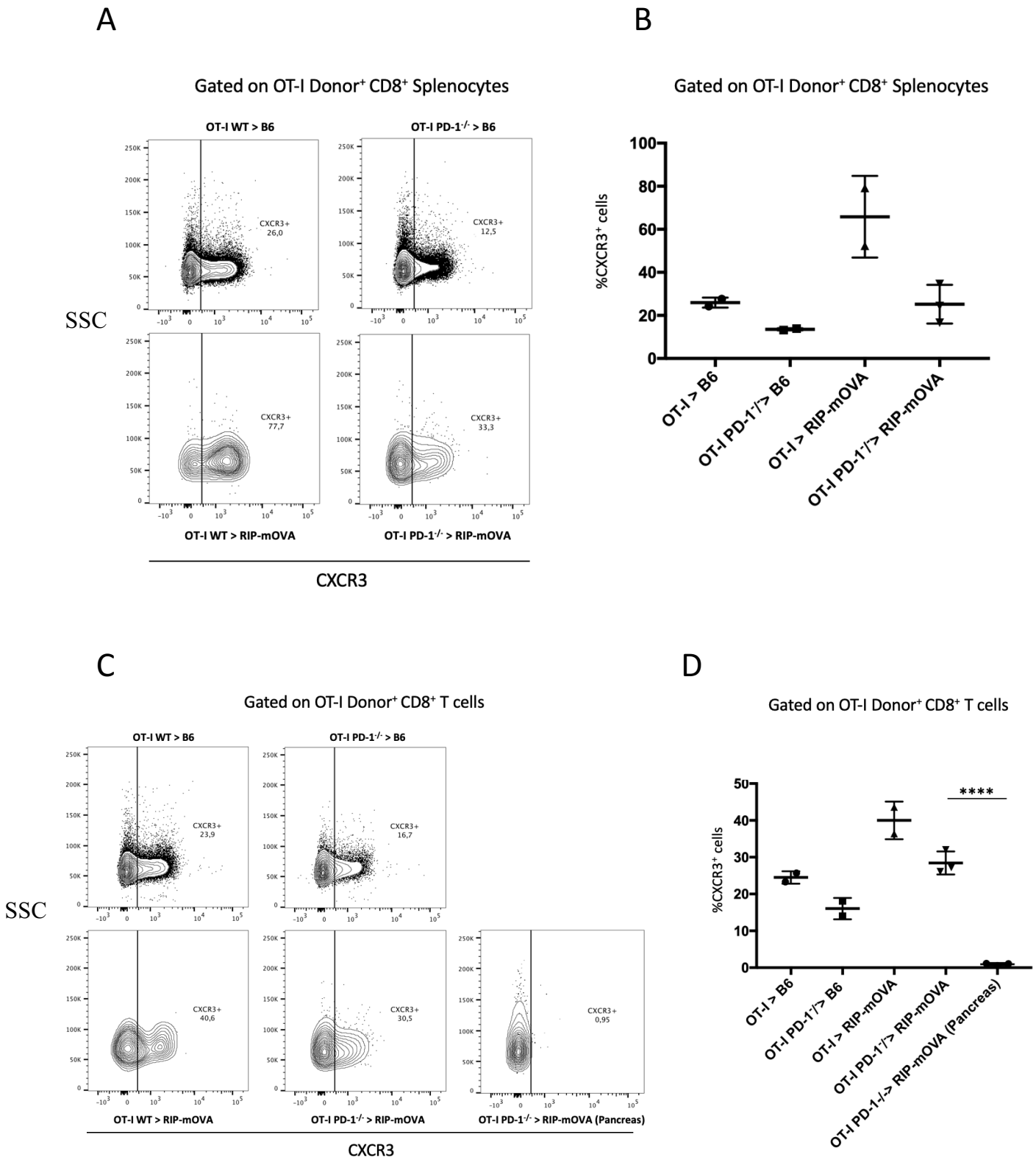

A
Gated on OT-I Donor+ CD8+ Splenocytes
OT-I WT > B6
OT-I PD-1-/- > B6
CXCR3+ 26,0
CXCR3+ 12,5
SSC
CXCR3+ 77,7
CXCR3+ 33,3
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
CXCR3
B
Gated on OT-I Donor+ CD8+ Splenocytes
%CXCR3+ cells
100
80
60
40
20
0
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
C
Gated on OT-I Donor+ CD8+ T cells
OT-I WT > B6
OT-I PD-1-/- > B6
CXCR3+ 23,9
CXCR3+ 16,7
SSC
CXCR3+ 40,6
CXCR3+ 30,5
CXCR3+ 0,95
OT-I WT > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA (Pancreas)
CXCR3
D
Gated on OT-I Donor+ CD8+ T cells
%CXCR3+ cells
50
40
30
20
10
0
****
OT-I > B6
OT-I PD-1-/-> B6
OT-I > RIP-mOVA
OT-I PD-1-/-> RIP-mOVA
OT-I PD-1-/-> RIP-mOVA (Pancreas)

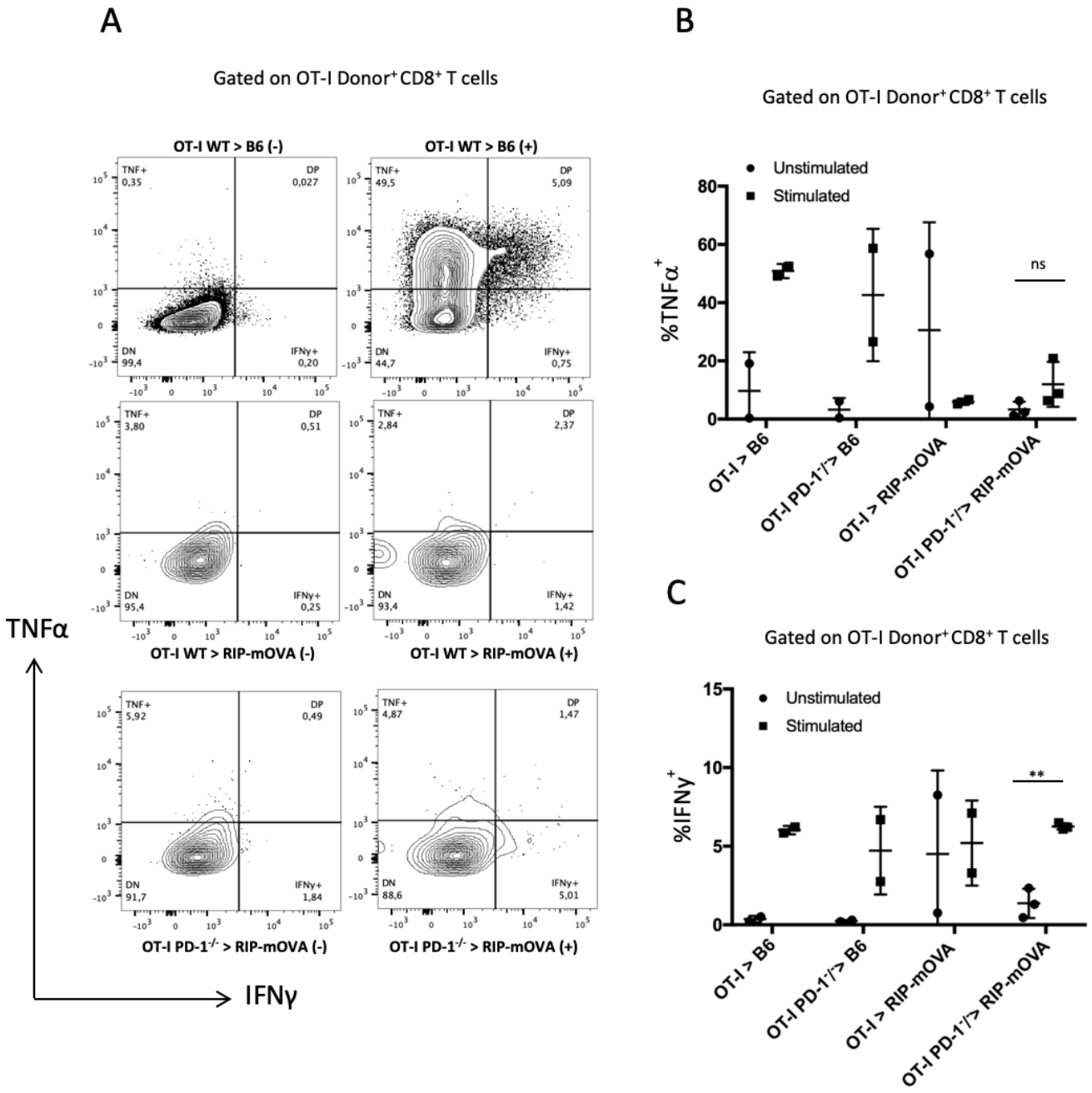
Figure 3-14. **OT-I PD-1$^{-/-}$ > RIP-mOVA/B6 chimeras express less CXCR3 than OT-I WT > RIP-mOVA/B6 controls**

Black dots represent data points collected using antibodies specific for Vα2 and CD45.2. **A.** Representative FACS plot of CXCR3 expression from OT-I splenocytes. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3) **B.** Fraction of OT-I CXCR3^{+} splenocytes. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3) **C.** Representative FACS plot of CXCR3 expression from OT-I T cells from pLn and pancreas. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3). **D.** Fraction of OT-I CXCR3^{+} T cells from pLn and pancreas. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3). Data was collected over two separate cohorts. P values were calculated using two-tailed unpaired *t* test.

Pancreatic Vα2$^+$ CD8$^+$ T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras consistently respond to re-stimulation with cognate antigen and produce IFNγ

Lastly, we hypothesized that pancreatic Vα2$^+$ CD8$^+$ T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras should be able to respond to a short *in vitro* OVA-peptide stimulation and produce cytokines that would ostensibly contribute to islet destruction pathogenesis. We isolated Vα2$^+$ CD8$^+$ T cells from the spleen, pLn and pancreas of OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA chimeras, stimulated for four hours with OVA-peptide and assessed cytokine production. We found that few Vα2$^+$ CD8$^+$ splenocytes from OT-I WT/PD-1$^{-/-}$ > B6 chimeras were capable of producing IFNγ but many were TNFα$^+$ after restimulation **(Fig. 3-15A)**. This was in contrast to Vα2$^+$ CD8$^+$ splenocytes from OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras, which were not TNFα$^+$ **(Fig. 3-15B)**. Interestingly, there was a larger fraction of Vα2$^+$ CD8$^+$ splenocytes from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA chimeras that were able to express IFNγ after restimulation, suggesting that PD-1 signals restrain the ability of OT-I T cells to produce IFNγ **(Fig. 3-15C)**. We observed similar trends in Vα2$^+$ CD8$^+$ T cells in the pLn and pancreas: we found that few Vα2$^+$ CD8$^+$ splenocytes from OT-I WT/PD-1$^{-/-}$ > B6 chimeras were capable of producing IFNγ but many were TNFα$^+$ after restimulation **(Fig. 3-15D)**. This was in contrast to Vα2$^+$ CD8$^+$ splenocytes from OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras, which were not TNFα$^+$ **(Fig. 3-15E)**. While not statistically significant, there was a fraction of Vα2$^+$ CD8$^+$ splenocytes from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras but not OT-I WT > RIP-mOVA chimeras that were able to produce IFNγ after

restimulation **(Fig. 3-15F)**. Finally, we observed that pancreatic $V\alpha2^+$ $CD8^+$ T cells from OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras were more $IFN\gamma^+$ compared to the same cells but from the pLn. These data suggest that a lack of PD-1 signals may enhance the ability of OT-I PD-$1^{-/-}$ > RIP-mOVA T cells to produce IFNγ which may contribute to diabetes pathogenesis.


A
Gated on OT-I Donor+ CD8+ T cells
OT-I WT > B6 (-)
OT-I WT > B6 (+)
TNF+ 0,35
DP 0,027
DN 99,4
IFNγ+ 0,20
TNF+ 49,5
DP 5,09
DN 44,7
IFNγ+ 0,75
TNF+ 3,80
DP 0,51
DN 95,4
IFNγ+ 0,25
TNF+ 2,84
DP 2,37
DN 93,4
IFNγ+ 1,42
OT-I WT > RIP-mOVA (-)
OT-I WT > RIP-mOVA (+)
TNF+ 5,92
DP 0,49
DN 91,7
IFNγ+ 1,84
TNF+ 4,87
DP 1,47
DN 88,6
IFNγ+ 5,01
OT-I PD-1-/- > RIP-mOVA (-)
OT-I PD-1-/- > RIP-mOVA (+)
TNFα
IFNγ
B
Gated on OT-I Donor+ CD8+ T cells
Unstimulated
Stimulated
%TNFα+
ns
OT-I > B6
OT-I PD-1-/- > B6
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
C
Gated on OT-I Donor+ CD8+ T cells
Unstimulated
Stimulated
%IFNγ+
**
OT-I > B6
OT-I PD-1-/- > B6
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA

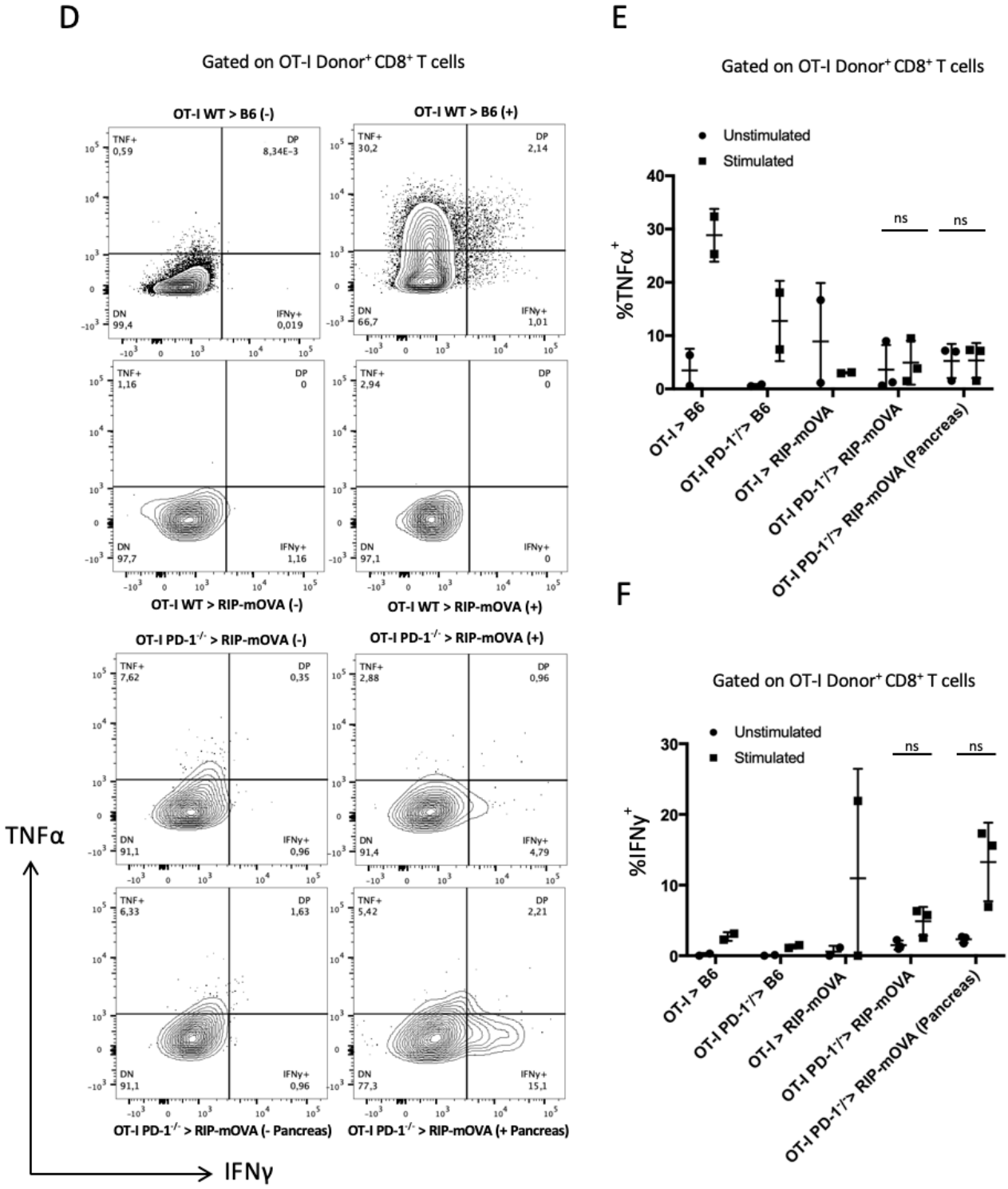

D
Gated on OT-I Donor+ CD8+ T cells
OT-I WT > B6 (-)
OT-I WT > B6 (+)
OT-I WT > RIP-mOVA (-)
OT-I WT > RIP-mOVA (+)
OT-I PD-1-/- > RIP-mOVA (-)
OT-I PD-1-/- > RIP-mOVA (+)
OT-I PD-1-/- > RIP-mOVA (- Pancreas)
OT-I PD-1-/- > RIP-mOVA (+ Pancreas)
TNF+
IFNγ+
DP
DN
TNFα
IFNγ
E
Gated on OT-I Donor+ CD8+ T cells
Unstimulated
Stimulated
%TNFα+
ns
OT-I > B6
OT-I PD-1-/- > B6
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA (Pancreas)
F
Gated on OT-I Donor+ CD8+ T cells
Unstimulated
Stimulated
%IFNγ+
ns

Figure 3-15. **T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras produce IFNγ after restimulation**

Peripheral T cells from OT-I WT/PD-1$^{-/-}$ > B6/RIP-mOVA chimeras were restimulated with 100 nM OVA-peptide (SIINFEKL) in RP-10 treated with 3 ug of Brefeldin A per mL. Black dots represent data points collected using antibodies specific for Vα2 and CD45.2. **A.** Representative FACS plot of cytokine expression from OT-I splenocytes. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3) **B.** Fraction of OT-I TNFα^{+} splenocytes. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3) **C.** Fraction of OT-I IFNγ^{+} splenocytes. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3) **D**. Representative FACS plot of cytokine expression from OT-I T cells from the pLn and pancreas. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3). **E.** Fraction of OT-I TNFα^{+} T cells from the pLn and pancreas. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3)**,** pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3). **E.** Fraction of OT-I IFNγ^{+} T cells from the pLn and pancreas. OT-I WT > B6 (n=2), OT-I PD-1$^{-/-}$ > B6 (n=2), OT-I WT > RIP-mOVA (n=2), OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3), pancreatic OT-I PD-1$^{-/-}$ > RIP-mOVA (n=3). Data was collected over two separate cohorts. P values were calculated using two-tailed paired *t* test.

Conclusion

We determined that PD-1 is necessary for T cell tolerance to TRA, though the precise underlying mechanism remains unclear as the expression of activation markers such as CD69 and CD44 and the expression of Ki67 were relatively similar between OT-I WT > RIP-mOVA and OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras, though the latter may yield differences pending further replicates. In contrast, we observed significant differences in the expression of CXCR3 between V$\alpha2^{+}$ $CD8^{+}$ T cells from OT-I PD-$1^{-/-}$ > RIP-mOVA and OT-I WT > RIP-mOVA chimeras and we found that V$\alpha2^{+}$ $CD8^{+}$ T cells from OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras have an enhanced ability to produce IFNγ compared to OT-I WT > RIP-mOVA controls. Ultimately, differences in proliferative capacity, trafficking potential and cytokine production due to a lack of PD-1 signals may synergize to induce autoimmune diabetes in OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras, however more replicates are needed to verify these preliminary findings.

CHAPTER 4: DISCUSSION

Summary of Results

We used the OT-I TCR transgenic mouse model in bone marrow chimeras where cognate antigen OVA is expressed under the control of the rat insulin promoter (RIP-mOVA) or where OVA is not expressed (B6) to investigate the role of PD-1 in T cell tolerance to TRA. We first sought to determine if the addition of blocking PD-1 antibodies could break tolerance in functionally impaired OT-I $Bim^{-/-}$ > RIP-mOVA $CD8^+$ T cells *in vitro* and *in vivo*. We determined that OT-I $Bim^{-/-}$ > RIP-mOVA $CD8^+$ T cells were intrinsically impaired but were not functionally rescued by PD-1 blockade. Subsequently, we generated OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras to investigate if a genetic loss of PD-1 would abrogate T cell tolerance. We discovered that 100% of OT-I PD-$1^{-/-}$ > RIP-mOVA mice develop autoimmunity, compared to 0% of control OT-I WT > RIP-mOVA mice. We then isolated OT-I thymocytes and peripheral T cells from the spleen, pancreatic lymph nodes (pLn), and pancreas from OT-I WT or PD-$1^{-/-}$ > RIP-mOVA chimeras, and phenotyped each chimera *ex vivo*. In the thymus, clonal deletion was intact in OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras, suggesting that PD-1 signals impact T cell tolerance outside of deletional mechanisms. In the periphery, we found a significant increase in the number of OT-I $CD8^+$ splenocytes in OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls. This increase in cell number of OT-I PD-$1^{-/-}$ $CD8^+$ T cells is associated with an altered expression of chemokine receptor CXCR3, a modest increase in proliferative capacity as measured by Ki67 expression, and the

ability of OT-I PD-$1^{-/-}$ $CD8^{+}$ T cells to produce IFNγ after a four-hour restimulation assay. Collectively, this data suggests that PD-1 plays a significant role in establishing T cell tolerance to TRA via non-deletional tolerance mechanisms **(Table 4-1)**.

	OT-I Bim$^{-/-}$ > RIP-mOVA	OT-I WT > RIP-mOVA	OT-I PD-1$^{-/-}$ > RIP-mOVA
Intact PD-1 signaling?	Yes	Yes	No
Autoimmune diabetes?	No	No	Yes
***In vitro* PD-1 blockade success?**	No	N/A	N/A
***In vivo* PD-1 blockade success?**	No	No	N/A
Clonal deletion?	No	Yes	Yes
Significant number of T cells in pancreas?	N/A	No	Yes
Activation markers?	Impaired	Yes	Yes*
Trafficking potential?	N/A	Yes	Altered
Proliferation?	Impaired	Yes	Enhanced**
***In vitro* cytokine production?**	Impaired	No	Yes

Table 4-1. **Summary of key findings with OT-I Bim$^{-/-}$ / WT / PD-1$^{-/-}$ > RIP-mOVA chimeras**

Table depicting key findings from results section. N/A - denotes incomplete information or information that was not directly tested. * - denotes that there may be differences between OT-I WT > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras as observed with CD69 and CD44 expression and CD69 MFI. ** - denotes that there may be a greater fraction of cells that are Ki67^{+} in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras, but further replicates are needed to find statistical significance.

Why Was PD-1 Blockade of OT-I Bim$^{-/-}$ > RIP-mOVA Chimeras Ineffective?

Vα2^{+} CD8^{+} T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras upregulate PD-1, are functionally impaired and do not induce autoimmune diabetes. As previously mentioned, we were unable to rescue the activation, proliferation and effector function *in vitro* or *in vivo* using PD-1 blockade. This is in contrast to evidence from other groups who have shown it is possible to augment the functionality of CD4^{+} and CD8^{+} T cells *in vitro* and *in vivo* after PD-1 blockade [81,108,118]. One possible reason for this difference is that there are significant discrepancies in PD-1 blockade protocols amongst different groups.

Protocol Diversity in PD-1 Blockade

There are three major murine αPD-1 clones used to block PD-1 signaling: J43, RMP1-14 and 29F.1A12, in addition to PD-L1 and PD-L2 blocking monoclonal antibodies. Given this diversity, we performed preliminary replicates of *in vitro* PD-1 blockade of OT-I Bim$^{-/-}$ > RIP-mOVA splenocytes using J43, RMP1-14, 29F.1A12 and αPD-L1, such that we would be confident that our results were not confounded by clone-specific effects. We did not observe significant differences between the four blocking antibodies tested, as none of the clones were able to significantly enhance functionality of OT-I Bim$^{-/-}$ > RIP-mOVA splenocytes. We ultimately settled on using both J43 and RMP1-14 for our main replicates as these clones are most commonly cited in the literature.

In the literature, there are also discrepancies in the amount of antibody used for both *in* vitro and *in vivo* PD-1 blockade. To address this, we used comparable amounts of antibody *in vitro* and *in* vivo compared to other groups who have shown significant functional differences in T cell functionality with PD-1 blockade. However, this approach did not lead to a functionally successful PD-1 blockade of $V\alpha2^+$ $CD8^+$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras.

Epigenetic Modification of pdcd1

Protocol differences notwithstanding, the failure of PD-1 blockade to significantly improve the functionality of $V\alpha2^+$ $CD8^+$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras may reflect the idea that these splenocytes have undergone epigenetic modification.

Some groups have suggested that only a subset of T cells are susceptible to PD-1 blockade: most often the T cells that have not been in continual contact with antigen such as recent thymic emigrants that have freshly seeded the periphery [110,113]. Indeed, there is strong evidence to suggest that T cells perpetually engaged with cognate antigen undergo epigenetic modification at the PD-1 genetic locus, *pdcd1* [111]. Typically, naïve T cells have been shown to have only small amounts *Pdcd1* methylation, which allows them to upregulate PD-1 after encounter with cognate antigen and become tolerized; there exists an inverse correlation between DNA methylation and PD-1 expression. Interestingly, one group studying chronic HIV infections give evidence that there is no re-methylation mechanism at the *pdcd1* locus after chronic antigen clearance, which may

explain why some "older", more antigen-experienced T cells (that express PD-1 and are demethylated) may not be susceptible to PD-1 blockade [119]. Furthermore, there is also strong evidence that additional epigenetic modifications such as histone modification, transcription factors and *cis*-DNA elements may modulate PD-1 expression. While the relative importance of each mechanism in the grand scheme PD-1 expression and tolerance is unclear, it is quite plausible that various epigenetic modifications may ultimately combine to cause resistance to PD-1 blockade.

Timing of PD-1 Blockade

From a whole system perspective, there is evidence in the literature to suggest that PD-1 signaling is particularly important in the context of lymphopenia; polyclonal PD-1$^{-/-}$ recent thymic emigrants (RTE) induce autoimmunity in lymphopenic Rag$^{-/-}$ recipients only if there is ample "space" for expansion [120]. Given that we treated OT-I Bim$^{-/-}$ > RIP-mOVA chimeras with PD-1 blockade *in vivo* at eight weeks post-generation, the initial wave of RTEs will have signaled through PD-1, thus restraining their autoimmune potential in the lymphopenic environment. To that end, we hypothesize that we may observe the induction of autoimmune diabetes in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras if we would treat with PD-1 blockade beginning from bone marrow chimera generation. With this kinetic alteration, we hypothesize that the initial wave of periphery settling T cells (RTEs), which may be most critical for the establishment of tolerance, will not have signaled through PD-1 and will thereby exhibit enhanced global autoimmune potential leading to diabetes in our model system.

Anatomical Dispersion of PD-1 Antibody

The lack of autoimmune diabetes induction in OT-I $Bim^{-/-}$ > RIP-mOVA chimeras treated with PD-1 blockade may reflect non-ideal anatomical dispersion of PD-1 blocking antibody. In other words, despite administration of αPD-1 antibody, there may still be some PD-1 signaling in the chimera, such as in the thymus during development which may ultimately be critical for T cell tolerance. Unfortunately, there are no easy ways to investigate this potential issue, though there is also no evidence in the literature to suggest that this type of antibody would not disperse evenly and completely *in vivo*.

Autoimmune Potential of OT-I $Bim^{-/-}$ T Cells

One caveat to the OT-I $Bim^{-/-}$ > B6/RIP-mOVA chimeras is that $Bim^{-/-}$ T cells are highly artificial, so the results we obtain may not be physiologically relevant. In other words, OT-I $Bim^{-/-}$ > RIP-mOVA chimeras may not have autoimmune potential independent of PD-1 signals. However, data from our group indicates that adoptive transfer of OT-I $Bim^{-/-}$ > B6 thymocytes and splenocytes can induce autoimmunity in sub-lethally irradiated RIP-mOVA recipients, suggesting that these cells have functionality and that the absence of Bim itself does not account for the ineffectiveness of PD-1 blockade.

Establishment vs. Maintenance of T Cell Tolerance

There continues to be a debate in the literature regarding when PD-1 signals are important for conferring tolerance, practically speaking, if they are most important for the establishment and/or maintenance of T cell tolerance.

PD-1 is Not Important for The Maintenance of Tolerance

We treated previously tolerized Vα2$^+$ CD8$^+$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA with PD-1 blockade *in vitro* to see if PD-1 signals were important for the maintenance of T cell tolerance. As previously mentioned, PD-1 blockade was not able to rescue the functionality of these cells. To explain these findings, we hypothesized that splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras would have previously been subjected to PD-1 signals that provided long lasting tolerance as RTEs or thymocytes during development; thus, thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras may not have already undergone this initial tolerizing event and may be susceptible to PD-1 blockade. However, we observed that thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras were also not sensitive to PD-1 blockade; it is likely that these thymocytes cultured *in vitro* had already signaled through PD-1. We hypothesize that in our OT-I Bim$^{-/-}$ > RIP-mOVA chimeras, PD-1 signals during initial encounter with antigen in the thymus are sufficient for long-lasting tolerance and therefore, PD-1 is not important for the maintenance of tolerance. To that end, unpublished data from our group using thymic transplant chimeras

have determined that continued antigen encounter, not PD-1 signaling, is required to maintain tolerance of splenocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras.

PD-1 is Important for The Establishment of Tolerance

The strongest evidence that PD-1 is crucial for the establishment of tolerance is that all OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras develop autoimmune diabetes. As mentioned in the results, the few OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA T cells that manage to escape clonal deletion and enter the periphery are able to induce autoimmunity, even in situations where there are antigen specific and non-antigen specific T cell competitors. However, in order to identify comprehensively and precisely when PD-1 signals are important for conferring T cell tolerance, the following adoptive transfer experiment should be performed:

We will use RIP-mOVA mice that express OVA-peptide but are unable to induce PD-1 signaling in OT-I T cells through the PD-1/PD-L1 axis ($PD\text{-}L1^{-/-}$ RIP-mOVA). We would obtain thymocytes from OT-I WT > RIP-mOVA chimeras that have undergone PD-1 signaling and adoptively transfer them into $PD\text{-}L1^{-/-}$ RIP-mOVA mice to observe if they can induce autoimmune diabetes. If early PD-1 signals are sufficient for providing long-lasting tolerance, we hypothesize that these OT-I WT > RIP-mOVA → $PD\text{-}L1^{-/-}$ RIP-mOVA mice will not become diabetic. One potential caveat is that OT-I cells would still be able to signal through the PD-1/PD-L2 axis, though the literature suggests these interactions may not be a major factor for immune tolerance [121]. To address this problem, we could treat $PD\text{-}L1^{-/-}$ RIP-mOVA mice with αPD-L2 concurrently with

adoptive transfer of OT-I cells. Overall, while our group and others provide strong evidence that PD-1 is more important for the establishment rather than the maintenance of tolerance, this adoptive transfer experiment would be extremely helpful in identifying precisely when PD-1 signals are important for establishment of tolerance.

How Exactly Do a Lack of PD-1 Signals Induce Autoimmunity?

Our OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras develop autoimmune diabetes at a 100% incidence rate, owing to a lack of tolerizing PD-1 signaling. This finding leads us to another question: how exactly are OT-I PD-1$^{-/-}$ > RIP-mOVA T cells functionality distinct from OT-I WT > RIP-mOVA T cells, given that they cannot signal through PD-1?

Differences in Strength of Signal

To begin, OT-I PD-1$^{-/-}$ T cells ostensibly receive a signal that is different in magnitude through the TCR compared to OT-I WT T cells, as we would hypothesize that OT-I PD-1$^{-/-}$ T cells would receive a stronger signal owing to the absence of SHP-2 mediated dephosphorylation of TCR-proximal signaling proteins. But how could we test if the difference in signal strength could equate to a functional difference, in our case leading to autoimmune diabetes induction? To address this question, we could make use of OT-I WT/PD-1$^{-/-}$ > RIP-mOVA variant chimeras, that express altered peptide ligands that are slightly below, equal to, and above the negative selection threshold when presented to

OT-I thymocytes. We hypothesize that if there are functional repercussions related to the difference in signal strength received through the TCR by a lack of PD-1 signaling, OT-I WT thymocytes would not be negatively selected to the weaker peptide ligand, but OT-I PD-$1^{-/-}$ thymocytes would undergo negative selection. In other words, we would test if differences in TCR signaling (digital signal – wide spectrum of signaling strength) would be sufficient to change the fate decision of OT-I DP thymocytes (functionally analog – positive or negative selection). In sum, while this proposed experiment directly pertains to fate decisions in the thymus, it may indirectly provide an explanation for increased autoimmune potential of OT-I PD-$1^{-/-}$ > RIP-mOVA T cells, as the strength of signal difference may result in the survival of OT-I PD-$1^{-/-}$ T cells after encounter with OVA-peptide as opposed to peripheral deletion, which may be considered an analog fate decision. We recognize that RIP-mOVA variant chimeras may be difficult to obtain, thereby we could alternatively make use of altered peptide ligands in conjunction with fetal thymic organ culture (FTOC) to assess if fate decisions are impacted by a lack of PD-1 signaling *in situ*.

Differences in Quality of Signal

PD-1 signals mediated by SHP-2 are able to dampen the T cell response by a variety of different mechanisms. PD-1 is able to dephosphorylate TCR proximal signaling proteins such as ZAP-70, ultimately preventing the activity of transcription factors important for activation, proliferation, cell survival and effector function. This direct control over the signaling capacity of the TCR signalosome is most often what implicates PD-1 in its

ability to establish T cell tolerance. However, there are also indirect inhibitory mechanisms over TCR signaling that may play a significant role in determining the autoimmune potential of an individual T cell.

Recently, one group provided evidence that PD-1 preferentially dephosphorylates proteins essential for CD28 signaling rather than TCR proximal proteins [122]. The basic signaling pathway of CD28 is as follows: PI3K associates with the intracellular tail of CD28 and mediates the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which activates AKT8 virus oncogene cellular homologue (AKT) leading to T cell growth and survival. Purportedly, PD-1 signals, through the activity of SHP-2, reduce Casein Kinase 2 (CK2) expression and activity, which in turn leads to the degradation of PIP3. In this way, essential CD28 co-stimulatory signals are inhibited by the activity of SHP-2 after simultaneous TCR and PD-1 engagement.

PD-1 has also been shown to modulate the activity of E3 ubiquitin ligases such as Cbl-b, that are able to terminate TCR signal transduction through TCR regulation [123]. Mechanistically, TCR and PD-1 co-ligation leads to the recruitment of SHP-2, which is able to indirectly induce the transcription of Cbl-b, though the exact mechanism is unclear. Cbl-b, along with other E3 ubiquitin ligases, then can ubiquitylate the intracellular motifs of the TCR leading to receptor internalization. PD-1 can thereby reduce the activity of a given T cell through indirect downregulation of the TCR.

Lastly, PD-1 signals can restrain the activation of T cells by preventing the metabolic switch from fatty acid β-oxidation to glycolysis; this process is thought to be caused by SHP-2 mediated dysfunction of PI3K/AKT and ERK signaling pathways [124]. Practically speaking, if a particular T cell encounters its cognate antigen in the periphery and attempts to transition from a naïve to effector cell type, it requires an extensive amount of intracellular energy. Therein, when PD-1 is ligated during initial T cell activation, it may prevent the engagement of these glycolytic pathways.

In sum, when a T cell undergoes PD-1 signaling, it receives qualitatively different signals through alterations in CD28, Cbl-b, and metabolic pathways. These qualitative differences, in conjunction with aforementioned differences in the strength of signal received through the TCR after PD-1 ligation, may work together prevent potentially autoreactive T cells from causing autoimmune disease.

Functionality of OT-I PD-1$^{-/-}$ T cells

Irrespective of the differential strength and/or quality of signals received by V$\alpha 2^{+}$ CD8^{+} PD-1$^{-/-}$ T cells, we observed differences in the fraction of Ki67^{+}, IFNγ^{+} and CXCR3^{+} expressing CD8^{+} T cells from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras.

There is a large amount of evidence suggesting that PD-1 signals restrain proliferation in both human and animal models. For example, proliferation as measured by Ki67 expression of blood CD8^{+} T cells from non-small cell lung cancer (NSCLC) patients is

enhanced after PD-1 blockade; BDC2.5 T effector cells adoptively transferred into pre-diabetic NOD mice treated with PD-1 blockade show enhanced proliferation as measured by CFSE labelling [125,126]. In the latter paper, the authors suggest that only a subset of newly generated effector $CD4^+$ and $CD8^+$ T cells respond to PD-1 blockade, unlike established tolerant and anergic T cells. These observations are similar to our data, as established tolerant $V\alpha2^+$ $CD8^+$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras do not show enhanced proliferation after PD-1 blockade *in vitro*; however, $V\alpha2^+$ $CD8^+$ T cells from OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras that have not been able to receive tolerizing PD-1 signals have a greater fraction of $Ki67^+$ T cells as compared to OT-I WT controls, though further replicates are necessary to confirm these trends. Given that PD-1 does not seem to regulate deletion, this increase in proliferation capacity in the absence of PD-1 signals may explain the modest increase in the number of OT-I PD-$1^{-/-}$ T cells in the periphery compared to OT-I WT controls.

There is also a significant body of literature that suggests PD-1 blockade can enhance the frequency of IFNγ producing cells, in addition to the amount of IFNγ produced [113]. Our experiments provide further supporting evidence that this is the case, as more OT-I PD-$1^{-/-}$ > RIP-mOVA $CD8^+$ T cells are $IFN\gamma^+$ compared to OT-I WT > RIP-mOVA controls after *ex vivo* restimulation, though we did not attempt to quantify the amount of IFNγ produced. Furthermore, additional replicates are required to confirm these trends, given the low number of replicates and the high variability of $IFN\gamma^+$ T cells from OT-I WT > RIP-mOVA chimeras, perhaps owing to the overall paucity of antigen specific T cells from these chimeras. We speculate that the enhanced ability of OT-I PD-$1^{-/-}$ > RIP-

mOVA $CD8^+$ T cells to produce $IFN\gamma^+$ may directly contribute to autoimmune diabetes induction as IFNγ has been shown to have cytotoxic effects on β cells *in vitro* and studies have also shown that IFNγ blockade in NOD mice reduces the incidence of diabetes, though the evidence for this is inconsistent.

Lastly, there is a body of diverse evidence that suggests CXCR3 upregulation is important for T cell entry into inflammatory sites, such as the pancreas in the NOD model system [127]. The expression pattern of CXCR3 is not entirely clear, though there are studies that suggest the following mechanism: CXCR3 is initially upregulated after antigen encounter during activation and is expressed on the cell surface before proliferation; after initial expression, interactions with CXCR3 and any of its ligands (CXCL9-11) allow it to traffic to inflammatory sites, though further exposure leads to internalization and degradation of CXCR3; *de novo* CXCR3 synthesis is possible in target tissues, though this process is inhibited by chronic antigen exposure [117,128–130]. In our model system, we observed fewer $CXCR3^+$ cells in OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls. We speculate that in the absence of PD-1 signals, OT-I T cells that have encountered cognate antigen and subsequently upregulated CXCR3 are better able to traffic pancreas than OT-I WT T cells, though expression of CXCR3 is completely downregulated in the pancreas of OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras. This may be due to the prevalence of cognate antigen in the pancreas of diabetic RIP-mOVA mice, preventing the *de novo* expression of CXCR3. Strangely, this expression pattern is different than what is seen in the NOD model system, as $CD8^+$ T cells in the late-insulitic legion express high levels of CXCR3. In this case,

there may be quantitatively less cognate antigen for a particular TCR, given the polyclonal array of T cells and the diversity of autoantigens present in the NOD model, such that *de novo* synthesis of CXCR3 is not blocked. Irrespective of these discrepancies, both CXCR3 and IFNγ have been reported to act in an inflammatory loop where IFNγ is produced in the islet microenvironment which stimulates islets to produce chemokines which ultimately attract $CXCR3^+$ T cells that further perpetuate the cycle. Thereby, it may be prudent to disrupt CXCR3 and/or IFNγ functionality in OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras to investigate their relative importance in facilitating autoimmune diabetes in our model system; the relative importance of altered proliferation, trafficking potential and cytokine production in the absence of PD-1 signals is unclear and warrants further investigation.

Deletional vs. Non-Deletional Tolerance Mechanisms

As shown clearly from our *ex vivo* analysis of OT-I WT/$PD\text{-}1^{-/-}$ > B6/RIP-mOVA chimeras, OT-I WT/$PD\text{-}1^{-/-}$ > RIP-mOVA thymocytes undergo robust clonal deletion, yet the few antigen specific OT-I $PD\text{-}1^{-/-}$ T cells that manage to escape the thymus are able to induce autoimmunity. This data suggests that deletional mechanisms of tolerance, at least in the thymus during development, are not critical for the successful establishment of tolerance to TRA. Conversely, OT-I $Bim^{-/-}$ > RIP-mOVA chimeras do not develop autoimmunity, despite massive emigration of OVA-specific T cells from the thymus given the abrogation of clonal deletion [91]. Our group has also shown that these OT-I $Bim^{-/-}$ T cells that escape clonal deletion have intact trafficking potential and are able to

infiltrate the pancreas and induce peri-insulitis, yet not islet destruction. Taken together, this juxtaposition brings into question the idea the deletional tolerance mechanisms are important for T cell tolerance to TRA, as it suggests that non-deletional tolerance mechanisms are more important. This finding is relatively unique as most published literature on tolerance mechanisms for $CD8^+$ T cells emphasize the importance of deletional tolerance mechanisms, though this may be due to how easily deletional tolerance mechanisms can be examined compared to non-deletional mechanisms.

Methodological Limitations of OT-I WT/PD-$1^{-/-}$ > RIP-mOVA Chimeras

One of the major limitations in using the bone marrow chimera methodology is that there is a relative paucity of peripheral OT-I T cells in both OT-I PD-$1^{-/-}$ > RIP-mOVA and OT-I WT > RIP-mOVA chimeras. This is owing to intact thymic clonal deletion, though peripheral deletion also plays a role; peripheral deletion of adoptively transferred OT-I splenocytes in RIP-mOVA recipients has been well-documented in the literature. Case in point, only ~0.13% of total splenocytes in OT-I WT > RIP-mOVA chimeras are $V\alpha2^+$ $CD8^+$ T cells on average. Furthermore, intracellular stains require treatment with fixation/permeabilization buffers, in which there may be a total loss of up to half of the cells. These factors culminate in a very small number of OT-I WT > RIP-mOVA T cells, which may be the underlying cause of the variability seen in some data from OT-I WT > RIP-mOVA chimeras. Additionally, as there are virtually no OT-I T cells in the pancreas of OT-I WT > RIP-mOVA chimeras, we are unable to include an organ-specific comparison to diabetogenic OT-I PD-$1^{-/-}$ T cells from diabetic RIP-mOVA mice.

Another factor that begets careful consideration is that our RIP-mOVA recipients were not on a $Rag^{-/-}$ background. Functionally, this means two-fold: that there were recipient derived T cells that were $V\alpha2^{+}$ but were not OVA-peptide specific; that were from the donor marrow, there may also be endogenous rearrangement of the TCR, also providing a source of T cells that have dual specificities. Given these factors, it was of utmost importance that we used an appropriate method of detection for OT-I $CD8^{+}$ T cells. We began these experiments by using OVA-specific tetramers to identify the antigen specific T cells. However, we found there were significant problems in the amount of background with the tetramer staining and that the tetramer was incompatible with biotin-streptavidin conjugate antibodies, which were often important in our panels. Instead, we used an antibody for Vα2 expressing T cells in conjunction with CD45 congenics such that we could eliminate recipient derived $Va2^{+}$ $CD8^{+}$ T cells from our analysis, though we could not entirely remove the few OT-I T cells with dual specificities. Despite this potential problem, the replicates obtained with the tetramer and congenic/Vα2 methodologies yielded similar results, giving us strong evidence that we were appropriately identifying OVA-peptide specific T cells regardless of the method used. Furthermore, pancreatic OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA T cells isolated using the congenic/Vα2 method were able to produce IFNγ after restimulation with OVA peptide, which gave us conclusive evidence that those T cells were antigen specific.

Additional Future Directions

We were able to successfully isolate OT-I WT/PD-1$^{-/-}$ > RIP-mOVA T cells and compare functionality *ex vivo*, though assessment of *in vitro* functionality may also produce significant data. Early in this project, we attempted to perform *in vitro* stimulation assays with OT-I WT/PD-1$^{-/-}$ > RIP-mOVA T cells to assess functionality when PD-1 signaling was absent. However, these experiments proved to be fruitless as cells from both OT-I WT/PD-1$^{-/-}$ > RIP-mOVA chimeras died quickly in culture. To get around this issue, we are currently generating apoptosis resistant OT-I PD-1$^{-/-}$ Bim$^{-/-}$ mice such that we investigate the functionality of these cells *in vitro* with OT-I Bim$^{-/-}$ T cells that do signal through PD-1.

Lastly, while ours and others' data suggests that PD-1 is critical for T cell tolerance to TRA, there are many other co-inhibitory molecules that may have similarly powerful effects on T cell tolerance. Given that LAG-3 and BTLA are both expressed on OT-I WT splenocytes after OVA-peptide stimulation, we plan to generate OT-I LAG-3$^{-/-}$ and OT-I BTLA$^{-/-}$ > RIP-mOVA chimeras to examine if a lack of BTLA or LAG-3 signaling would be sufficient to induce autoimmunity.

Final Conclusions

The data presented in this thesis demonstrates the importance of PD-1 signals in T cell tolerance. While we were not able to rescue the functionality of established-tolerant/functionally impaired $V\alpha2^{+}$ $CD8^{+}$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras with PD-1 blockade, we showed that the genetic loss of PD-1 in OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA chimeras induces autoimmunity. These OT-I $PD\text{-}1^{-/-}$ T cells exhibit enhanced proliferation capacity, trafficking potential and cytokine production, which is consistent with data from other groups.

From a therapeutics perspective, PD-1 checkpoint blockade therapy may have the potential to revolutionize cancer treatment: the idea is simple, make slight alterations to your body's own immune system such that it is better equipped to fight the cancer itself. Indeed, the Nobel Prize was recently awarded to two scientists who pioneered the use of CTLA-4 and PD-1 checkpoint blockade for treating certain types of cancer. However, the literature also suggests that this success has not been without some limitations and serious concerns [92]. In sum, a fraction of cancer patients respond well to PD-1 blockade, however the cancer remission is refractory upon cessation of blockade. Furthermore, some patients do not respond at all to PD-1 blockade, even if their tumors express high levels of PD-L1. This difficulty highlights the extreme complexity in cancer pathophysiology and emphasizes the need for improved biomarkers for successful blockade treatment. Some patients who are treated with PD-1 blockade, particularly when treated concurrently with CTLA-4 blockade, develop immune related adverse effects

(IRAE). These patients develop autoimmune disease during the treatment which may be long lasting and not refractory after treatment and/or recovery from cancer.

Globally, ours and other groups' research investigating when PD-1 signals are important for conferring immune tolerance are critical for the improvement of cancer immunotherapies. Based upon our data and others' suggesting that PD-1 is most important in the establishment of T cell tolerance, we could speculate that the thymocytes/RTEs in cancer patients treated with PD-1 blockade that have not undergone PD-1 signaling may be the most directly involved with IRAE development in patients. While it is beyond the scope of experiments we can perform, it may be worthwhile for pharmaceutical companies to develop a mechanism of localized administration of PD-1 blockade such that PD-1 interactions are not interrupted outside of the tumor milieu, limiting IRAE development in patients.

Ultimately, we still do not understand precisely when PD-1 signals are most important for conferring T cell tolerance, though we aim to find out in future experiments. To that end, below is a model depicting our current understanding of when PD-1 signals are important **(Fig. 4-1)**. We hope that by unraveling the intricate details of PD-1 signalling, we will contribute to improved immunotherapeutics for treating cancer and autoimmune disease in the very near future.

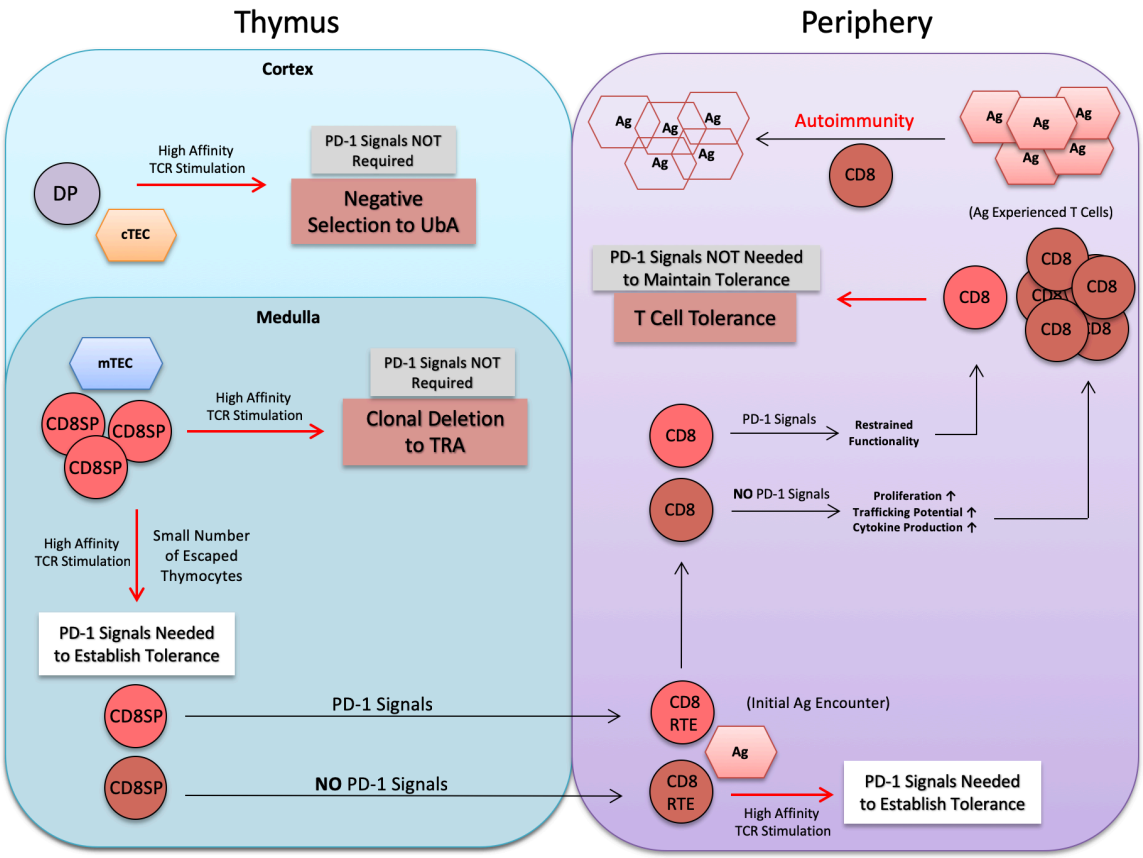


Figure 4-1. **Model for how PD-1 signals are important for the establishment but not maintenance of T cell tolerance**

In the cortex, PD-1 signals have been shown to be unimportant for negative selection to UbA. In the medulla, PD-1 signals are not required for clonal deletion to TRA, however they may be important for establishing tolerance in T cells that have escaped deletion after high affinity TCR stimulation. Though the precise kinetics remain unclear, PD-1 signals on RTEs may also be important for establishing tolerance. Based upon our model system, $CD8^+$ T cells that do not receive PD-1 signals as thymocytes or RTEs display evidence of enhanced functionality (increased proliferation capacity, trafficking potential and cytokine production) which may ultimately lead to autoimmunity. Lastly, PD-1 signals in the periphery are not needed to maintain T cell tolerance; initial tolerizing PD-1 signals seem to have long-lasting effects and be sufficient for long-term tolerance.

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