**Title:** Establishment of CD8+ T cell thymic central tolerance to tissue-restricted antigen requires PD-1

**Running Title:** PD-1 regulates CD8+ T cell thymic central tolerance

**Authors:** Julia F. May*[1], Rees G. Kelly*[1], Alexander Y.W. Suen*, Gina Rayat[$#], Colin C. Anderson[$]*, Troy A. Baldwin*

**Affiliations:**

*Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada.

[$]Department of Surgery, University of Alberta, Edmonton, Alberta, Canada.

[#]Ray Rajotte Surgical-Medical Research Institute, Alberta Diabetes and Transplant Institutes, University of Alberta, Edmonton, Alberta, Canada.

[1] Equal contribution.

**Corresponding Author:**

Troy A. Baldwin

6-25 Heritage Medical Research Centre

University of Alberta

Edmonton, AB T6G 2S2

Canada

Email: tbaldwin@ualberta.ca

Phone: 780-492-7553

Fax: 780-492-7521

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## Abstract

Highly self-reactive T cells are censored from the repertoire by both central and peripheral tolerance mechanisms upon receipt of high-affinity TCR signals. Clonal deletion is considered a major driver of central tolerance; however, other mechanisms such as induction of regulatory T cells and functional impairment have been described. An understanding of the interplay between these different central tolerance mechanisms is still lacking. We previously showed that impaired clonal deletion to a model tissue-restricted antigen (TRA) did not compromise tolerance. In this study, we determined that T cells that failed clonal deletion in this model were rendered functionally impaired in the thymus. PD-1 was induced in the thymus and established cell-intrinsic tolerance to TRA in $CD8^+$ thymocytes independently of clonal deletion. PD-1 signaling in developing thymocytes was sufficient to induce tolerance but was dispensable for the initial maintenance of tolerance in the periphery. We showed that chronic exposure to high affinity antigen supported the long-term maintenance of tolerance in this model. Taken together, our study identifies a role for PD-1 in establishing central tolerance in autoreactive T cells that escape clonal deletion and sheds light on potential mechanisms of action of anti-PD-1 pathway immune checkpoint blockade and the development of immune-related adverse events.

## Significance Statement

The establishment of T cell tolerance is critical to prevent autoimmune diseases. Apoptosis of highly self-reactive thymocytes is an important mechanism that enforces central tolerance. However, not all self-reactive thymocytes undergo apoptosis during development, and the fate of cells that evade this process is under-examined. Using bone marrow chimera, adoptive transfer, and thymic transplant experiments, we found acute PD-1 signaling is required to establish tolerance to tissue-restricted antigen (TRA) independently of clonal deletion. This tolerance is maintained by chronic exposure to tolerizing antigen but persists in the absence of PD-1 until late time points. Overall, this study identifies a role for PD-1 in establishing central tolerance, and it provides insight into the mechanism of PD-1 pathway-targeting cancer immunotherapies.

## Introduction

The outcomes of thymocyte selection shape the peripheral T cell repertoire. The affinity of the TCR for self-peptide-MHC (self-pMHC) plays a dominant role in regulating these selection outcomes, but signals derived from other receptors are also important. T cell central tolerance mechanisms include processes such as clonal deletion, anergy, and agonist selection(1). However, much is left to understand regarding the limits and interplay between these processes.

Nascent αβ TCR-committed thymocytes transition from the $CD4^-CD8^-$ double-negative (DN) stage to the $CD4^+CD8^+$ double-positive (DP) stage and acquire the ability to respond to self-pMHC by virtue of expressing a TCR. While in the thymic cortex, low affinity interactions between the αβ TCR and self-pMHC expressed by cortical thymic epithelial cells result in positive selection and further transition into $CD4^-CD8^+$ or $CD4^+CD8^-$ single-positive (SP) thymocytes (2). Conversely, strong TCR signals at the DP or SP thymocyte stage during thymic development result in several negative selection outcomes, through which autoreactive thymocytes are censored from the repertoire. Peptides from both ubiquitously expressed proteins (UbA) as well as proteins that are expressed in a tissue-restricted fashion (TRA) can induce negative selection.

Although clonal deletion, wherein autoreactive thymocytes are instructed to undergo apoptosis, has long been considered the primary central tolerance mechanism, the fate of autoreactive thymocytes that escape clonal deletion is not yet fully understood (3). Studies by Ramsdell and Fowlkes used a superantigen model of negative selection to determine that a population of superantigen-reactive CD4SP and CD8SP could escape clonal deletion but were unresponsive to superantigen stimulation (4). Similarly using TCR transgenic models, we and others recently showed that TRA-specific CD8SP and CD4SP thymocytes that encountered their high affinity antigen during development, but were unable to undergo apoptosis, continued maturation and emigrated into the periphery (5, 6) but showed functional impairment (7). More recent work using polyclonal models demonstrated that CD4SP thymocytes that encountered their high affinity antigen in the cortex were mostly deleted, but those that received a high affinity signal in the medulla were largely selected into the Treg lineage rather than being deleted or were functionally impaired (8, 9). Collectively, these studies highlight the complex interplay between different central tolerance mechanisms.

Programmed cell death protein 1 (PD-1) is a co-inhibitory receptor expressed on T cells, B cells, and some subsets from the myeloid lineage (10). PD-1 ligation impairs T cell responses and thus PD-1 blockade forms the basis for some extremely promising cancer immunotherapies by improving the function of anti-tumour $CD8^+$ T cells rendered dysfunctional in the tumour microenvironment, reviewed in (11). PD-1 is thought to function through its recruitment of the phosphatase SHP2 which negatively

regulates the activation of TCR-proximal signaling molecules (12, 13) and CD28-driven co-stimulation (14, 15). On lymphocytes at steady state, PD-1 expression is limited to a small fraction of thymic and peripheral cells, but is induced on thymocytes and peripheral T cells following strong TCR interactions (16, 17). The ligands for PD-1, PD-L1 and PD-L are expressed on the thymic epithelium and professional antigen presenting cells in the thymus (18, 19). PD-1 deficiency results in autoimmunity in pre-clinical models (20) and single nucleotide polymorphisms in PD-1 are linked to autoimmunity in humans (21–23). In the thymus, transgenic overexpression of PD-1 has been shown to modulate positive selection (24, 25), while PD-1 deficiency in UbA-mediated models of negative selection showed little or no impact (26, 27). Studies examining a role for PD-1 in thymic tolerance induced by TRA are currently lacking.

In this study we explored the fate of MHCI-restricted thymocytes specific for a TRA that were unable to undergo clonal deletion. We found that PD-1 was critical to establish cell-intrinsic tolerance in the thymus but only appeared to support the maintenance of tolerance at later time points, which contrasts with the current paradigm for PD-1-targeting blockade immunotherapies. Additionally, chronic exposure to high affinity antigen supports the long-term maintenance of tolerance in this model. Taken together, our study identifies a role for PD-1 in establishing central tolerance in TRA-specific thymocytes that have escaped clonal deletion. Furthermore, it supports a model where cancer immunotherapies that target the PD-1 pathway do not ‘rescue’ already dysfunctional anti-tumour T cells, but rather act to prevent anti-tumour T cells from becoming dysfunctional.

## Results

*Cell-intrinsic functional impairment is induced in antigen-specific CD8$^+$ T cells in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras*

We and others previously showed that Bim is required for thymic clonal deletion to tissue-restricted antigen (TRA) using the OT-I TCR transgenic system (5–7, 28). OT-I Bim$^{-/-}$ T cells that escaped thymic clonal deletion in RIP-mOVA (29) recipients did not go on to cause autoimmunity due to functional impairments characterized by defects in activation, proliferation, and cytokine production. The precise mechanism(s) behind this impairment was unclear but may be due to cell-extrinsic suppression or cell-intrinsic anergy (30–32). We hypothesized that if a population of dominant suppressors existed in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras, they should suppress the activation and proliferation of wildtype OT-I T cells. CFSE labelled splenocytes from CD45.1$^+$ OT-I mice were mixed at a 1:1 ratio with splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras and stimulated with OVA peptide-pulsed splenocytes *in vitro*. General gating strategies are outlined in figure S1. As we previously found, on day 2 post-stimulation, the induction of activation markers CD69 and CD25 on OT-I splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras was diminished compared to that of splenocytes from wildtype OT-I CD45.1$^+$ mice. Activation marker induction on splenocytes from OT-I CD45.1$^+$ mice was not significantly lower in mixed cell culture compared to unmixed OT-I splenocytes (Fig. 1A and B). Similarly, on day 2 (Fig. S2A) and day 3, while OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras showed impaired proliferative responses, the presence of OT-I Bim$^{-/-}$ > RIP-mOVA splenocytes did not significantly impact the proliferation of wildtype OT-I splenocytes, as measured by the division index (DI) and the proliferation index (PI) (Fig. 1C-E).

To assay functional impairment *in vivo*, we co-adoptively transferred either whole spleen or enriched CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras with splenocytes from CD45.1$^+$ OT-I WT mice (1:1 ratio) into sub-lethally irradiated RIP-mOVA recipients. Neither whole spleen nor enriched CD8$^+$ OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras were diabetogenic, suggesting that a dominant suppressor population of non-T cell origin is not responsible for functional impairment in these chimeras (Fig. 1F). Enriched CD8$^+$ OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras did not have a dominant suppressive effect on the diabetogenicity or expansion of wildtype OT-I T cells following 1:1 co-adoptive transfer into sub-lethally irradiated RIP-mOVA recipients (Fig. 1F-G). Functional impairment of OT-I Bim$^{-/-}$ T cells from RIP-mOVA chimeras was also demonstrated by a reduced capacity for lymphopenia-induced proliferation compared to control cells from OT-I Bim$^{-/-}$ > WT chimeras (Fig. S2B). These data suggest that a population of Ag-specific suppressors are not

generated in OT-I Bim$^{-/-}$ > RIP-mOVA mice and that the functional impairment of OT-I Bim$^{-/-}$ T cells resulting from cognate antigen encounter in RIP-mOVA recipients is cell-intrinsic.

*TRA-specific T Cells in OT-I Bim$^{-/-}$ >RIP-mOVA chimeras are rendered functionally impaired in the thymus.*

After determining that the functional impairment of OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras was cell-intrinsic, we investigated whether this functional impairment was established during development in the thymus or in the periphery. The thymus is the initial site of high affinity antigen encounter and induction of non-responsiveness in thymocytes has been reported previously (33). To examine this question, we stimulated CFSE labelled thymocytes from OT-I Bim$^{-/-}$ > WT and OT-I Bim$^{-/-}$ > RIP-mOVA chimeras with peptide pulsed splenocytes as stimulators and assessed OT-I thymocyte activation and proliferation. On day 2, expression of activation markers CD69 and CD25 was impaired in OT-I Bim$^{-/-}$ > RIP-mOVA thymocytes compared to OT-I Bim$^{-/-}$ > WT controls (Fig. 2B). Additionally, we assessed proliferation on day 3 and found that OT-I Bim$^{-/-}$ > RIP-mOVA thymocytes exhibited a defect in proliferation compared to OT-I Bim$^{-/-}$ > WT thymocytes (Fig. 2C-E).

To determine if the observed *in vitro* defects in activation and proliferation translated into functional *in vivo* impairment, we adoptively transferred either thymocytes or splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA or OT-I Bim$^{-/-}$ > WT chimeras into sub-lethally irradiated RIP-mOVA recipients to assess diabetogenicity. Most recipients of thymocytes and all recipients of splenocytes from OT-I Bim$^{-/-}$ > WT chimeras resulted in diabetes as expected. In contrast, none of the sub-lethally irradiated RIP-mOVA recipients that received thymocytes or splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras became diabetic (Fig. 2F). Collectively, these data suggest that functional impairment of OT-I Bim$^{-/-}$ > RIP-mOVA T cells is established in the thymus.

*PD-1 signals are required for T cell tolerance in OT-I > RIP-mOVA chimeras.*

The functional impairment seen in OT-I Bim$^{-/-}$ >RIP-mOVA thymocytes and splenocytes may be driven by co-inhibitory molecules and/or other factors that inhibit T cell activity. As such, we investigated whether various known co-inhibitors were differentially expressed in OT-I > RIP-mOVA thymocytes and splenocytes that escaped clonal deletion compared to OT-I > WT controls. We found that PD-1 was more highly expressed on both OT-I thymocytes and OT-I T cells from peripheral immune organs in OT-I > RIP-mOVA (Fig. 3A) and OT-I Bim$^{-/-}$ > RIP-mOVA (Fig. S4A) organs compared to OT-I cells from WT recipients. Previous literature supports a role for PD-1 in enforcing

tolerance (34). Given this increased PD-1 expression, we hypothesized that PD-1 expression was playing an active role in establishing and/or maintaining the functional impairment seen in TRA-specific T cells that escaped clonal deletion in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras.

We intercrossed OT-I and PD-1$^{-/-}$ mice to generate OT-I PD-1$^{-/-}$ mice to determine if PD-1 regulated tolerance in OT-I > RIP-mOVA chimeras. Bone marrow chimeras using OT-I PD-1$^{-/-}$ BM and either WT or RIP-mOVA recipients were generated. 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. 3B). Next, we investigated if autoimmunity in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras was dependent on all donor CD8$^{+}$ T cells being PD-1$^{-/-}$. 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/OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras developed diabetes (Fig. 3B). These data suggested that only a fraction of the overall pool of OT-I T cells needed to be PD-1$^{-/-}$ to induce autoimmunity. This finding gives additional support against the presence of dominant suppressors precluding diabetes in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras. To ensure that the absence of Bim itself was not preventing autoimmunity, we generated OT-I PD-1$^{-/-}$ Bim$^{-/-}$ > RIP-mOVA chimeras and again observed a 100% incidence of diabetes, suggesting that PD-1 signals with or without the presence of Bim are necessary for tolerance. To further solidify that the deletion of PD-1 specifically impairs tolerance in this context, we investigated whether deletion of another co-inhibitory receptor impaired tolerance. B and T lymphocyte attenuator (BTLA) is a co-inhibitory receptor expressed on thymocytes following positive selection (35), and has been thought to play a similar role to PD-1 (36, 37). To test whether the loss of BTLA impaired tolerance, we generated OT-I BTLA$^{-/-}$ > WT or RIP-mOVA chimeras. None of the OT-I BTLA$^{-/-}$ > RIP-mOVA chimeras developed diabetes, suggesting that elimination of any co-inhibitory receptor does not impair tolerance (Fig. S3).

Finally, to determine whether PD-1 signaling specifically within the T cell compartment was necessary to induce tolerance, we conditionally deleted PD-1 in CD8$^{+}$ T cells using *pdcd1* floxed mice. CD8-Cre mice were intercrossed with OT-I, constitutive PD-1$^{-/-}$, and *pdcd1* floxed mice to generate OT-I CD8-Cre PD-1$^{fl/ko}$ mice. In OT-I CD8-Cre PD-1$^{fl/ko}$ > RIP-mOVA chimeras, the loss of PD-1 became evident at the SM-M1 CD8SP thymocyte stages (Fig. S4B). Additionally, OT-I CD8-Cre PD-1$^{fl/ko}$ > RIP-mOVA chimeras experienced rapid onset of diabetes (Fig. 3C), suggesting that PD-1 signaling specifically within the CD8$^{+}$ T cell compartment is required to establish tolerance in this model. Here and in subsequent diabetes analyses, we euthanized mice from some groups early for the purpose of *ex vivo* analysis (indicated by tick marks). Thus, mice from some groups were not followed to the end of the study, and therefore in some cases our reported incidence of diabetes may be underestimates. The

Kaplan-Meier graphs depicting the percentage of mice remaining diabetes free is accurate as the data points censored early are not included in this calculation.

*OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras exhibit a mild impairment in clonal deletion and increased numbers of OT-I CD8$^{+}$ T Cells in the periphery.*

Because 100% of the OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras developed autoimmune diabetes, we next investigated the mechanism by which PD-1 signals contribute to tolerance to TRA. We first determined if PD-1 modulated clonal deletion of OT-I thymocytes after high-affinity antigen encounter in the thymus of RIP-mOVA recipients. We examined the CD4 by CD8 profile of thymocytes from OT-I WT > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras and observed a modest reduction in the frequency of CD8SP thymocytes and a similar CD8 dulling in this compartment compared to WT recipient controls (Fig. 4A, Top). Furthermore, we observed a significant reduction in the frequency and total number of mature (V$\alpha$2$^{+}$ CD24$^{lo}$) CD8SP thymocytes in both OT-I WT and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to WT recipient controls, indicating intact clonal deletion in the absence of PD-1 (Fig. 4A, bottom; B). However, we did observe a small increase in the number of mature CD8SP thymocytes from OT-I PD-1$^{-/-}$ >RIP-mOVA chimeras compared to OT-I > RIP-mOVA controls, but this was not statistically significant (Fig. 4B). Overall, these data suggest that while the vast majority of OT-I thymocytes encountered cognate antigen and underwent clonal deletion in OT-I PD-1$^{-/-}$ > RIP-mOVA recipients, the remaining OT-I T cells in these chimeras still went on to induce autoimmune diabetes.

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. In PD-1-sufficient animals, the number of V$\alpha$2$^{+}$ CD8$^{+}$ T cells in the spleen and pancreatic lymph nodes demonstrated a significant overall reduction in RIP-mOVA chimeras compared to WT recipient controls, again suggesting intact clonal deletion. Like what was observed in the thymus, there was an increase in the number of OT-I T cells in the spleen and pancreatic LNs from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls, but this was not statistically significant (Fig. 4C, D). However, OT-I T cell numbers were not restored to levels in WT recipients. Overall, in this model, clonal deletion appears largely intact in the absence of PD-1. Thus, we conclude PD-1 contributes to tolerance in this context by a mechanism distinct from thymic clonal deletion.

*Interruption of PD-1:PD-L1 interactions does not reverse established tolerance in peripheral or thymic OT-I T cells.*

We next investigated the specific requirements for the PD-1-driven tolerance induced in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras. Given that OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras expressed PD-1 and are ostensibly impaired in a cell-intrinsic manner, we asked whether PD-1-dependent tolerance required continued PD-1 signaling or was stable even when PD-1 signaling was removed. To address this question, we stimulated splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras with OVA-peptide pulsed splenocytes *in vitro* in the absence or presence of well-characterized blocking anti-PD-1 monoclonal antibodies. On day 2, we observed that PD-1 blockade did not result in an increase in CD69 and CD25 expression on OT-I Bim$^{-/-}$ splenocytes from RIP-mOVA recipients (Fig. 5A). Additionally, both the division index and proliferation index of V$\alpha$2$^{+}$ CD8$^{+}$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras was not altered by PD-1 blockade on day 3 (Fig. 5B). PD-1 blockade also did not restore activation of OT-I Bim$^{-/-}$ thymocytes from RIP-mOVA recipients (Fig. 5C). Similarly, *in vivo* administration of anti-PD-1 blocking antibodies to OT-I > RIP-mOVA or OT-I Bim$^{-/-}$ > RIP-mOVA bone marrow chimeras failed to break tolerance, suggesting tolerance was stable even when PD-1 signaling was presumably interrupted (Fig. 5D).

Given some potential limitations with the use of antibody-mediated blockade, we moved to an adoptive transfer approach with PD-L1$^{-/-}$ recipients. Since PD-L2 is another ligand for PD-1, we first determined if PD-L2 could compensate for the loss of PD-L1 and enforce tolerance in PD-L1$^{-/-}$ recipients. We generated OT-I Bim$^{-/-}$ > RIP-mOVA PD-L1$^{-/-}$ bone marrow chimeras and found these chimeras rapidly became diabetic, while those heterozygous for PD-L1 did not, suggesting PD-L2 could not compensate for the loss of PD-L1 in inducing or maintaining tolerance in this setting (Fig. S5). We then adoptively transferred splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras into sub-lethally irradiated RIP-mOVA PD-L1$^{-/-}$ recipients to cease PD-1 signaling (detailed in Fig. 5E). In contrast to non-tolerant controls, not all RIP-mOVA PD-L1$^{-/-}$ recipients that received OT-I Bim$^{-/-}$ > RIP-mOVA splenocytes became diabetic, and in the recipients in which diabetes was observed, disease was late-onset (Fig. 5F). 4 of the 12 recipients for this group were removed from the study at 8 weeks (indicated by tick-marks); while they may have become diabetic at later time points, they nevertheless remained healthy past the time where all positive control mice became diabetic. This suggests that a continuous PD-1 signal is not required for the maintenance of tolerance, but in the absence of PD-1 signals, tolerance wanes over extended periods of time.

Since functional impairment in our chimeras is observable in thymocytes, we hypothesized that PD-1 signaling within the thymic compartment may be sufficient to establish tolerance in this model. To address our hypothesis, we collected thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras and

transferred them into sub-lethally irradiated RIP-mOVA PD-L1$^{-/-}$ recipients. In this experiment, the adoptively transferred thymocytes only experienced PD-1 signaling during thymic development. Like the splenocyte adoptive transfer, adoptively transferred thymocytes remained tolerant for an extended period of time in contrast to non-tolerant controls, with tolerance waning at late time points in some cases (Fig. 5G). These data indicate that acute PD-1 signaling in the thymus is critically important to the establishment of tolerance, but continued PD-1 signals only support the maintenance of tolerance over the long-term.

*Continuous exposure to antigen in the periphery is required to maintain functional impairments in OT-I Bim$^{-/-}$ >RIP-mOVA chimeras*

With our finding that acute PD-1 signaling in the thymus was sufficient to establish tolerance, but dispensable for the initial maintenance of tolerance in the periphery, we wondered whether continued exposure to high affinity antigen in the periphery was required to maintain tolerance. To tackle this question, we transplanted either a WT or RIP-mOVA neonatal thymus under the kidney capsule of nude (athymic) recipients and at least 8 weeks later lethally-irradiated the thymic transplant recipients and transplanted OT-I Bim$^{-/-}$ donor bone marrow. In recipients of a RIP-mOVA thymus, exposure to high affinity OVA is limited to the thymus and withdrawn from OT-I CD8$^{+}$ cells upon emigration from the thymus (Fig. 6A).

We harvested the transplanted thymus from both groups and examined the CD4/CD8 profile of OT-I thymocytes. We observed similar frequencies of CD8SP thymocytes and CD8 dulling between the thymus transplant recipients and the corresponding chimeric controls (Fig. 6B). Stimulation of thymocytes from the thymic transplant bone marrow chimeras and bone marrow chimera groups also demonstrated equivalent functional impairments as assessed by CD69 induction on day 2 post-activation (Fig. 6C). Splenocytes from these chimeras were stimulated *in vitro* with OVA peptide-pulsed stimulators. As expected, activation marker expression (CD69), proliferation, and cytokine production (TNFα and IFNγ) were diminished *in vitro* in OT-I Bim$^{-/-}$ > RIP-mOVA splenocytes compared to the corresponding WT control (Fig. 6D-G). However, in OT-I Bim$^{-/-}$ > Nude (RT) chimeras, proliferation and cytokine production were not significantly different from the OT-I Bim$^{-/-}$ > Nude (WT) control, suggesting restored function in OT-I T cells when exposure to high affinity antigen is withdrawn after thymic egress (Fig. 6D-G). Overall, it appears that, in contrast to PD-1 signaling, persistent exposure to high affinity antigen is required to maintain the functional impairments we see in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras.

## Discussion

Clonal deletion of strongly self-reactive thymocytes is a well-described mechanism of T cell central tolerance, however, potent non-deletional mechanisms also exist. Previously, we and others showed that while Bim is required for clonal deletion to TRA in OT-I Bim$^{-/-}$ > RIP-mOVA bone marrow chimeras (5), it is dispensable for tolerance (6, 7). In the present study, we demonstrated that TRA-specific T cells that escape clonal deletion in these chimeras are rendered functionally impaired in a cell-intrinsic manner during antigen encounter in the thymus. PD-1 expression during negative selection in the thymus was required to establish tolerance, but continued PD-1 signaling in the periphery only contributed to the maintenance of this tolerance at much later time points. Additionally, we found antigen persistence in the periphery was needed to maintain a state of tolerance, as has previously been reported (38–40). Collectively, these data highlight the critical window during which PD-1 signaling results in the establishment of tolerance and shed light on potential mechanisms by which PD-1 blockade functions in the context of cancer immunotherapy and development of immune-related adverse events.

While the removal of autoreactive T cell clones via thymic clonal deletion has long been considered a primary mechanism for establishing tolerance in the T cell compartment, conventional T cells that recognize self-peptides with high affinity can be readily found in healthy individuals (41). Like in the case of regulatory T cells, some of these T cells are actively selected for, while in other cases these T cells may be products of failed clonal deletion. By deleting Bim and blocking clonal deletion, a large population of highly self-reactive T cells are released into the periphery, yet tolerance is maintained (6, 7). Similarly, recent studies in polyclonal mice have revealed limited deletion of TRA-specific T cells and instead, tolerance was enforced by Treg-mediated suppression (8), or T cell-intrinsic functional impairment (9, 42). We find that functional impairment is observed both in thymocytes and peripheral T cells suggesting that tolerance is first established in the thymus. These data are consistent with the recent findings of Malhotra *et al.* where MHCII-restricted thymocytes specific for a TRA showed reduced responsiveness (9). Additionally, in an allo-reactive MHCI-restricted TCR transgenic model, thymocytes were rendered non-responsive (33) and similar findings were reported in a super-antigen mediated model of tolerance (4).

Given our finding of reduced responsiveness in thymocytes and T cells that escaped clonal deletion, we assessed whether the dysfunction was cell-intrinsic or cell-extrinsic in nature. In previous

studies where thymocytes were found to be functionally impaired, it was not determined if the impairment was due to cell-extrinsic dominant suppression or through cell-intrinsic mechanisms. In addition to $CD4^+$ $Foxp3^+$ Treg, there is mounting evidence supporting the existence of a $CD8^+$ T regulatory subset, but the precise signals controlling differentiation into this fate are not well understood (43). Using a classical in vitro suppression assay with cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras as a source of potential suppressors, we did not observe a reduction in the activation of wildtype OT-I T cells. Additionally, in both of our co-adoptive transfer experiments, neither the total splenocyte nor the $CD8^+$ T cell compartments appeared to contain dominant suppressors. These findings indicate that the functional impairment observed when clonal deletion is prevented is cell-intrinsic in nature. Furthermore, it would suggest that simple impairment of clonal deletion does not lead to the generation of $CD8^+$ T regulatory cells and that other factors are involved. However, it remains possible that the mechanisms of tolerance vary with the antigen, quantity of antigen, and APC in question, as has been previously suggested (8, 9).

Since we demonstrated a cell-intrinsic functional impairment induced during thymocyte development, we were interested in determining which proteins were responsible for this outcome. It is known that the co-inhibitory receptor PD-1 plays a role in regulating T cell function and is induced in DP thymocytes following strong TCR signaling. In the 2C TCR transgenic model of negative selection, PD-1 deficiency resulted in enhanced generation of DN thymocytes (26) while no such effect was observed in MHCI- or MHCII-restricted male antigen reactive TCR transgenic models (27). We found that PD-1 was similarly induced on OT-I CD8SP thymocytes that survived deletion in RIP-mOVA recipients, and that PD-1 expression was required for tolerance to TRA. Deletion of PD-1 did not dramatically affect the number of OT-I CD8SP thymocytes in RIP-mOVA chimeras, although there were increased numbers of OT-I T cells in the spleen and pancreatic lymph nodes of these mice, as was similarly observed for MOG-specific $CD4^+$ T cells on a polyclonal background (44). We think it is unlikely that tolerance was broken due to the elevated numbers of Ag-specific cells, but rather that PD-1 signaling induced a tolerogenic program in the OT-I T cells since OT-I $Bim^{-/-}$ > RIP-mOVA chimeras contain substantially more OT-I T cells than OT-I $PD\text{-}1^{-/-}$ > RIP-mOVA counterparts and tolerance was still enforced in OT-I $Bim^{-/-}$ T cells (6, 7).

PD-1 has primarily been investigated for its role in peripheral and overall maintenance of tolerance by limiting reactivation, function, and trafficking in self-reactive T cells (45), but a role for PD-1 in establishing tolerance in the thymus has not be reported. Through an adoptive transfer approach using thymocytes from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras as a source of auto-reactive T cells and sub-

lethally irradiated PD-L1$^{-/-}$ RIP-mOVA recipients, we found that acute PD-1 signaling during development was sufficient to establish stable tolerance. Furthermore, PD-1 blockade in tolerant OT-I Bim$^{-/-}$ > RIP-mOVA chimeras was unable to break tolerance. These findings are supported by studies where PD-1-deficient polyclonal thymocytes or recent thymic emigrants, but not established peripheral T cells precipitated a lethal, multi-organ autoimmunity when adoptively transferred into Rag$^{-/-}$ recipients (27). It was also recently shown that PD-1 could delay lethal autoimmunity in *Aire*$^{-/-}$ mice (46); in the absence of AIRE in this model, many TRA would not be expressed in the thymus and instead would be first encountered in the periphery, where PD-1 signals could provide protection against autoimmunity. Finally, intestinal inflammation and mortality driven by naïve OT-I T cells in iFABP-OVA mice was shown to be restricted by PD-L1 blockade only when the treatment was administered the day of adoptive transfer, but not 30 days later (47). Given observations made by us and others, we thus hypothesize that it is upon primary high affinity antigen exposure that PD-1 signals are required to induce a durable tolerant state that resists reinvigoration. This is in contrast to previous studies (48–51) proposing only a minor role for PD-1 in early tolerance induction (see review (52)). However, many of these studies were performed on the NOD background, wherein native tolerance is not established (53).

While acute PD-1 signals received by OT-I T cells in the thymus provided a relatively durable tolerance when OT-I thymocytes were adoptively transferred into sub-lethally irradiated RIP-mOVA PD-L1$^{-/-}$ recipients, we cannot formally discount a role for PD-L2. However, it appears that PD-L2 does not exert a large effect on tolerance in this model. In our studies with OT-I Bim$^{-/-}$ > RIP-mOVA PD-L1$^{-/-}$ chimeras, tolerance is not established, and diabetes is induced rapidly even with unaltered PD-L2 expression. Similarly, the Sharpe group previously demonstrated in the NOD background a unique role for PD-L1 in restraining self-reactive T cells in the pancreas (54, 55). Additionally, thymocytes or splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA mice adoptively transferred into RIP-mOVA PD-L1$^{-/-}$ recipients did not maintain tolerance indefinitely. Since in PD-L1 sufficient recipients these adoptively transferred thymocytes remained tolerant throughout the entire course of the experiment, PD-1 signaling induced via interactions with PD-L1 likely aided in the maintenance of tolerance. It will be interesting to assess the necessity for thymic PD-1 signaling by limiting PD-1 interactions to the periphery (e.g adoptive transfer of thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA PD-L1$^{-/-}$ chimeras into PD-L1$^{+/+}$ recipients), in which case failure to establish tolerance would further suggest it is PD-1 signals received specifically during first encounter with Ag in the thymus that are necessary for establishing tolerance. Indeed, recent thymic emigrants are thought uniquely sensitive to tolerizing factors compared to more

mature circulating T cells, suggesting that PD-1 signals delivered in the thymus and periphery may lead to functionally different outcomes (56).

Here we have demonstrated that tolerance induced by acute PD-1 signaling during thymocyte development results in a relatively long-lasting tolerance in the absence of continued peripheral PD-1 signaling. Work done by the Fife group showed that established anergic peripheral T cells are not reinvigorated by PD-L1 blockade (47). They and others postulated that this is due to an epigenetic landscape resistant to reinvigoration in the context of "exhaustion" and tolerance (57–60). Given this, we hypothesize that acute PD-1 signaling supports this long-term tolerance by inducing a tolerant epigenetic memory. Using a double transgenic $TCR_{GAG}$ Alb:GAG model, the Greenberg group recently showed that self-reactive T cells that escape thymic deletion do not cause liver injury or respond to immunization with LM-GAG. These cells regained function through robust lymphopenia-induced proliferation (LIP) and responded to LM-GAG immunization at early time points, but 3-4 months post-adoptive transfer, functional impairment was reacquired. Their observations were similarly recorded following transfer into both high affinity Ag-bearing (Alb:GAG) and Ag-lacking (C57BL/6) recipients (57). Conversely, we failed to observe LIP by the functionally impaired $CD8^+$ T cells from OT-I $Bim^{-/-}$ > RIP-mOVA chimeras and instead observed a dependence on persistent high affinity antigen for this functional impairment. This difference may be because OT-I thymocytes developing in a RIP-mOVA thymus normally undergo efficient thymic deletion, while in the $TCR_{GAG}$ Alb:GAG model, deletion is only partial. Further, it is unclear whether the functional impairment observed in $TCR_{GAG}$ T cells was established in the thymus, as was the case for OT-I T cells; therefore, the states of dysfunction our groups documented may not be analogous (57). The tolerance-specific epigenetic program they and others propose drives tolerance in self-reactive T cells (57, 58) may also underlie our observations. This phenomenon is a clear obstacle for immunotherapies seeking to reinvigorate functionally impaired T cells, thus future investigation should aim to determine the mechanism by which this occurs and its relationship with PD-1.

Immune checkpoint blockade (ICB) therapies targeting the PD-1:PD-L1 signaling axis have received considerable attention in the last 10 years for their effectiveness in treating a number of different cancers. A recent study proposed that ICB targeting the PD-1:PD-L1 signaling axis promoted the activation and expansion of newly recruited clones to an anti-tumour response, rather than reinvigorating pre-existing tumour-infiltrating T lymphocytes as is often suggested to underlie the success of PD-1 blockade (61). In this study, we provide evidence to support this alternate mechanism of

anti-PD-1 ICB therapies. If PD-1 is indeed critical for the establishment of tolerance rather than its maintenance as we suggest, it follows that PD-1-targeting ICB would enhance recruitment of clones naïve to the PD-1 tolerogenic signal. Targets of these therapies may originate in the periphery as tumour-specific naïve T cells whose primary encounter with antigen is no longer rendered tolerogenic by PD-1, or in the thymus as tumour-harboured self-antigen-specific thymocytes whose native tolerance through PD-1 is prevented.

Despite the success of ICB therapies, clinical responses vary with different types of cancer, and immune-related adverse events (IRAE) are a persistent risk (11, 62). It is conceivable that should PD-1 blocking antibodies reach the thymus, normal selection processes may be hindered. While this provides a potential explanation for the development of some IRAEs, it may also be in some cases the mechanism behind successful cancer immunotherapies. This highlights the need for a greater understanding of the mechanism by which PD-1 supports normal tolerance and how this relates to ICB therapies. Overall, we provide evidence for a novel role for PD-1 in the establishment of tolerance to TRA in the thymus in escapees from clonal deletion. Depending on the conditions of antigen presentation (antigen density, presenting cells, location of first encounter), PD-1 signals may induce states of tolerance or simply aid in its maintenance, potentially with different functional outcomes.

## Methods

*Mice*

C57BL/6j (WT, strain 000664), C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA, strain 005431), NOD.B6-*Cd274*$^{tm1Shr}$/J (PD-L1$^{-/-}$, strain 018307), C57BL/6-Tg(Cd8a-cre)1Itan/J (CD8-cre, strain 008766), B6.Cg-*Foxn1*$^{nu}$/J (Nude, strain 000819) mice were sourced from Jackson Laboratory. C57BL/6-*Pdcd1*$^{tm1.1Mrl}$ (PD-1$^{fl/fl}$, strain 13976) were purchased from Taconic Biosciences. The PD-L1$^{-/-}$ mice were backcrossed to C57BL/6j mice at least 5 generations prior to intercrossing with RIP-mOVA mice. OT-I Bim$^{-/-}$ mice were provided by Dr. Maureen McGargill (St. Jude Children's Research Hospital, Memphis, TN, USA). PD-1$^{-/-}$ mice were provided by Dr. Tasuku Honjo (Kyoto University, Kyoto, Japan) and BTLA$^{-/-}$ were obtained from Dr. Kenneth Murphy (Washington University, St. Louis, MO). Mice with compound genotypes were intercrossed in-house. All mice were maintained via protocols approved by the University of Alberta Animal Care and Use Committee.

*Bone marrow chimeras*

T cells were depleted from donor bone marrow by the I.P. administration of 100 µg anti-Thy1.2 (clone 30H12) -2 and -1 d prior to harvest. 1 d prior to transplant, recipients were irradiated by 2 doses of 450 rad, separated by a 4 hour rest period. Bone marrow was flushed from the femurs and tibias of donor mice with PBS (2 % FBS, 2 mM EDTA) and filtered through a 70 µm nylon strainer. $5x10^6$ – $10x10^6$ HSCs were transplanted to recipients via tail vein injection. On day 1 post-transplant, 100 µg of anti-Thy1.2 antibody (clone 30H12) was administered I.P. to the recipients. For the first 4 wk post-transplant, recipient mice were given water containing Novo-Trimel (via University of Alberta Health Sciences Lab Animal Services). Chimeras were allowed to recover for at least 8 wk before further manipulation or analysis.

*Tissue collection*

Mice were anaesthetized with isoflurane and euthanized via $CO_2$ asphyxiation. Organs were harvested and made into single-cell suspensions by grinding through either sterile wire or nylon mesh screens in petri dishes containing RP10 media (RPMI 1640, 2.05 mM L-glutamine [Hyclone], 10 % FBS, 5 mM HEPES, 50 mM 2-mercaptoethanol, 50 µg/mL penicillin/streptomycin, 50 µg/mL gentamycin sulfate). Cells were treated with ammonium-chloride-potassium lysis buffer to lyse RBCs. Viable cell numbers were determined using Trypan Blue exclusion dye and a hemocytometer (Fisher Scientific) under light microscopy (Zeiss).

*Cell culture and in vitro stimulation assays*

Cells were cultured at $2.5x10^6$ cells/well in 48-well plates in RP10 media at 37˚C in a tissue culture incubator. Whole spleen cell stimulators from C57BL/6 mice were resuspended in RP10 media at $20x10^6$ cells/mL pulsed with or without 100 nM OVA peptide sourced from AnaSpec (SIINFEKL, AS-60193-1) for 1 h at 37˚C with gentle shaking every 15-20 minutes. Stimulator cells were washed three times before culture. Responder cells were either resuspended in PBS at $10x10^6$ cells/mL and stained with 1.25 µM CFSE or at $5x10^6$ cells/mL stained with 5µM CellTrace Violet. Staining was conducted for 20 min at 37ºC with regular mixing before quenching with at least 4 volumes of RP10 media. Responder cells were mixed with stimulator cells at a ratio of 4:1 and incubated at 37˚C for indicated time points.

*Abs and flow cytometry*

All staining was carried out in round-bottom 96 well plates using FACS buffer (PBS, 1 % FBS, 0.02 % sodium azide, 1 mM EDTA [pH 7.2]). Prior to staining, samples were pre-incubated with anti-Fc receptor blocking solution (24G.2 hybridoma supernatant) at a 1:20 dilution in FACS buffer. Surface staining was carried out at 4˚C for 15 min. Antibodies were sourced from either BD Biosciences, ThermoFisher, or BioLegend. Clones used were as follows: CD4 (RM4.5), CD8 (53-6.7), CD69 (H1.2F3), PD-1 (J43), CD19 (1D3), CD25 (7D4), Vα2 (B20.1), H-$2K^b$ (AF6-88.5.5.3), CD24 (M1/69), IFN-γ (XMG1.2), TNFα (MP6-XT22), CD45.1 (A20), and CD45.2 (104). Live/Dead staining was performed using LIVE/DEAD Fixable Aqua, Lime, or Blue (Thermo FisherScientific) or Fixable Viability Dye eFluor 780 (eBioscience). Biotinylated H-$2K^b$-OVA monomers were obtained from the NIH tetramer facility and tetramerized in house. Intracellular staining was performed using BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). Data was collected on either BD LSRFortessa SORP/X-20, or a Cytek Aurora spectral cytometer. Analysis was conducted using Flowjo software (TreeStar).

*Adoptive transfers*

Whole spleen and thymus cell suspensions from adoptive transfer donors were analyzed by flow cytometry to determine the frequency of $CD8^+$ $Vα2^+$ (splenic) and CD8SP $Vα2^+$ $CD24^{Lo}$ cells (thymic), respectively. Unfractionated cell suspensions or enriched $CD8^+$ T cells were transferred I.V. $CD8^+$ T cell enrichment was performed using the StemCell EasySep Mouse $CD8^+$ T cell Isolation Kit (Cat. # 19853A) on pooled lymph nodes and spleen. The purities of isolated fractions were > 70 % in all cases.

Unless otherwise stated, $5x10^6$ splenic or $4x10^6$ thymic mature $CD8^+$ $V\alpha2^+$ OT-I T cells were adoptively transferred I.V. into sub-lethally irradiated recipients (450 rad). Co-adoptive transfers involved $5x10^6$ cells per donor for a total of $10x10^6$ $CD8^+$ $V\alpha2^+$ T cells transferred.

*Blood glucose monitoring*

Blood glucose concentrations of bone marrow chimeras were monitored weekly starting at 3 wk post-transplantation using a OneTouch Ultra2 blood glucose meter and OneTouch Ultra Test Strips. Adoptive transfer recipients were monitored every 3-4 days starting 4 d post-transfer for the first month, and every week subsequently. Blood samples were acquired by tail vein bleeding. When blood glucose readings exceeded 15 mM, another reading was taken 24 h later. Mice were confirmed diabetic upon two consecutive readings above 15 mM.

*PD-1 blockade*

For *in vitro* blockade experiments, cultured cells were treated continuously with 5 µg/mL of αPD-1 (clones: J43 or RMP1-14, BioXCell). For *in vivo* experiments, mice were treated I.P. with 250 µg RMP1-14 every second day for 2 wk. The blood glucose concentration of treated mice was monitored for at least 4 wk following the final antibody injection.

*Thymic transplants*

Thymic lobes were isolated from 1- to 3- day old neonatal WT or RIP-mOVA mice and were cultured for 7-10 days on a filter-strainer stack (filter: MF-Millipore Membrane Filter, mixed cellulose esters, hydrophilic, 0.45 µm, 13 mm, white, gridded, HAWG01300; strainer: 70 µm, nylon, FisherScientific, #22363548) in a 6 well plate containing 1.35 mM 2'-deoxyguanosine (2-dG, Sigma-Aldrich) in complete DMEM-10 (DMEM, 4.5 g/L glucose, 0.584 g/L glutamine and 3.7 g/L $NaHCO_3$) to deplete cells of hematopoietic origin. Media was changed as needed. Thymi were washed for 2 h in DMEM-10 before transplantation under the kidney capsule of recipient mice following the transplantation protocol described by Mok *et al.* 2019 (63). Reconstitution was confirmed from the presence of $CD4^+$/$CD8^+$ T cells in peripheral blood 8 wk post-transplant by flow cytometry. Subsequent bone marrow chimera experiments were conducted 4 mo following thymic transplant.

*Statistical analysis*

Statistical analyses were conducted using Prism software (Graph-Pad). Specific tests used are listed in figure legends.

Figure 1: TRA-Specific T Cells in OT-I Bim$^{-/-}$ > RIP-mOVA Chimeras Are Functionally Impaired Via Cell-Intrinsic Mechanisms.

A, Whole splenocytes from OT-I CD45.2$^{+}$ Bim$^{-/-}$ > RIP-mOVA chimeras showed impaired activation in vitro relative to OT-I CD45.1$^{+}$ WT C57BL/6 controls (day 2 *in vitro*). Rows represent cultures with $10^{-7}$ M SIINFEKL (OVA) peptide (+, stimulated) presented by pulsed splenocytes, no-peptide control splenocytes (-, unstimulated), and cultures wherein responding isolates were mixed or cultured separately. B, Compilation of data from A (n = 3). C, Day 3 proliferation of cultures described in A indicated by dilution of CFSE dye. D-E, Compilation of division and proliferation indices from cultures described in A (n = 4). F, Incidence of diabetes in sub-lethally irradiated RIP-mOVA recipients of adoptively transferred splenic CD8$^{+}$ Vα2$^{+}$ OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA and/or OT-I WT. Note that 7 recipients with OT-I Bim$^{-/-}$ > RIP-mOVA transfers received whole spleen while all other recipients in this experiment received CD8-enriched isolates from pooled spleen and lymph nodes (SPL+LN). Log-rank (Mantel-Cox) test results are relative to the OT-I WT → RIP-mOVA group. Data are representative of 4 separate cohorts. G, Adoptive transfer recipients described in F were euthanized upon confirmation of diabetes and analyzed for expansion of either donor cell population within the spleen. Total donor cells were identified by OVA-K$^{b}$ tetramer staining. For each assay described above, the number of independent experiments (IE) ≥ 3. Data are mean ± SD. Asterisks represent statistical significance as determined by a two-way ANOVA with Sidak's multiple comparisons test (B, D-E), a paired t test (G) or Log-rank test (F). Statistical comparisons between other groups of interest are indicated. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 2: Splenic and Thymic TRA-Specific T Cells in OT-I Bim$^{-/-}$ > RIP-mOVA Chimeras Are Functionally Impaired.

A, Schematic representing experiments carried out in B-F. Activation marker induction (B) and Proliferation (C) in OT-I Bim$^{-/-}$ > RIP-mOVA (n = 4) or OT-I Bim$^{-/-}$ > WT thymocytes (n = 3) following *in vitro* stimulation. Open histograms represent culture with OVA peptide-pulsed stimulators, while greyed histograms represent no-peptide control stimulators. D-E, Compilation of data described in C. F, From either OT-I Bim$^{-/-}$ > RIP-mOVA or > WT bone marrow chimeras, 2-5x$10^{6}$ Vα2$^{+}$ CD24$^{Lo}$ thymocytes (THY) or Vα2$^{+}$ OT-I T splenocytes (SPL) from whole spleen were transferred to sub-lethally irradiated RIP-mOVA recipients, which were then monitored for the induction of diabetes. Log-rank (Mantel-Cox) test results are relative to organ transfer groups. Data are representative of 7 separate cohorts. For each assay described above, the number of independent experiments (IE) ≥ 4. Data are

mean ± SD. Asterisks represent statistical significance as determined by a two-way ANOVA with Sidak's multiple comparisons test (B), unpaired t test with Welch's correction (D-E), or Log-rank test (F). Statistical comparisons between other groups of interest are indicated. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 3: *PD-1 signals in the T cell compartment necessarily support tolerance to TRA.*
A, Comparison of PD-1 expression across organs in OT-I > RIP-mOVA (open histogram, n = 3) and OT-I > WT (greyed histogram, n = 2) bone marrow chimeras (IE = 1). B, Incidence of diabetes in RIP-mOVA chimeras with OT-I WT, OT-I PD-1$^{-/-}$, a 1:1 mix of the former and latter, and OT-I Bim$^{-/-}$ PD-1$^{-/-}$ bone marrow. Data are representative of 3 separate cohorts. C, Incidence of diabetes in RIP-mOVA or WT chimeras with CD8-cre OT-I PD-1$^{fl/ko}$ or PD-1$^{fl/WT}$ bone marrow; PD-1$^{fl/WT}$ > RIP-mOVA chimeras were sacrificed alongside their PD-1$^{fl/ko}$ counterparts to confirm the timing of PD-1 ablation (IE = 4). Data are representative of 4 separate cohorts. Asterisks represent statistical significance as determined by Log-rank test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 4: OT-I PD-1$^{-/-}$ > RIP-mOVA Chimeras Exhibit a Mild Impairment in Clonal Deletion and Increased Numbers of OT-I Vα2$^{+}$ CD8$^{+}$ T Cells in the Periphery.
A, (Top) Thymic profiles of WT or PD-1$^{-/-}$ OT-I > RIP or B6 chimeras; (Bottom) Frequency of mature thymocytes within the CD8SP subset. B, Absolute number of CD8$^{+}$ Vα2$^{+}$ CD24$^{Lo}$ mature OT-I thymocytes in chimeras from A. A data point from the PD-1$^{-/-}$ > B6 group was determined to be an outlier using the Grubb's test ($p < 0.05$) and was therefore omitted. C, Absolute number of CD8$^{+}$ splenic OT-I. D, Absolute number of CD8$^{+}$ OT-I in the pancreatic lymph nodes. Data above represent ≥ 5 independent experiments (IE). Data are mean ± SD. Asterisks represent statistical significance as determined by a two-way ANOVA with Sidak's multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 5: Acute PD-1 signaling within the thymic compartment is sufficient to induce tolerance to TRA.
Splenocytes from either OT-I Bim$^{-/-}$ > RIP or WT chimeras were cultured *in vitro* with peptide pulsed whole-spleen stimulators +/- αPD-1 antagonist antibody. A, Day 2 activation marker expression for responding OT-I splenocytes (stimulated groups shown). B, Day 3 proliferation and division indices for responding OT-I splenocytes. A data point in RIP-mOVA (αPD-1, proliferation index) group was determined to be an outlier by the Grubb's test ($p < 0.05$) and removed from the dataset. C, Day 2

activation marker expression for responding OT-I thymocytes (n > 3). D, Incidence of diabetes in chimeras treated with αPD-1 antagonist antibody or isotype control (IE = 4). E, Incidence of diabetes in sub-lethally irradiated RIP-mOVA PD-L1$^{-/-}$ recipients of adoptively transferred CD8-enriched CD8$^{+}$ Vα2$^{+}$ splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras. F, Incidence of diabetes in sub-lethally irradiated RIP-mOVA PD-L1$^{-/-}$ recipients of adoptively transferred CD8$^{+}$ Vα2$^{+}$ CD24$^{lo}$ thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras. For each assay described above, the number of independent experiments (IE) ≥ 4 unless otherwise stated. Data are mean ± SD. Asterisks represent statistical significance as determined by one-way ANOVA with Sidak's multiple comparisons tests (A-C) or Log-rank tests (E-F). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 6: Continuous Exposure to Antigen in the Periphery is Required to Maintain Functional Impairments in OT-I Bim$^{-/-}$ > RIP-mOVA Chimeras

A, Schematic showing experiments conducted in B-G. OT-I Bim$^{-/-}$ bone marrow was transferred into lethally-irradiated WT or RIP-mOVA recipients, or nude recipients. Nude recipients were previously grafted with either a WT or RIP-mOVA thymus under the kidney capsule. B, Thymic CD8 by CD4 profile for each chimera described in A. C, Thymocytes from the chimeras described in A were cultured *in vitro* with peptide-pulsed whole-spleen cell stimulators and analyzed on day 2 for activation marker expression (n = 3); values are adjusted relative to WT or Nude (WT) controls within independent experiments. D, *In vitro* cultures were analyzed on day 3 for proliferation; open histogram represents stimulated treatment (peptide pulsed-stimulators), greyed histogram represents no-peptide control stimulators E, Compilation of proliferation data described in D; OT-I Bim$^{-/-}$ > WT (n = 5), OT-I Bim$^{-/-}$ > RIP-mOVA (n = 5), OT-I Bim$^{-/-}$ > Nude (WT) (n = 4), OT-I Bim$^{-/-}$ > Nude (RT) (n = 4). F, *In vitro* cultures were re-stimulated for 4 hours on day 5 in the presence of Brefeldin A and analyzed for TNFα and IFNγ production. G, Compilation of data described in F; OT-I Bim$^{-/-}$ > WT (n = 4), OT-I Bim$^{-/-}$ > RIP-mOVA (n = 4), OT-I Bim$^{-/-}$ > Nude (WT) (n = 4). For the OT-I Bim$^{-/-}$ > Nude (RT) group, only 3 replicates are listed; a fourth data point was determined to be an outlier with Grubb's test ($p < 0.05$) and was thus omitted. For each assay described above, the number of independent experiments (IE) ≥ 3. Data are mean ± SD. Asterisks represent statistical significance as determined by a paired t test (C) or one-way ANOVA with Sidak's multiple comparisons tests (E, G). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

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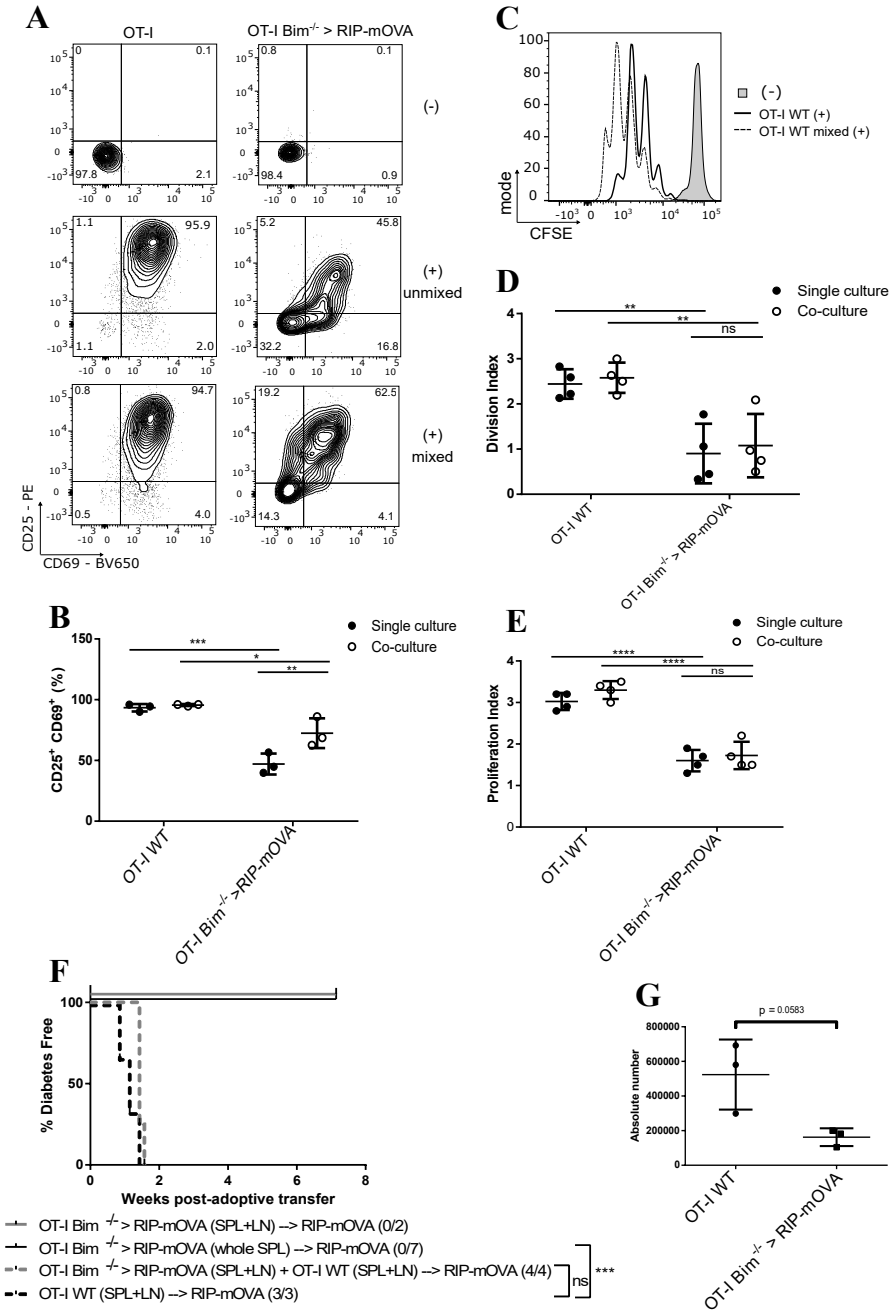
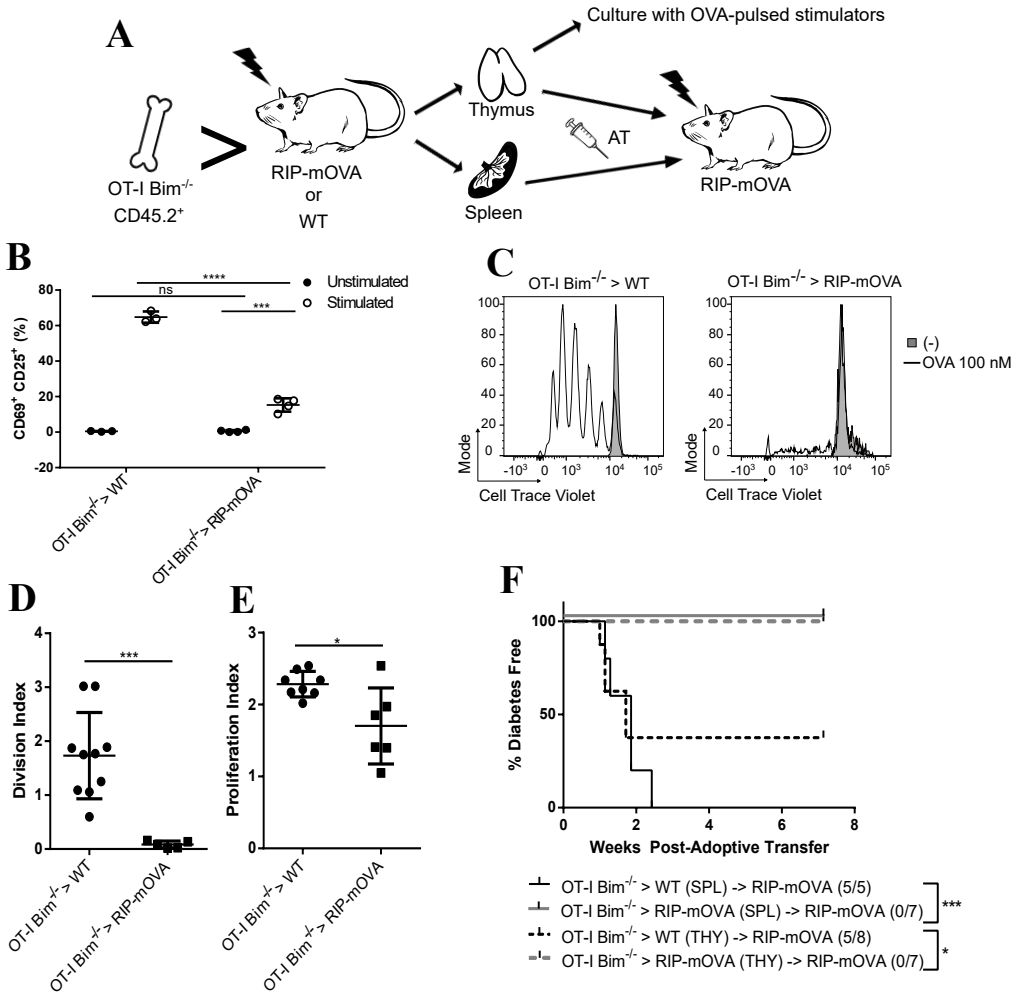
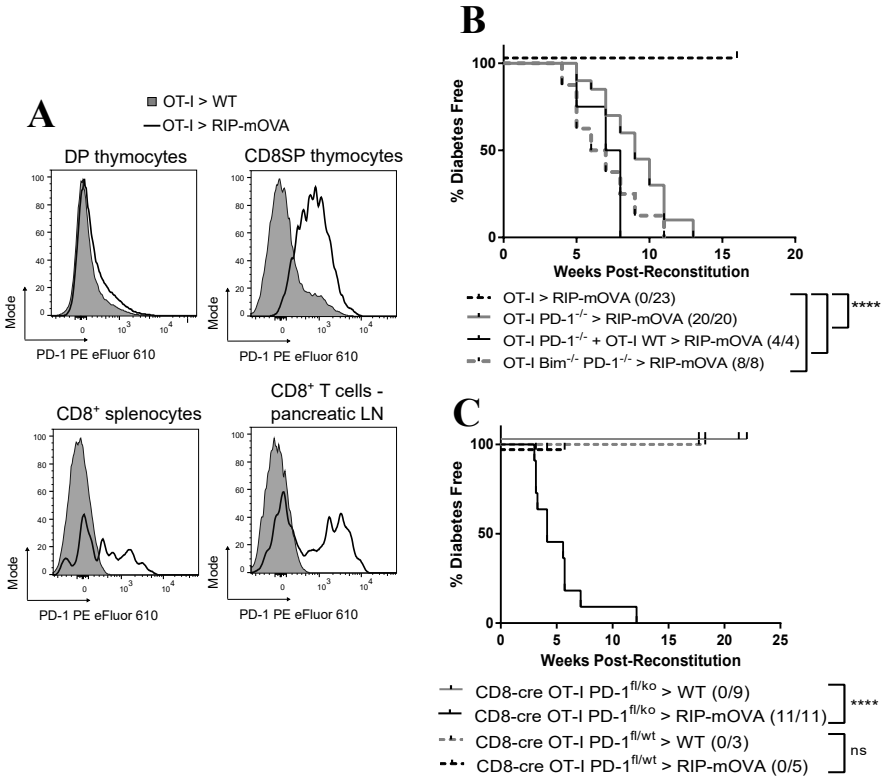
# Figure 1


A
OT-I
OT-I Bim-/- > RIP-mOVA
(-)
(+) unmixed
(+) mixed
CD25 - PE
CD69 - BV650
C
(-)
OT-I WT (+)
OT-I WT mixed (+)
mode
CFSE
D
Single culture
Co-culture
Division Index
OT-I WT
OT-I Bim-/- > RIP-mOVA
B
Single culture
Co-culture
CD25+ CD69+ (%)
OT-I WT
OT-I Bim-/- >RIP-mOVA
E
Single culture
Co-culture
Proliferation Index
OT-I WT
OT-I Bim-/- >RIP-mOVA
F
% Diabetes Free
Weeks post-adoptive transfer
OT-I Bim -/- > RIP-mOVA (SPL+LN) --> RIP-mOVA (0/2)
OT-I Bim -/- > RIP-mOVA (whole SPL) --> RIP-mOVA (0/7)
OT-I Bim -/- > RIP-mOVA (SPL+LN) + OT-I WT (SPL+LN) --> RIP-mOVA (4/4)
OT-I WT (SPL+LN) --> RIP-mOVA (3/3)
ns
***
G
p = 0.0583
Absolute number
OT-I WT
OT-I Bim-/- > RIP-mOVA

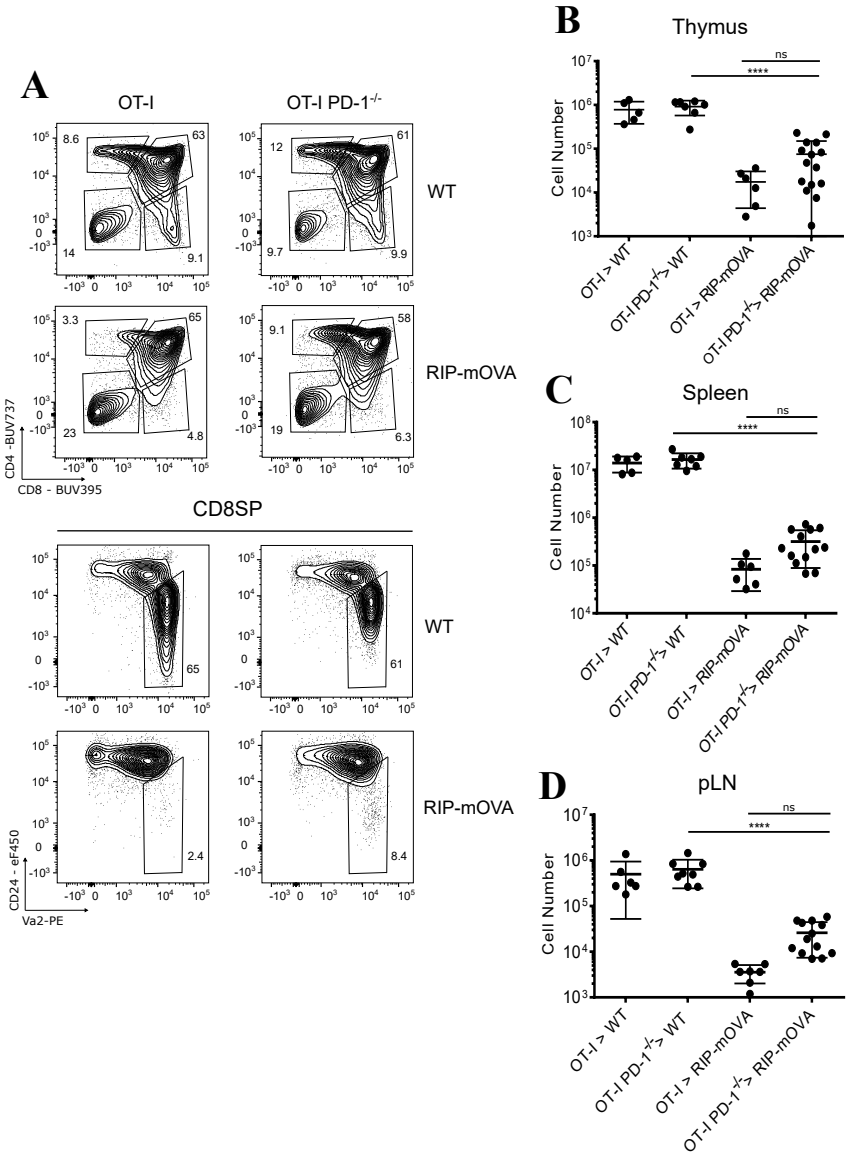
# Figure 2


A
Culture with OVA-pulsed stimulators
Thymus
AT
OT-I Bim-/-
CD45.2+
RIP-mOVA
or
WT
Spleen
RIP-mOVA
B
Unstimulated
Stimulated
ns
****
***
CD69+ CD25+ (%)
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
C
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
(-)
OVA 100 nM
Mode
Cell Trace Violet
D
***
Division Index
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
E
*
Proliferation Index
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
F
% Diabetes Free
Weeks Post-Adoptive Transfer
OT-I Bim-/- > WT (SPL) -> RIP-mOVA (5/5)
OT-I Bim-/- > RIP-mOVA (SPL) -> RIP-mOVA (0/7)
***
OT-I Bim-/- > WT (THY) -> RIP-mOVA (5/8)
OT-I Bim-/- > RIP-mOVA (THY) -> RIP-mOVA (0/7)
*

# Figure 3

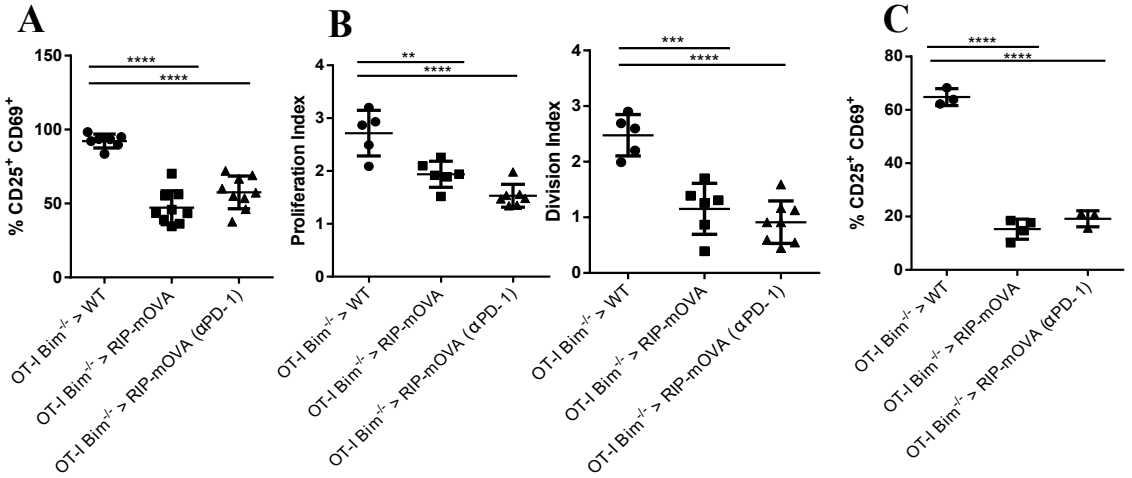

A
OT-I > WT
OT-I > RIP-mOVA
DP thymocytes
CD8SP thymocytes
CD8+ splenocytes
CD8+ T cells - pancreatic LN
Mode
PD-1 PE eFluor 610
B
% Diabetes Free
Weeks Post-Reconstitution
OT-I > RIP-mOVA (0/23)
OT-I PD-1-/- > RIP-mOVA (20/20)
OT-I PD-1-/- + OT-I WT > RIP-mOVA (4/4)
OT-I Bim-/- PD-1-/- > RIP-mOVA (8/8)
****
C
% Diabetes Free
Weeks Post-Reconstitution
CD8-cre OT-I PD-1fl/ko > WT (0/9)
CD8-cre OT-I PD-1fl/ko > RIP-mOVA (11/11)
CD8-cre OT-I PD-1fl/wt > WT (0/3)
CD8-cre OT-I PD-1fl/wt > RIP-mOVA (0/5)
****
ns

# Figure 4


A
OT-I
OT-I PD-1-/-
WT
RIP-mOVA
CD4 - BUV737
CD8 - BUV395
CD8SP
WT
RIP-mOVA
CD24 - eF450
Va2-PE

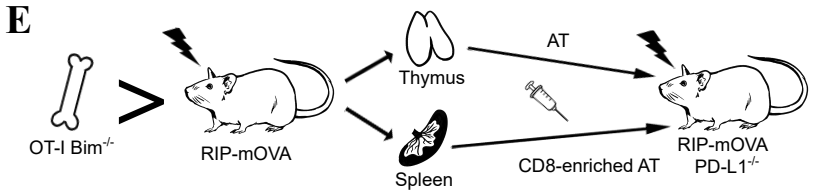

B
Thymus
Cell Number
ns
****
OT-I > WT
OT-I PD-1-/- > WT
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
C
Spleen
Cell Number
ns
****
OT-I > WT
OT-I PD-1-/- > WT
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA
D
pLN
Cell Number
ns
****
OT-I > WT
OT-I PD-1-/- > WT
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA

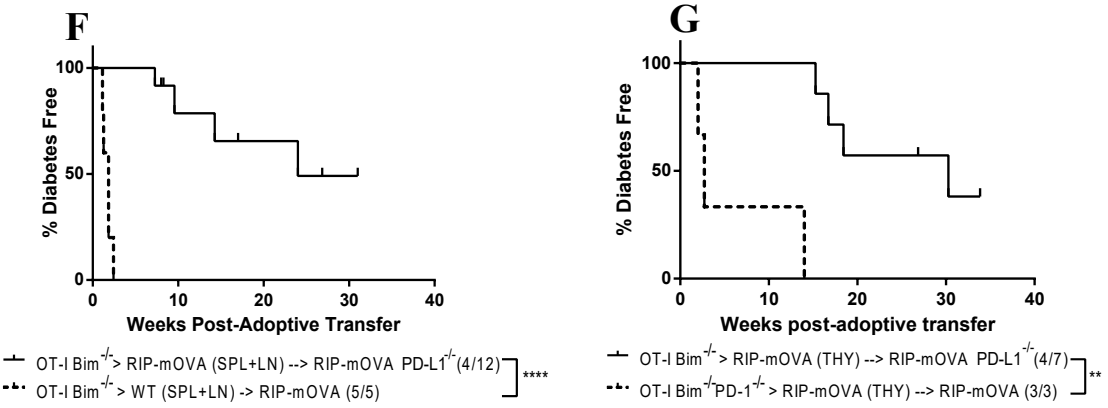
# Figure 5



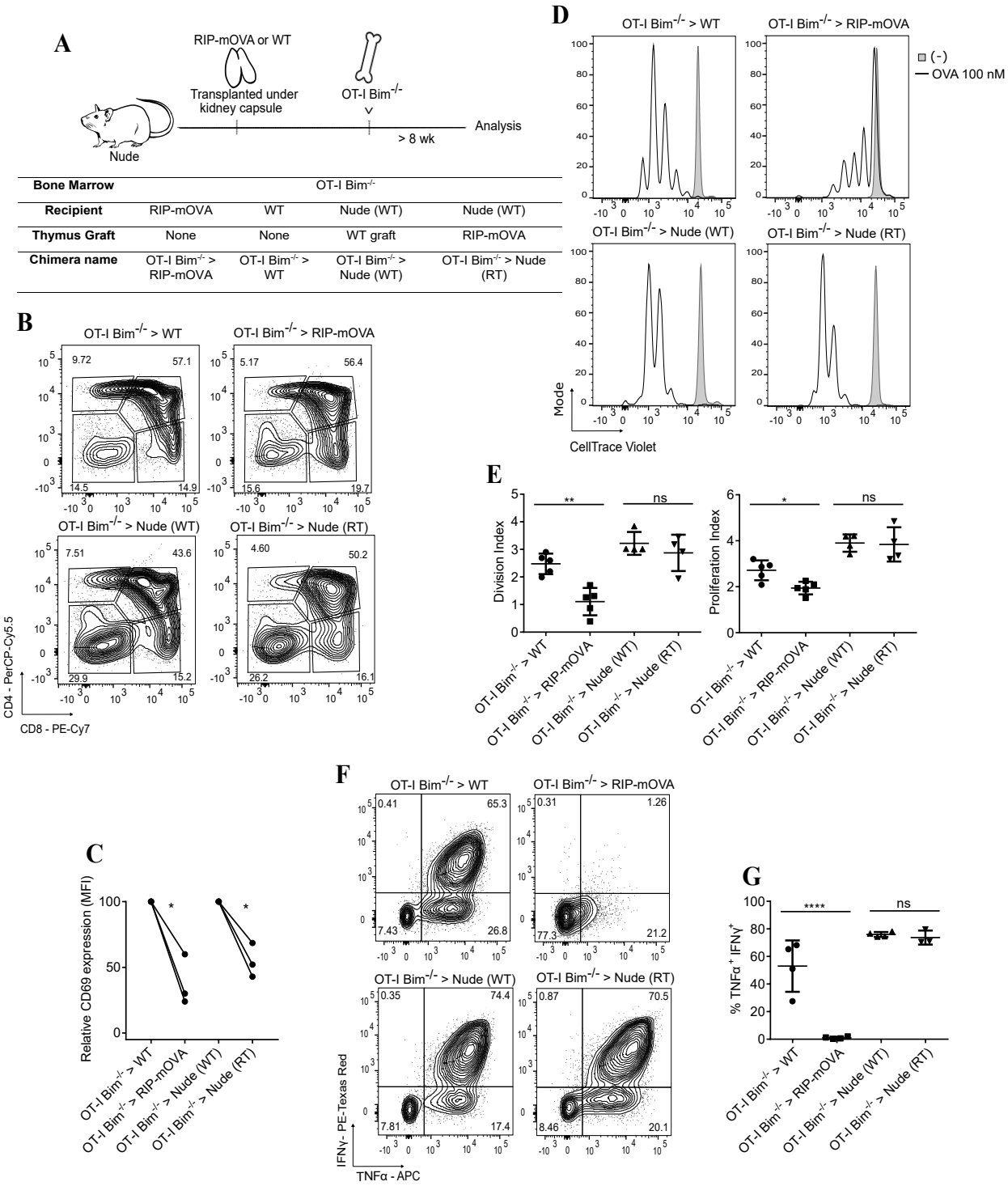

D

| Chimera | OT-I > RIP-mOVA | | | OT-I Bim$^{-/-}$ > RIP-mOVA | | |
|---|---|---|---|---|---|---|
| Treatment | None | αPD-1 (J43) | Isotype | None | αPD-1 (J43) | Isotype |
| Diabetes Incidence | 0/23 | 0/4 | 0/2 | 0/33 | 0/4 | 0/2 |



# Figure 6


A
RIP-mOVA or WT
Transplanted under kidney capsule
OT-I Bim-/-
Nude
> 8 wk
Analysis
Bone Marrow
OT-I Bim-/-
Recipient
RIP-mOVA
WT
Nude (WT)
Nude (WT)
Thymus Graft
None
None
WT graft
RIP-mOVA
Chimera name
OT-I Bim-/- > RIP-mOVA
OT-I Bim-/- > WT
OT-I Bim-/- > Nude (WT)
OT-I Bim-/- > Nude (RT)
B
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
OT-I Bim-/- > Nude (WT)
OT-I Bim-/- > Nude (RT)
CD4 - PerCP-Cy5.5
CD8 - PE-Cy7
C
Relative CD69 expression (MFI)
D
(-)
OVA 100 nM
Mode
CellTrace Violet
E
Division Index
Proliferation Index
ns
F
IFNγ - PE-Texas Red
TNFα - APC
G
% TNFα+ IFNγ+
****