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

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23 **Pre-print server:** BioRxiv,

24 - **Licensing information:**

25 **Classification:** Biological Sciences, Immunology and Inflammation

26 **Keywords:** PD-1, Bim, T cell, tolerance, thymus, clonal deletion, thymic negative selection,

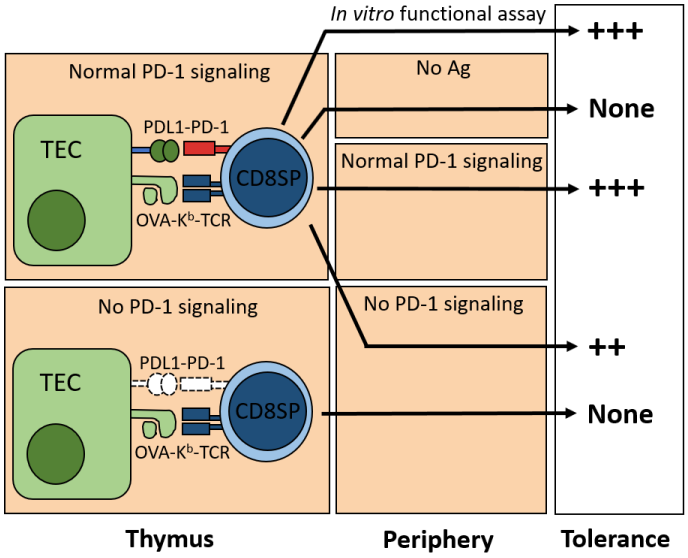
27 autoimmunity

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 murine T cells that failed clonal deletion were rendered functionally impaired in the thymus. PD-1 was induced in the thymus and was required to establish cell-intrinsic tolerance to TRA in $CD8^+$ thymocytes independently of clonal deletion. PD-1 signaling in developing thymocytes was sufficient for tolerance induction, however in some cases the absence of PD-L1-mediated signaling in the periphery resulted in broken tolerance at late time points. Finally, we showed that chronic exposure to high affinity antigen supported the long-term maintenance of tolerance. Taken together, our study identifies a critical role for PD-1 in establishing central tolerance in autoreactive T cells that escape clonal deletion. It also sheds light on potential mechanisms of action of anti-PD-1 pathway immune checkpoint blockade and the development of immune-related adverse events.

46 **Graphical Abstract**

47



48

49

50 **Introduction**

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

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

65 Although clonal deletion, wherein autoreactive thymocytes are instructed to undergo apoptosis,
66 has long been considered the primary central tolerance mechanism, the fate of autoreactive thymocytes
67 that escape clonal deletion is not yet fully understood (3). Studies by Ramsdell and Fowlkes used a
68 superantigen model of negative selection to determine that a population of superantigen-reactive CD4SP
69 and CD8SP could escape clonal deletion but were unresponsive to superantigen stimulation (4).
70 Similarly using TCR transgenic models, we and others recently showed that TRA-specific CD8SP and
71 CD4SP thymocytes that encountered their high affinity antigen during development, but were unable to
72 undergo apoptosis, continued maturation and emigrated into the periphery (5, 6) but showed functional
73 impairment (7). More recent work using polyclonal models demonstrated that CD4SP thymocytes that
74 encountered their high affinity antigen in the cortex were mostly deleted, but those that received a high

75 affinity signal in the medulla were largely selected into the Treg lineage or were functionally impaired
76 rather than being deleted (8, 9). These studies highlight the complex interplay between different central
77 tolerance mechanisms.

78 Programmed cell death protein 1 (PD-1) is a co-inhibitory receptor expressed on T cells, B cells,
79 and some subsets from the myeloid lineage (10). PD-1 ligation impairs T cell responses and thus PD-1
80 blockade forms the basis for some promising cancer immunotherapies by improving the function of anti-
81 tumour $CD8^+$ T cells in the tumour microenvironment, reviewed in (11). PD-1 is thought to function
82 through its recruitment of the phosphatase SHP2, which negatively regulates the activation of TCR-
83 proximal signaling molecules (12) and CD28-driven co-stimulation (13, 14). On lymphocytes at steady
84 state, PD-1 expression is limited to a small fraction of thymic and peripheral cells, but is induced on
85 thymocytes and peripheral T cells following strong TCR interactions (15, 16). The ligands for PD-1,
86 PD-L1 and PD-L2 are expressed on the thymic epithelium and professional antigen presenting cells in
87 the thymus and throughout the periphery (17, 18). PD-1 deficiency results in autoimmunity in pre-
88 clinical models (19) and single nucleotide polymorphisms in PD-1 are linked to autoimmunity in
89 humans (20–22). In the thymus, transgenic overexpression of PD-1 has been shown to modulate positive
90 selection (23), while PD-1 deficiency in UbA-mediated models of negative selection showed little or no
91 impact (24, 25). Studies examining a role for PD-1 in thymic tolerance induced by TRA are currently
92 lacking.

93 In this study we explored the fate of MHCI-restricted thymocytes specific for a TRA in the
94 absence of clonal deletion. We found that PD-1 was sufficient to establish cell-intrinsic tolerance in the
95 thymus and continued PD-L1-mediated signaling in the periphery was dispensable until late time points.
96 Additionally, chronic exposure to high affinity antigen supports the long-term maintenance of tolerance
97 in this model. Taken together, our study identifies a role for PD-1 in establishing central tolerance in
98 TRA-specific thymocytes that have escaped clonal deletion. Furthermore, it supports a model where

99 cancer immunotherapies that target the PD-1 pathway act to prevent anti-tumour T cells from becoming

100 dysfunctional in addition to rescuing them from the dysfunctional state.

101

102 **Methods**

103

104 *Mice*

105 C57BL/6j (WT, strain 000664), C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/WehiJ (RIP-mOVA, strain
106 005431) (26), NOD.B6-*Cd274*^{tm1Shr}/J (PD-L1$^{-/-}$, strain 018307) (27), C57BL/6-Tg(Cd8a-cre)1Itan/J
107 (CD8-cre, strain 008766) (28), B6.Cg-*Foxn1*^{nu}/J (Nude, strain 000819) (29) mice were sourced from
108 Jackson Laboratory. C57BL/6-*Pdcd1*$^{tm1.1Mrl}$ (PD-1$^{fl/fl}$, strain 13976) were purchased from Taconic
109 Biosciences. The PD-L1$^{-/-}$ mice were backcrossed to C57BL/6j mice at least 5 generations prior to
110 intercrossing with RIP-mOVA mice. OT-I Bim$^{-/-}$ mice were provided by Dr. Maureen McGargill (St.
111 Jude Children's Research Hospital, Memphis, TN, USA). PD-1$^{-/-}$ mice were provided by Dr. Tasuku
112 Honjo (Kyoto University, Kyoto, Japan) (30) and BTLA$^{-/-}$ were obtained from Dr. Kenneth Murphy
113 (Washington University, St. Louis, MO) (31). Mice with compound genotypes were intercrossed in-
114 house. Across this study, both male and female mice were used and age-matched where possible. All
115 mice were maintained via protocols approved by the University of Alberta Animal Care and Use
116 Committee.

117

118 *Bone marrow chimeras*

119 T cells were depleted from donor bone marrow by I.P. administration of 100 µg anti-Thy1.2 (clone
120 30H12) -2 and -1 d prior to harvest. 1 d prior to transplant, recipients were irradiated with 2 doses of 450
121 rad, separated by a 4-hour rest period. Bone marrow was flushed from the femurs and tibias of donor
122 mice with PBS (2 % FBS, 2 mM EDTA) and filtered through a 70 µm nylon strainer. $5x10^6$ – $10x10^6$
123 total BM cells were transplanted to recipients via tail vein injection. On day 1 post-transplant, 100 µg of
124 anti-Thy1.2 antibody (clone 30H12) was administered I.P. to the recipients. For the first 4 wk post-
125 transplant, recipient mice were given water containing Novo-Trimel (via University of Alberta Health

126 Sciences Lab Animal Services). Chimeras were allowed to recover for at least 8 wk before further
127 manipulation or analysis.
128
129 *Tissue collection*
130 Mice were anaesthetized with isoflurane and euthanized via CO_2 asphyxiation. Organs were harvested
131 and made into single-cell suspensions by grinding through either sterile wire or nylon mesh screens in
132 petri dishes containing RP10 media (RPMI 1640, 2.05 mM L-glutamine [Hyclone], 10 % FBS, 5 mM
133 HEPES, 50 mM 2-mercaptoethanol, 50 µg/mL penicillin/streptomycin, 50 µg/mL gentamycin sulfate).
134 Cells were treated with ammonium-chloride-potassium lysis buffer to lyse RBCs. Viable cell numbers
135 were determined using Trypan Blue exclusion dye and a hemocytometer (Fisher Scientific) under light
136 microscopy (Zeiss).
137
138 *Adoptive transfers*
139 Whole spleen and thymus cell suspensions from adoptive transfer donors were analyzed by flow
140 cytometry to determine the frequency of $CD8^+$ $V\alpha2^+$ (splenic) and CD8SP $V\alpha2^+$ $CD24^{lo}$ cells (thymic),
141 respectively. $CD8^+$ T cell enrichment was performed using the StemCell EasySep Mouse $CD8^+$ T cell
142 Isolation Kit (Cat. # 19853A) on pooled lymph nodes and spleen. The purities of isolated fractions were
143 > 70 % in all cases. Unless otherwise stated, $5x10^6$ splenic or $4x10^6$ thymic mature $CD8^+$ $V\alpha2^+$ OT-I T
144 cells were adoptively transferred I.V. into sub-lethally irradiated recipients (450 rad). Co-adoptive
145 transfers involved $5x10^6$ cells per donor for a total of $10x10^6$ $CD8^+$ $V\alpha2^+$ T cells transferred.
146
147 *Cell culture and in vitro stimulation assays*
148 Cells were cultured at $2.5x10^6$ cells/well in 48-well plates in RP10 media at 37˚C in a tissue culture
149 incubator. Whole spleen cell stimulators from C57BL/6 mice were resuspended in RP10 media at
150 $20x10^6$ cells/mL pulsed with or without 100 nM OVA peptide sourced from AnaSpec (SIINFEKL, AS-

151 60193-1) for 1 h at 37˚C with gentle shaking every 15-20 minutes. Stimulator cells were washed three
152 times before culture. Responder cells were either resuspended in PBS at $10x10^6$ cells/mL and stained
153 with 1.25 µM CFSE or at $5x10^6$ cells/mL stained with 5µM CellTrace Violet. Staining was conducted
154 for 20 min at 37ºC with regular mixing before quenching with at least 4 volumes of RP10 media.
155 Responder cells were mixed with stimulator cells at a ratio of 4:1 and incubated at 37˚C for indicated
156 time points. Proliferation and division indices were calculated using FlowJo. Proliferation index is
157 calculated as the number of divisions within the dividing cellular population, while the division index is
158 calculated as the average number of divisions undergone by each cell in the starting culture.
159
160 *Abs and flow cytometry*
161 All staining was carried out in round-bottom 96 well plates using FACS buffer (PBS, 1 % FBS, 0.02 %
162 sodium azide, 1 mM EDTA [pH 7.2]). Prior to staining, samples were pre-incubated with anti-Fc
163 receptor blocking solution (24G.2 hybridoma supernatant) at a 1:20 dilution in FACS buffer. Surface
164 staining was carried out at 4˚C for 15 min. Antibodies were sourced from either BD Biosciences,
165 ThermoFisher, or BioLegend. Clones used were as follows: CD4 (RM4.5), CD8 (53-6.7), CD69
166 (H1.2F3), PD-1 (J43), CD19 (1D3), CD25 (7D4), Vα2 (B20.1), H-$2K^b$ (AF6-88.5.5.3), CD24 (M1/69),
167 IFN-γ (XMG1.2), TNFα (MP6-XT22), CD45.1 (A20), and CD45.2 (104). Live/Dead staining was
168 performed using LIVE/DEAD Fixable Aqua, Lime, or Blue (Thermo FisherScientific) or Fixable
169 Viability Dye eFluor 780 (eBioscience). Biotinylated H-$2K^b$-OVA monomers were obtained from the
170 NIH tetramer facility and tetramerized in house. Intracellular staining was performed using BD
171 Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). Data was collected on
172 either BD LSRFortessa SORP/X-20, or a Cytek Aurora spectral cytometer. Analysis was conducted
173 using Flowjo software (TreeStar).
174
175 *Blood glucose monitoring*

176 Blood glucose concentrations of bone marrow chimeras were monitored weekly starting at 3 wk post-
177 transplantation using a OneTouch Ultra2 blood glucose meter and OneTouch Ultra Test Strips. Adoptive
178 transfer recipients were monitored every 3-4 days starting 4 d post-transfer for the first month, and every
179 week subsequently. Blood samples were acquired by tail vein bleeding. When blood glucose readings
180 exceeded 15 mM, another reading was taken 24 h later. Mice were confirmed diabetic upon two
181 consecutive readings above 15 mM.
182
183 *PD-1 blockade*
184 For *in vitro* blockade experiments, cultured cells were treated continuously with 5 µg/mL of αPD-1
185 (clones: J43 or RMP1-14, BioXCell). For *in vivo* experiments, mice were treated I.P. with 250 µg
186 RMP1-14 every second day for 2 wk. The blood glucose concentration of treated mice was monitored
187 for at least 4 wk following the final antibody injection.
188
189 *Thymic transplants*
190 Thymic lobes were isolated from 1- to 3-day old neonatal WT or RIP-mOVA mice and cultured for 7-10
191 days on a filter-strainer stack (filter: MF-Millipore Membrane Filter, mixed cellulose esters, hydrophilic,
192 0.45 µm, 13 mm, white, gridded, HAWG01300; strainer: 70 µm, nylon, FisherScientific, #22363548) in
193 a 6 well plate containing 1.35 mM 2'-deoxyguanosine (2-dG, Sigma-Aldrich) in complete DMEM-10
194 (DMEM, 4.5 g/L glucose, 0.584 g/L glutamine and 3.7 g/L $NaHCO_3$) to deplete cells of hematopoietic
195 origin. Media was changed as needed. Thymi were washed for 2 h in DMEM-10 before transplantation
196 under the kidney capsule of recipient mice following our standard protocol (32). T cell reconstitution
197 was confirmed by the presence of $CD4^+/CD8^+$ T cells in peripheral blood 8 wk post-transplant by flow
198 cytometry. Subsequent bone marrow transplantation was conducted 4 mo. following thymic transplant.
199
200 *Statistical analysis*

201 Statistical analyses were conducted using Prism software (Graph-Pad). Specific tests used are listed in
202 figure legends.
203
204

205 **Results**
206

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

209 We and others previously showed that Bim is required for thymic clonal deletion to tissue-
210 restricted antigen (TRA) using the OT-I TCR transgenic system (5–7). OT-I Bim$^{-/-}$ T cells that escaped
211 thymic clonal deletion in RIP-mOVA recipients did not go on to cause autoimmunity due to functional
212 impairments characterized by defects in activation, proliferation, and cytokine production. The
213 mechanism(s) behind this impairment was unclear but may be due to cell-extrinsic suppression or cell-
214 intrinsic anergy (33–35). We hypothesized that if a population of dominant suppressors existed in OT-I
215 Bim$^{-/-}$ > RIP-mOVA chimeras, they should suppress the activation and proliferation of wildtype OT-I T
216 cells. CFSE labelled splenocytes from CD45.1$^+$ OT-I mice were mixed at a 1:1 ratio with splenocytes
217 from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras and stimulated with OVA peptide-pulsed splenocytes *in vitro*.
218 General gating strategies are outlined in Fig. S1. Activation marker induction (Fig. 1A, B) and
219 proliferation measured by proliferation index (Fig. 1C-D, Fig. S2A) in WT OT-I T cells was not
220 significantly reduced by co-culture with splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras. As we
221 previously found, on day 2 post-stimulation, the induction of activation markers CD69 and CD25 on
222 OT-I splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras was diminished compared to that of
223 splenocytes from wildtype (WT) OT-I CD45.1$^+$ mice.

224 To assay functional impairment *in vivo,* we co-adoptively transferred either whole spleen or
225 enriched CD8$^+$ T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras with splenocytes from CD45.1$^+$ OT-I
226 WT mice (1:1 ratio) into sub-lethally irradiated RIP-mOVA recipients. Neither whole spleen nor
227 enriched CD8$^+$ OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras alone were diabetogenic,
228 suggesting that a dominant suppressor population of non-T cell origin was not responsible for functional
229 impairment in these chimeras (Fig. 1E). Enriched CD8$^+$ OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA
230 chimeras co-adoptively transferred with WT OT-I T cells did not have a dominant suppressive effect on
231 diabetogenicity (Fig. 1E). In these co-adoptive transfer recipients, WT OT-I T cells also expanded to a
232 greater extent than those from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras (Fig. 1F). Functional impairment of
233 OT-I Bim$^{-/-}$ T cells from RIP-mOVA chimeras was also demonstrated by a reduced capacity for
234 lymphopenia-induced proliferation compared to control cells from OT-I Bim$^{-/-}$ > WT chimeras (Fig.
235 S2B). Collectively, these data suggest that a population of Ag-specific suppressors are not generated in
236 OT-I Bim$^{-/-}$ > RIP-mOVA mice and that functional impairment of OT-I Bim$^{-/-}$ T cells from these
237 chimeras is cell-intrinsic.

238

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

241 After determining that the functional impairment of OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA
242 chimeras was cell-intrinsic, we investigated whether this state was established during development in the
243 thymus or in the periphery. The thymus is the initial site of high affinity antigen encounter and induction
244 of non-responsiveness in thymocytes has been reported previously (36). We stimulated CFSE-labelled
245 thymocytes from OT-I Bim$^{-/-}$ > WT and OT-I Bim$^{-/-}$ > RIP-mOVA chimeras with OVA peptide pulsed
246 splenocytes and examined activation and proliferation. On day 2, expression of activation markers CD69
247 and CD25 was impaired in OT-I Bim$^{-/-}$ > RIP-mOVA thymocytes compared to OT-I Bim$^{-/-}$ > WT
248 controls (Fig. 2B). Additionally on day 3, thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras
249 exhibited a defect in proliferation compared to OT-I Bim$^{-/-}$ > WT thymocytes (Fig. 2C-D). These
250 impaired responses were not likely to be due to a reduction in more mature CD8SP thymocyte subsets in
251 OT-I Bim$^{-/-}$ > RIP-mOVA recipients since the thymi of these mice demonstrated enhanced frequencies
252 of mature (M2; CD69^{lo} H-2K$^{b\ hi}$) thymocytes over WT controls (Fig. 2E).

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

261

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

263 The functional impairment seen in OT-I Bim$^{-/-}$ > RIP-mOVA thymocytes and splenocytes may
264 be driven by co-inhibitory molecules and/or other factors that inhibit T cell activity. As such, we
265 investigated whether various known co-inhibitors were differentially expressed in OT-I > RIP-mOVA
266 thymocytes and splenocytes that escaped clonal deletion compared to OT-I > WT controls. We found
267 enhanced PD-1 expression on OT-I T cells from both the thymus and periphery of OT-I > RIP-mOVA
268 (Fig. 3A) and OT-I Bim$^{-/-}$ > RIP-mOVA chimeras compared to OT-I cells from WT recipients, while the
269 expression of other co-inhibitory molecules was not appreciably altered (Fig. S3A). Given the increased
270 PD-1 expression, and that previous literature supports a role for PD-1 in enforcing tolerance (10), we

271 hypothesized that PD-1 was playing an active role in establishing and/or maintaining the functional
272 impairment seen in TRA-specific T cells that escaped clonal deletion in OT-I Bim$^{-/-}$ > RIP-mOVA
273 chimeras.

274 We intercrossed OT-I and PD-1$^{-/-}$ mice to generate OT-I PD-1$^{-/-}$ mice to determine if PD-1
275 regulated tolerance in OT-I > RIP-mOVA chimeras. Bone marrow (BM) chimeras using OT-I WT or
276 OT-I PD-1$^{-/-}$ BM and either WT or RIP-mOVA recipients were generated. We observed a 100%
277 incidence of diabetes in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras, compared to 0% incidence in OT-I WT >
278 RIP-mOVA controls (Fig. 3B). Next, we investigated if autoimmunity in OT-I PD-1$^{-/-}$ > RIP-mOVA
279 chimeras was dependent on all donor OT-I T cells being PD-1$^{-/-}$. We generated mixed bone marrow
280 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
281 WT/OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras developed diabetes (Fig. 3B). These data suggested that only a
282 fraction of the overall pool of OT-I T cells needed to be PD-1$^{-/-}$ to induce autoimmunity. This finding
283 further supports the case that functional impairment of OT-I T cells in OT-I Bim$^{-/-}$ > RIP-mOVA was
284 cell intrinsic and not due to a population of dominant suppressors. To ensure that the absence of Bim
285 itself was not preventing autoimmunity, we generated OT-I PD-1$^{-/-}$ Bim$^{-/-}$ > RIP-mOVA chimeras and
286 again observed a 100% incidence of diabetes, suggesting that PD-1 signals with or without the presence
287 of Bim are necessary for tolerance.

288 To further solidify that the deletion of PD-1 specifically and not any co-inhibitory receptor
289 impairs tolerance in this context, we investigated whether deletion of B and T lymphocyte attenuator
290 (BTLA) impaired tolerance. BTLA is a co-inhibitory receptor expressed on thymocytes following
291 positive selection (37), and has been thought to play a similar role to PD-1 in regulating T cell tolerance
292 (31). We generated OT-I BTLA$^{-/-}$ > WT or RIP-mOVA chimeras and found that none of the OT-I
293 BTLA$^{-/-}$ > RIP-mOVA chimeras developed diabetes, suggesting that elimination of any co-inhibitory
294 receptor does not impair tolerance (Fig. S4).

295 Finally, to determine whether PD-1 signaling specifically within the T cell compartment was
296 necessary to induce tolerance, we conditionally deleted PD-1 in CD8^{+} T cells using *pdcd1* floxed mice.
297 CD8-Cre mice were intercrossed with OT-I, constitutive PD-1$^{-/-}$, and *pdcd1* floxed mice to generate OT-
298 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
299 evident at the SM-M1 CD8SP thymocyte stages (Fig. S3B) and all OT-I CD8-Cre PD-1$^{fl/ko}$ > RIP-
300 mOVA chimeras experienced rapid onset of diabetes while OT-I CD8-Cre PD-1$^{fl/wt}$ > RIP-mOVA
301 chimeras were protected (Fig. 3C). Therefore, PD-1 signaling specifically within the CD8^{+} T cell
302 compartment is required to establish tolerance in this model. Here and in subsequent diabetes analyses,
303 we euthanized mice from some groups early for the purpose of *ex vivo* analysis (indicated by tick

304 marks). Thus, mice from some groups were not followed to the end of the study, and therefore in some
305 cases our reported incidence of diabetes may be underestimates. The Kaplan-Meier graphs depicting the
306 percentage of mice remaining diabetes-free is accurate as the data points censored early are not included
307 in this calculation.
308
309 *OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras exhibit a mild impairment in clonal deletion and increased numbers*
310 *of OT-I CD8^{+} T Cells in the periphery.*
311 Because 100% of the OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras developed autoimmune diabetes, we next
312 investigated the mechanism by which PD-1 signals contribute to tolerance to TRA. We first determined
313 if PD-1 modulated clonal deletion of OT-I thymocytes after high-affinity antigen encounter in the
314 thymus of RIP-mOVA recipients. We examined the CD4 by CD8 profile of thymocytes from OT-I WT
315 > RIP-mOVA and OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras and observed a modest reduction in the
316 frequency of CD8SP thymocytes and a similar CD8 dulling in this compartment compared to OT-I WT
317 > RIP-mOVA controls (Fig. 4A, Left). Furthermore, we observed a significant reduction in the
318 frequency and total number of mature (Vα2^{+} CD24^{lo}) CD8SP thymocytes in both OT-I WT and OT-I
319 PD-1$^{-/-}$ > RIP-mOVA chimeras compared to WT recipient controls, indicating intact clonal deletion in
320 the absence of PD-1 (Fig. 4A, Right; B). We did observe a small increase in the number of mature
321 CD8SP thymocytes from OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I > RIP-mOVA
322 controls, but this was not statistically significant (Fig. 4B). Overall, these data suggest that while the vast
323 majority of OT-I thymocytes encountered cognate antigen and underwent clonal deletion in OT-I PD-1$^{-/-}$
324 > RIP-mOVA recipients, the remaining PD-1-deficient OT-I T cells in these chimeras still went on to
325 induce autoimmune diabetes.
326 We next sought to determine if PD-1 deficiency had an impact on the number of peripheral OT-I
327 CD8^{+} T cells in OT-I PD-1$^{-/-}$ > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls.
328 With both OT-I WT and OT-I PD-1$^{-/-}$ BM, the number of Vα2^{+} CD8^{+} T cells in the spleen and
329 pancreatic lymph nodes demonstrated a significant overall reduction in RIP-mOVA chimeras compared
330 to WT recipient controls, again suggesting intact clonal deletion. Like what was observed in the thymus,
331 there was an increase in the number of OT-I T cells in the spleen and pancreatic LNs from OT-I PD-1$^{-/-}$
332 > RIP-mOVA chimeras compared to OT-I WT > RIP-mOVA controls, but this was not statistically
333 significant (Fig. 4C, D). Overall, in this model, clonal deletion appears largely intact in the absence of
334 PD-1. Thus, we conclude PD-1 contributes to tolerance in this context by a mechanism distinct from
335 thymic clonal deletion.
336

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

339 We next investigated the specific requirements for the PD-1-driven tolerance induced in OT-I
340 Bim$^{-/-}$ > RIP-mOVA chimeras. Given that OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras
341 expressed PD-1 and are ostensibly impaired in a cell-intrinsic manner, we asked whether PD-1-
342 dependent tolerance required continued PD-1 signaling or was stable even when PD-1 signaling was
343 withdrawn. To address this question, we stimulated splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA
344 chimeras with OVA-peptide pulsed splenocytes *in vitro* with or without well-characterized blocking
345 anti-PD-1 monoclonal antibodies. On day 2, we observed that PD-1 blockade did not result in an
346 increase in CD69 and CD25 expression on OT-I Bim$^{-/-}$ splenocytes (Fig. 5A) or thymocytes (Fig. 5C)
347 from RIP-mOVA recipients. Additionally, the proliferation index of Vα2^{+} CD8^{+} splenocytes from OT-I
348 Bim$^{-/-}$ > RIP-mOVA chimeras was not altered by PD-1 blockade on day 3 (Fig. 5B). Similarly, *in vivo*
349 administration of anti-PD-1 blocking antibodies to OT-I > RIP-mOVA or OT-I Bim$^{-/-}$ > RIP-mOVA
350 bone marrow chimeras failed to break tolerance, suggesting tolerance was stable even when PD-1
351 signaling was presumably interrupted (Fig. 5D).

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

369 model. To address this, we collected thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras and
370 transferred them into sub-lethally irradiated RIP-mOVA PD-L1$^{-/-}$ recipients so that PD-1 signaling
371 would be limited to the thymus. Like the splenocyte adoptive transfer, transferred thymocytes remained
372 tolerant for an extended period in contrast to non-tolerant controls, with tolerance waning at late time
373 points in some cases (Fig. 5H). These data indicate that acute PD-1 signaling in the thymus is critically
374 important to the establishment of tolerance, but continued PD-L1-dependent signals only support the
375 maintenance of tolerance at late time points.
376
377 *Continuous exposure to antigen in the periphery is required to maintain functional impairments in OT-I*
378 *Bim$^{-/-}$ >RIP-mOVA chimeras*
379 With our finding that acute PD-1 signaling in the thymus was sufficient to establish tolerance,
380 but dispensable for the initial maintenance of tolerance in the periphery, we wondered whether
381 continued exposure to high affinity antigen in the periphery was required to maintain tolerance. To
382 tackle this question, we transplanted either a WT or RIP-mOVA neonatal thymus under the kidney
383 capsule of nude (athymic) recipients, and at least 8 weeks later lethally-irradiated the thymic transplant
384 recipients and transplanted OT-I Bim$^{-/-}$ donor bone marrow. In recipients of a RIP-mOVA thymus,
385 exposure to high affinity OVA is limited to the thymus and withdrawn from OT-I CD8^{+} cells upon
386 emigration from the thymus (Fig. 6A).
387 We harvested the transplanted thymus from both groups and examined the CD4/CD8 profile of
388 OT-I thymocytes. We observed similar frequencies of CD8SP thymocytes and CD8 dulling between the
389 thymus transplant recipients and the corresponding chimeric controls (Fig. 6B). Following *in vitro*
390 stimulation, thymocytes from these groups also demonstrated equivalent functional impairments as
391 assessed by CD69 induction on day 2 post-activation (Fig. 6C). Additionally, splenocytes from OT-I
392 Bim$^{-/-}$ > RIP-mOVA chimeras showed diminished proliferation and cytokine production (TNFα and
393 IFNγ) compared to the corresponding WT control following *in vitro* stimulation (Fig. 65D-G). However,
394 in OT-I Bim$^{-/-}$ > Nude (RT) chimeras, proliferation and cytokine production were not significantly
395 different from the OT-I Bim$^{-/-}$ > Nude (WT) control, suggesting restored function in OT-I T cells when
396 exposure to high affinity antigen is withdrawn after thymic egress (Fig. 6D-G). Overall, it appears that,
397 in contrast to PD-1 signaling, persistent exposure to high affinity antigen is required to maintain the
398 functional impairments we see in OT-I Bim$^{-/-}$ > RIP-mOVA chimeras.
399
400

401 **Discussion**
402

403 Clonal deletion of strongly self-reactive thymocytes is a well-described mechanism of T cell
404 central tolerance, however, potent non-deletional mechanisms also exist. Previously, we and others
405 showed that while Bim is required for clonal deletion to TRA in OT-I Bim$^{-/-}$ > RIP-mOVA bone marrow
406 chimeras (5), it is dispensable for tolerance (6, 7). In the present study, we demonstrated that TRA-
407 specific T cells that escape clonal deletion in these chimeras are rendered functionally impaired in a cell-
408 intrinsic manner during antigen encounter in the thymus. PD-1 expression during negative selection in
409 the thymus was required to establish tolerance, but continued PD-1 signaling in the periphery only
410 contributed to the maintenance of this tolerance at later time points. Additionally, we found antigen
411 persistence in the periphery was needed to maintain a state of tolerance, as has previously been reported
412 (38–41). Collectively, these data highlight the critical window during which PD-1 signaling results in
413 the establishment of tolerance and shed light on potential mechanisms by which PD-1 blockade
414 functions in the context of cancer immunotherapy and development of immune-related adverse events.
415 While the removal of autoreactive T cell clones via thymic clonal deletion has long been
416 considered a primary mechanism for establishing T cell tolerance, conventional T cells that recognize
417 self-peptides with high affinity can be readily found in healthy individuals (42). Like in the case of
418 regulatory T cells, some of these T cells are actively selected for, while in other cases these T cells may
419 be products of failed clonal deletion. By deleting Bim and blocking clonal deletion, a large population of
420 highly self-reactive T cells are released into the periphery, yet tolerance is maintained (6, 7). Similarly,
421 recent studies in polyclonal mice have revealed limited deletion of TRA-specific T cells; rather,
422 tolerance was enforced by Treg-mediated suppression (8), or T cell-intrinsic functional impairment (9,
423 43). Indeed, we and others previously observed an increased number and frequency of anergic-
424 phenotype CD4^{+} thymocytes and splenocytes when Bim was absent (44, 45). We find that functional
425 impairment is observed both in thymocytes and peripheral T cells suggesting tolerance is first
426 established in the thymus. These data are consistent with the recent findings of Malhotra *et al.* where
427 MHCII-restricted thymocytes specific for a TRA showed reduced responsiveness (9). Similar findings
428 were reported for thymocytes in an allo-reactive MHCI-restricted TCR transgenic model (36) and in a
429 super-antigen mediated model of tolerance (4).
430 In previous studies where thymocytes were found to be functionally impaired, it was not
431 determined if the impairment was due to cell-extrinsic dominant suppression or through cell-intrinsic
432 mechanisms. In addition to CD4^{+} Foxp3^{+} Treg, there is mounting evidence supporting the existence of a
433 CD8^{+} T regulatory subset, but the precise signals controlling differentiation into this fate are not well

434 understood (46). Using a classical in vitro suppression assay with cells from OT-I Bim$^{-/-}$ > RIP-mOVA
435 chimeras as a source of potential suppressors, we did not observe decreased activation of wildtype OT-I
436 T cells. Additionally, in our co-adoptive transfer experiments, neither the total splenocyte nor the CD8^{+}
437 T cell compartments appeared to contain dominant suppressors. These findings indicate that the
438 functional impairment observed when clonal deletion is prevented is cell-intrinsic in nature.
439 Furthermore, it would suggest that simple impairment of clonal deletion does not lead to the generation
440 of CD8^{+} T regulatory cells and that other factors are involved. However, it remains possible that the
441 mechanisms of tolerance vary with the antigen, quantity of antigen, and presenting APC, as has been
442 previously suggested (8, 9).
443 It is known that the co-inhibitory receptor PD-1 plays a role in regulating T cell function and is
444 induced in DP thymocytes following strong TCR signaling. In the 2C TCR transgenic model of negative
445 selection, PD-1 deficiency resulted in enhanced generation of DN thymocytes (24) while no such effect
446 was observed in MHCI- or MHCII-restricted male antigen reactive TCR transgenic models (25). We
447 found that PD-1 was similarly induced on OT-I CD8SP thymocytes that survived deletion in RIP-
448 mOVA recipients. Deletion of PD-1 did not dramatically affect the number of OT-I CD8SP thymocytes
449 in RIP-mOVA chimeras, although there were increased numbers of OT-I T cells in the spleen and
450 pancreatic lymph nodes of these mice, as was similarly observed for MOG-specific CD4^{+} T cells on a
451 polyclonal background (47). We think it is unlikely that tolerance was broken due to the elevated
452 numbers of Ag-specific cells, but rather that PD-1 signaling induced a tolerogenic program in the OT-I T
453 cells, since OT-I Bim$^{-/-}$ > RIP-mOVA chimeras contain substantially more OT-I T cells than OT-I PD-1^{-}
454 $^{/-}$ > RIP-mOVA counterparts and tolerance was still enforced in OT-I Bim$^{-/-}$ T cells (6, 7).
455 PD-1 has primarily been investigated for its role in peripheral tolerance by limiting reactivation,
456 function, and trafficking of self-reactive T cells (10), but a role for PD-1 in establishing tolerance in the
457 thymus has not be reported. Through an adoptive transfer approach using thymocytes from OT-I Bim$^{-/-}$
458 > RIP-mOVA chimeras as a source of auto-reactive T cells and sub-lethally irradiated PD-L1$^{-/-}$ RIP-
459 mOVA recipients, we found that acute PD-1 signaling during development was sufficient to establish
460 stable tolerance. Furthermore, PD-1 blockade in tolerant OT-I Bim$^{-/-}$ > RIP-mOVA chimeras was unable
461 to break tolerance. This may be due to the blockade treatment falling short of 100 % effectiveness; in
462 this case, even low-level PD-1 signals may filter through and impact tolerance. Additionally, since PD-
463 L1$^{-/-}$ recipients of OT-I Bim$^{-/-}$ > RIP-mOVA thymocytes only began to go diabetic ~ 15 weeks post-
464 adoptive transfer, it may be that PD-1 antibody blockade was too brief to observe a similar effect on
465 tolerance (Fig. 5H). That PD-1 signaling during thymic selection is sufficient to induce tolerance is
466 further supported by Thangavelu *et al.* where PD-1-deficiency in polyclonal thymocytes or recent

467 thymic emigrants, but not established peripheral T cells precipitated a lethal, multi-organ autoimmunity
468 when adoptively transferred into Rag$^{-/-}$ recipients (25). Together with our findings, this suggests our
469 conclusions are broadly applicable to thymic selection overall. It was also recently shown that PD-1
470 could delay lethal autoimmunity in *Aire*$^{-/-}$ mice (48); in the absence of AIRE in this model, many TRA
471 would not be expressed in the thymus and instead would be first encountered in the periphery, where
472 PD-1 signals could induce protection against autoimmunity. Finally, intestinal inflammation and
473 mortality driven by naïve OT-I T cells in iFABP-OVA mice was shown to be restricted by PD-L1
474 blockade only when the treatment was administered the day of adoptive transfer, but not 30 days later
475 (49). Given observations made by us and others, we thus hypothesize that it is upon the first high affinity
476 antigen exposure that PD-1 signals are required to induce a durable tolerant state that resists
477 reinvigoration. This is in contrast to previous studies (50–53) proposing only a minor role for PD-1 in
478 early tolerance induction (see review (54)). However, many of these studies were performed on the
479 NOD background, wherein native tolerance is not established (55).

480 While acute PD-1 signals received by OT-I T cells in the thymus provided a relatively durable
481 tolerance when OT-I thymocytes were adoptively transferred into sub-lethally irradiated RIP-mOVA
482 PD-L1$^{-/-}$ recipients, we cannot formally discount a role for PD-L2. However, it appears that PD-L2 does
483 not exert a large effect on tolerance in this model. In our studies with OT-I Bim$^{-/-}$ > RIP-mOVA PD-L1^{-}
484 $^{/-}$ chimeras, tolerance is not established, and diabetes is induced rapidly even with unaltered PD-L2
485 expression. Similarly, the Sharpe group previously demonstrated in the NOD background a unique role
486 for PD-L1 in restraining self-reactive T cells in the pancreas (56). Additionally, thymocytes or
487 splenocytes from OT-I Bim$^{-/-}$ > RIP-mOVA mice adoptively transferred into RIP-mOVA PD-L1$^{-/-}$
488 recipients did not maintain tolerance indefinitely. Since in PD-L1 sufficient recipients adoptively
489 transferred thymocytes remained tolerant throughout the entire course of the experiment, PD-1 signaling
490 induced via interactions with PD-L1 likely aided in the maintenance of tolerance. It will be interesting to
491 assess the necessity for thymic PD-1 signaling by limiting PD-1 interactions to the periphery (e.g
492 adoptive transfer of thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA PD-L1$^{-/-}$ chimeras into PD-L1$^{+/+}$
493 recipients), in which case failure to establish tolerance would further suggest it is PD-1 signals received
494 specifically during first encounter with Ag in the thymus that are necessary for establishing tolerance.
495 Indeed, recent thymic emigrants are thought uniquely sensitive to tolerizing factors compared to more
496 mature circulating T cells, suggesting that PD-1 signals delivered in the thymus and periphery may lead
497 to functionally different outcomes (57).

498 Work done by the Fife group showed that established anergic peripheral T cells are not
499 reinvigorated by PD-L1 blockade (49). They and others postulated that this is due to an epigenetic

500 landscape resistant to reinvigoration in the context of "exhaustion" and tolerance (58–61). Given this,
501 we hypothesize that acute PD-1 signaling supports long-term tolerance by inducing a tolerant epigenetic
502 memory. Using a double transgenic TCR_{GAG} Alb:GAG model, the Greenberg group recently showed
503 that self-reactive T cells that escape thymic deletion do not cause liver injury or respond to
504 immunization with LM-GAG. These cells regained function through robust lymphopenia-induced
505 proliferation (LIP) and responded to LM-GAG immunization at early time points, but 3-4 months post-
506 adoptive transfer, functional impairment was reacquired. Their observations were similarly recorded
507 following transfer into both high affinity Ag-bearing (Alb:GAG) and Ag-lacking (C57BL/6) recipients
508 (58). Conversely, we failed to observe LIP by the functionally impaired $CD8^+$ T cells from OT-I $Bim^{-/-}$ >
509 RIP-mOVA chimeras and instead observed a dependence on persistent high affinity antigen for this
510 functional impairment. This difference may be because OT-I thymocytes developing in a RIP-mOVA
511 thymus normally undergo efficient thymic deletion, while in the TCR_{GAG} Alb:GAG model, deletion is
512 only partial. Further, it is unclear whether the functional impairment observed in TCR_{GAG} T cells was
513 established in the thymus, as was the case for OT-I T cells; therefore, the states of dysfunction our
514 groups documented may not be analogous (58). The tolerance-specific epigenetic program they and
515 others propose drives tolerance in self-reactive T cells (58, 59) may also underlie our observations. This
516 phenomenon is a clear obstacle for immunotherapies seeking to reinvigorate functionally impaired T
517 cells, thus future investigation should aim to determine the mechanism by which this occurs and its
518 relationship with PD-1.

519 Immune checkpoint blockade (ICB) therapies targeting the PD-1:PD-L1 signaling axis have
520 received considerable attention in the last 10 years for their effectiveness in treating a number of
521 different cancers. A recent study proposed that ICB targeting the PD-1:PD-L1 signaling axis promoted
522 the activation and expansion of newly recruited clones to an anti-tumour response, rather than
523 reinvigorating pre-existing tumour-infiltrating T lymphocytes as is often suggested to underlie the
524 success of PD-1 blockade (62). In this study, we provide evidence to support this alternate mechanism of
525 anti-PD-1 ICB therapies. If PD-1 is indeed critical for the establishment of tolerance rather than its
526 maintenance as we suggest, it follows that PD-1-targeting ICB may enhance recruitment of clones naïve
527 to the PD-1 tolerogenic signal. Targets of these therapies may originate in the periphery as tumour-
528 specific naïve T cells whose primary encounter with antigen is no longer rendered tolerogenic by PD-1,
529 or in the thymus as tumour-harboured self-antigen-specific thymocytes whose native tolerance through
530 PD-1 is prevented. Reinvigoration of "exhausted" CD8 T cells is still possible through processes
531 independent from what we have observed in this study, but here we introduce new considerations for the
532 understanding of anti-PD-1 targeting blockade therapies.

533 Despite the success of ICB therapies, clinical responses vary with different types of cancer, and
534 immune-related adverse events (IRAE) are a persistent risk (11, 63). It is conceivable that should PD-1
535 blocking antibodies reach the thymus, normal selection processes may be hindered. While this provides
536 a potential explanation for the development of some IRAEs, it may also be in some cases the mechanism
537 behind successful cancer immunotherapies. This highlights the need for a greater understanding of the
538 mechanism by which PD-1 supports normal tolerance and how this relates to ICB therapies. Overall, we
539 provide evidence for a novel role for PD-1 in the establishment of tolerance to TRA in the thymus in
540 escapees from clonal deletion. Depending on the conditions of antigen presentation (antigen density,
541 presenting cells, location of first encounter), PD-1 signals may induce states of tolerance or simply aid in
542 its maintenance, potentially with different functional outcomes.
543

544 **Figure legends:**
545

546 Figure 1: TRA-Specific T Cells in OT-I Bim$^{-/-}$ > RIP-mOVA Chimeras Are Functionally Impaired Via
547 Cell-Intrinsic Mechanisms.
548 A, Splenocytes from OT-I WT (CD45.1), OT-I Bim$^{-/-}$ > RIP-mOVA or a 1:1 mixture of the above
549 splenocytes were cultured with un-pulsed stimulator splenocytes or stimulator splenocytes pulsed with
550 OVA peptide in vitro. Activation was measured by CD69 and CD25 upregulation on day 2. B,
551 Compilation of data from A (n = 3). C, Day 3 proliferation of cultures described in A measured by
552 dilution of CFSE dye. D, Compilation of proliferation indices from cultures described in A (n = 4). E,
553 Incidence of diabetes in sub-lethally irradiated RIP-mOVA recipients of adoptively transferred splenic
554 CD8^{+} Vα2^{+} OT-I T cells from OT-I Bim$^{-/-}$ > RIP-mOVA and/or OT-I WT. Note that 7 recipients with
555 OT-I Bim$^{-/-}$ > RIP-mOVA transfers received whole spleen while all other recipients in this experiment
556 received CD8-enriched isolates from pooled spleen and lymph nodes (SPL+LN). Log-rank (Mantel-
557 Cox) test results are relative to the OT-I WT → RIP-mOVA group. Data are representative of 4 separate
558 cohorts. F, Recipients of CD8-enriched isolates (SPL+LN) from OT-I Bim$^{-/-}$ > RIP-mOVA and OT-I
559 WT described in E were analyzed for expansion of either donor cell population within the spleen. Total
560 donor cells were identified by OVA-K^{b} tetramer staining. For each assay described above, the number of
561 independent experiments (IE) ≥ 3. Data are mean ± SD. Asterisks represent statistical significance as
562 determined by a two-way ANOVA with Sidak's multiple comparisons test (B, D), a paired t test (F) or
563 Log-rank test (E). Statistical comparisons between other groups of interest are indicated. * $p \leq 0.05$, ** p
564 ≤ 0.01, *** $p \leq 0.001$, **** $p \leq 0.0001$.
565

566 Figure 2: Splenic and Thymic TRA-Specific T Cells in OT-I Bim$^{-/-}$ > RIP-mOVA Chimeras Are
567 Functionally Impaired.
568 A, Schematic representing experiments carried out in B-F. Activation marker induction (B),
569 representative histograms for thymocyte proliferation (C), and compiled proliferation indices (D) of OT-
570 I Bim$^{-/-}$ > RIP-mOVA (n = 4) or OT-I Bim$^{-/-}$ > WT thymocytes (n = 3) following *in vitro* stimulation.
571 Open histograms represent culture with OVA peptide-pulsed stimulators, while greyed histograms
572 represent no-peptide control stimulators. E, Distribution of Vα2^{+} CD8SP OT-I thymocytes among semi-
573 mature (SM; H-2K$^{b\ lo}$), Mature 1 (M1; CD69^{hi} H-2K$^{b\ hi}$), and Mature 2 (M2; CD69^{lo} H-2K$^{b\ hi}$)
574 population. F, From either OT-I Bim$^{-/-}$ > RIP-mOVA or > WT bone marrow chimeras, 2-5x10^{6} Vα2^{+}
575 CD24^{lo} thymocytes (THY) or Vα2^{+} OT-I T splenocytes (SPL) from whole spleen were transferred to
576 sub-lethally irradiated RIP-mOVA recipients, which were then monitored for the induction of diabetes.

577 Log-rank (Mantel-Cox) test results are relative to organ transfer groups. For each assay described above,
578 the number of independent experiments (IE) ≥ 3. Data are mean ± SD. Asterisks represent statistical
579 significance as determined by a two-way ANOVA with Sidak's multiple comparisons test (B), unpaired
580 t test with Welch's correction (D), or Log-rank test (E). Statistical comparisons between other groups of
581 interest are indicated. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
582
583 Figure 3: *PD-1 signals in the T cell compartment necessarily support tolerance to TRA*.
584 A, PD-1 expression on OT-I T cells across organs in OT-I > RIP-mOVA (open histogram) and OT-I >
585 WT (greyed histogram) bone marrow chimeras. Data is representative of n ≥ 3. B, Incidence of diabetes
586 in RIP-mOVA chimeras with OT-I WT, OT-I PD-$1^{-/-}$, a 1:1 mix of the former and latter, and OT-I $Bim^{-/-}$
587 PD-$1^{-/-}$ bone marrow. Data are generated from 3 separate cohorts of chimeras. C, Incidence of diabetes
588 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-
589 mOVA chimeras were sacrificed alongside their PD-$1^{fl/ko}$ counterparts to confirm the timing of PD-1
590 ablation (IE = 4). Data are compiled from 4 separate chimera cohorts. Asterisks represent statistical
591 significance as determined by Log-rank test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
592
593 Figure 4: OT-I PD-$1^{-/-}$ > RIP-mOVA chimeras exhibit a mild impairment in clonal deletion and
594 increased numbers of OT-I $V\alpha2^{+}$ $CD8^{+}$ T cells in the periphery. A, (Left) Thymic profiles of WT or PD-
595 $1^{-/-}$ OT-I > RIP or B6 chimeras; (Right) Frequency of mature thymocytes within the CD8SP subset. B,
596 Absolute number of $CD8^{+}$ $V\alpha2^{+}$ $CD24^{lo}$ mature OT-I thymocytes in chimeras from A. A data point from
597 the PD-$1^{-/-}$ > B6 group was determined to be an outlier using the Grubb's test ($p < 0.05$) and was
598 therefore omitted. C, Absolute number of $CD8^{+}$ splenic OT-I. D, Absolute number of $CD8^{+}$ OT-I in the
599 pancreatic lymph nodes. Data above represent ≥ 5 independent experiments (IE). Data are mean ± SD.
600 Asterisks represent statistical significance as determined by a two-way ANOVA with Sidak's multiple
601 comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
602
603
604 Figure 5: Acute PD-1 signaling within the thymic compartment is sufficient to induce tolerance to TRA.
605 Splenocytes from either OT-I $Bim^{-/-}$ > RIP or WT chimeras were cultured *in vitro* with peptide pulsed
606 whole-spleen stimulators +/- αPD-1 antagonist antibody. A, Day 2 activation marker expression for
607 responding OT-I splenocytes (stimulated groups shown). B, Compilation of day 3 proliferation indices
608 for responding OT-I splenocytes. A data point in RIP-mOVA (aPD-1, proliferation index) group was

609 determined to be an outlier by the Grubb's test ($p < 0.05$) and removed from the dataset. C, Day 2
610 activation marker expression for OT-I thymocytes stimulated as in A ($n \geq 3$). D, Incidence of diabetes in
611 chimeras treated with αPD-1 antagonist antibody or isotype control. E, Incidence of diabetes in OT-I
612 Bim$^{-/-}$ > RIP-mOVA PD-L1$^{-/-}$ chimeras. The number of independent experiments ≥ 3. F, Schematic
613 demonstrating experiments conducted in G-H. G, Incidence of diabetes in sub-lethally irradiated RIP-
614 mOVA PD-L1$^{-/-}$ recipients of adoptively transferred CD8-enriched CD8^{+} Vα2^{+} splenocytes from OT-I
615 Bim$^{-/-}$ > RIP-mOVA chimeras. H, Incidence of diabetes in sub-lethally irradiated RIP-mOVA PD-L1$^{-/-}$
616 recipients of adoptively transferred thymocytes from OT-I Bim$^{-/-}$ > RIP-mOVA chimeras. For each assay
617 described above, the number of independent experiments (IE) ≥ 4 unless otherwise stated. Data are mean
618 ± SD. Asterisks represent statistical significance as determined by one-way ANOVA with Sidak's
619 multiple comparisons tests (A-C) or Log-rank tests (E, G, H). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$,
620 **** $p \leq 0.0001$.
621
622 Figure 6: Continuous Exposure to Antigen in the Periphery is Required to Maintain Functional
623 Impairments in OT-I Bim$^{-/-}$ > RIP-mOVA Chimeras
624 A, Schematic showing experiments conducted in B-G. OT-I Bim$^{-/-}$ bone marrow was transferred into
625 lethally-irradiated WT or RIP-mOVA recipients, or nude recipients. Nude recipients were previously
626 grafted with either a WT or RIP-mOVA thymus under the kidney capsule. B, Thymic CD8 by CD4
627 profile for each chimera described in A. C, Thymocytes from the chimeras described in A were cultured
628 *in vitro* with peptide-pulsed whole-spleen cell stimulators and analyzed on day 2 for activation marker
629 expression ($n = 3$); values are adjusted relative to WT or Nude (WT) controls within independent
630 experiments. D, Splenocytes from the chimeras described in A were stimulated as in C. *In vitro* cultures
631 were analyzed on day 3 for proliferation. E, Representative histograms of proliferation data described in
632 D. Open histogram represents stimulated treatment (peptide pulsed-stimulators), greyed histogram
633 represents no-peptide control stimulators; OT-I Bim$^{-/-}$ > WT ($n = 5$), OT-I Bim$^{-/-}$ > RIP-mOVA ($n = 5$),
634 OT-I Bim$^{-/-}$ > Nude (WT) ($n = 4$), OT-I Bim$^{-/-}$ > Nude (RT) ($n = 4$). F, *In vitro* cultures were re-
635 stimulated for 4 hours on day 5 in the presence of Brefeldin A and analyzed for TNFα and IFNγ
636 production. G, Compilation of data described in F; OT-I Bim$^{-/-}$ > WT ($n = 4$), OT-I Bim$^{-/-}$ > RIP-mOVA
637 ($n = 4$), OT-I Bim$^{-/-}$ > Nude (WT) ($n = 4$). For the OT-I Bim$^{-/-}$ > Nude (RT) group, only 3 replicates are
638 listed; a fourth data point was determined to be an outlier with Grubb's test ($p < 0.05$) and was thus
639 omitted. For each assay described above, the number of independent experiments (IE) ≥ 3. Data are
640 mean ± SD. Asterisks represent statistical significance as determined by a paired t test (C) or one-way

641 ANOVA with Sidak's multiple comparisons tests (D, G). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** p
642 ≤ 0.0001.

643 **Acknowledgements:**
644

645 The authors thank Dr. Heather Melichar and Stefanie Valbon for critical review of the manuscript. Flow
646 cytometry experiments were performed at The University of Alberta Faculty of Medicine & Dentistry
647 Flow Cytometry Facility, which receives financial support from the Faculty of Medicine & Dentistry
648 and Canada Foundation for Innovation (CFI) awards to contributing investigators. Health Sciences
649 Laboratory Animal Services and Mr. Bing Zhang provided animal husbandry and technical assistance.
650

651 **Footnotes:**
652 - This work was supported by a grant from the Canadian Institutes of Health Research (PS
653 156104) to T.A.B.
654 - Abbreviations used in this article:
655 WT; *Wildtype,* TRA; Tissue-Restricted Antigen, UbA; Ubiquitous Antigen, pMHC;
656 peptide-MHC, BM; Bone Marrow
657
658

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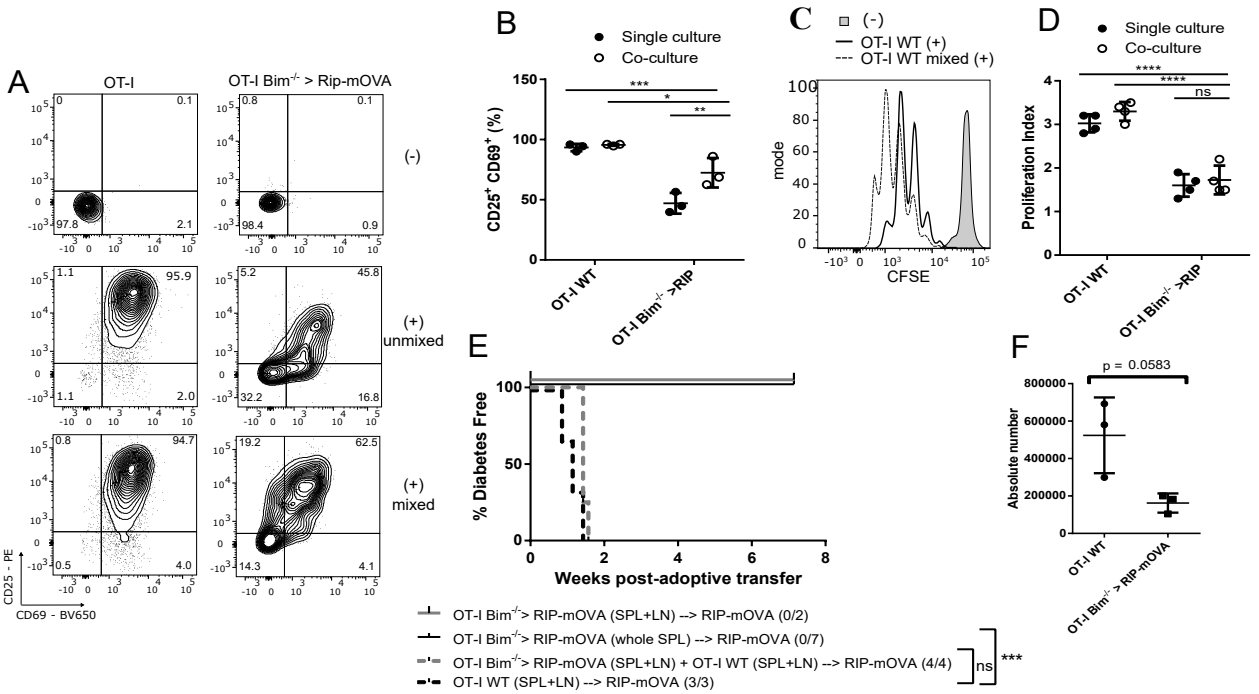
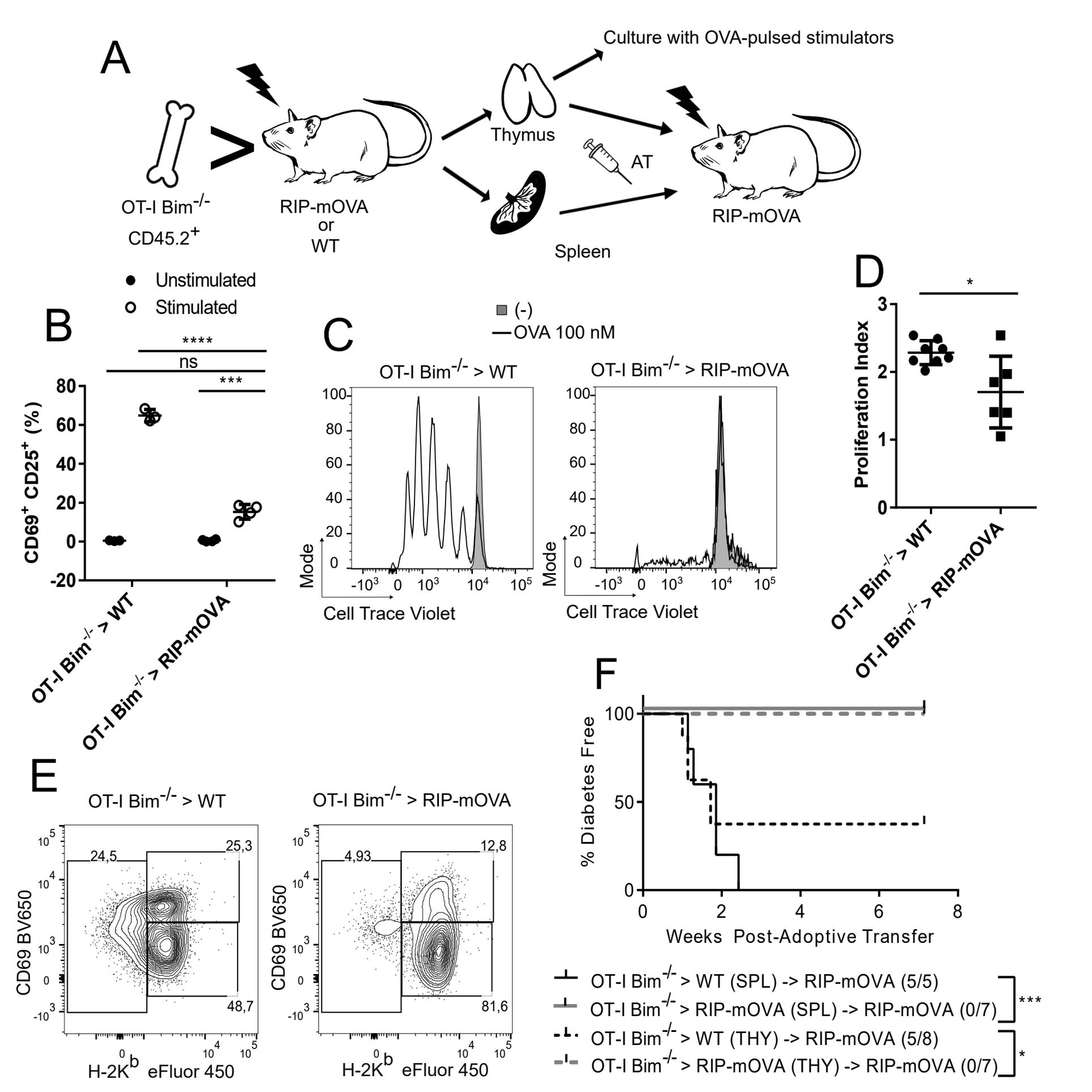
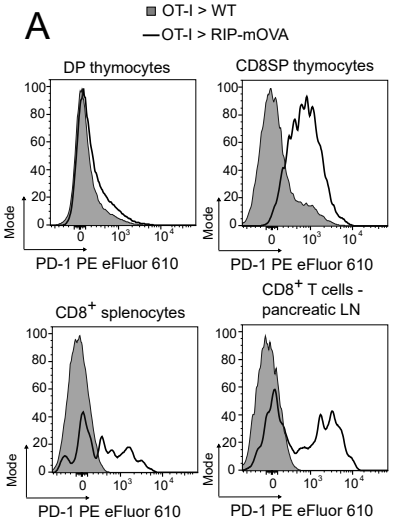
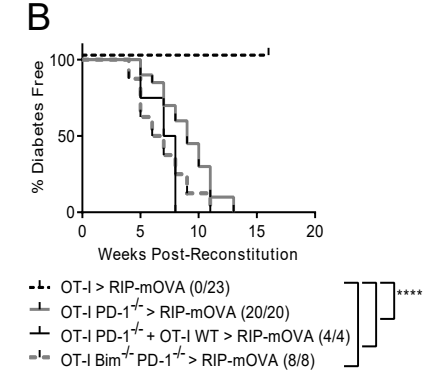
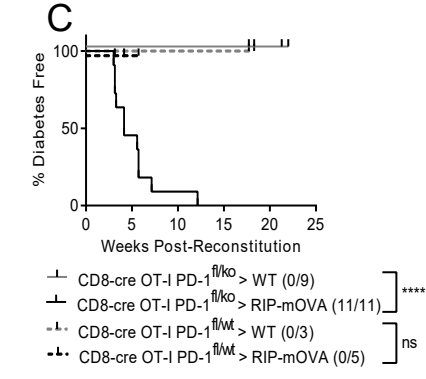
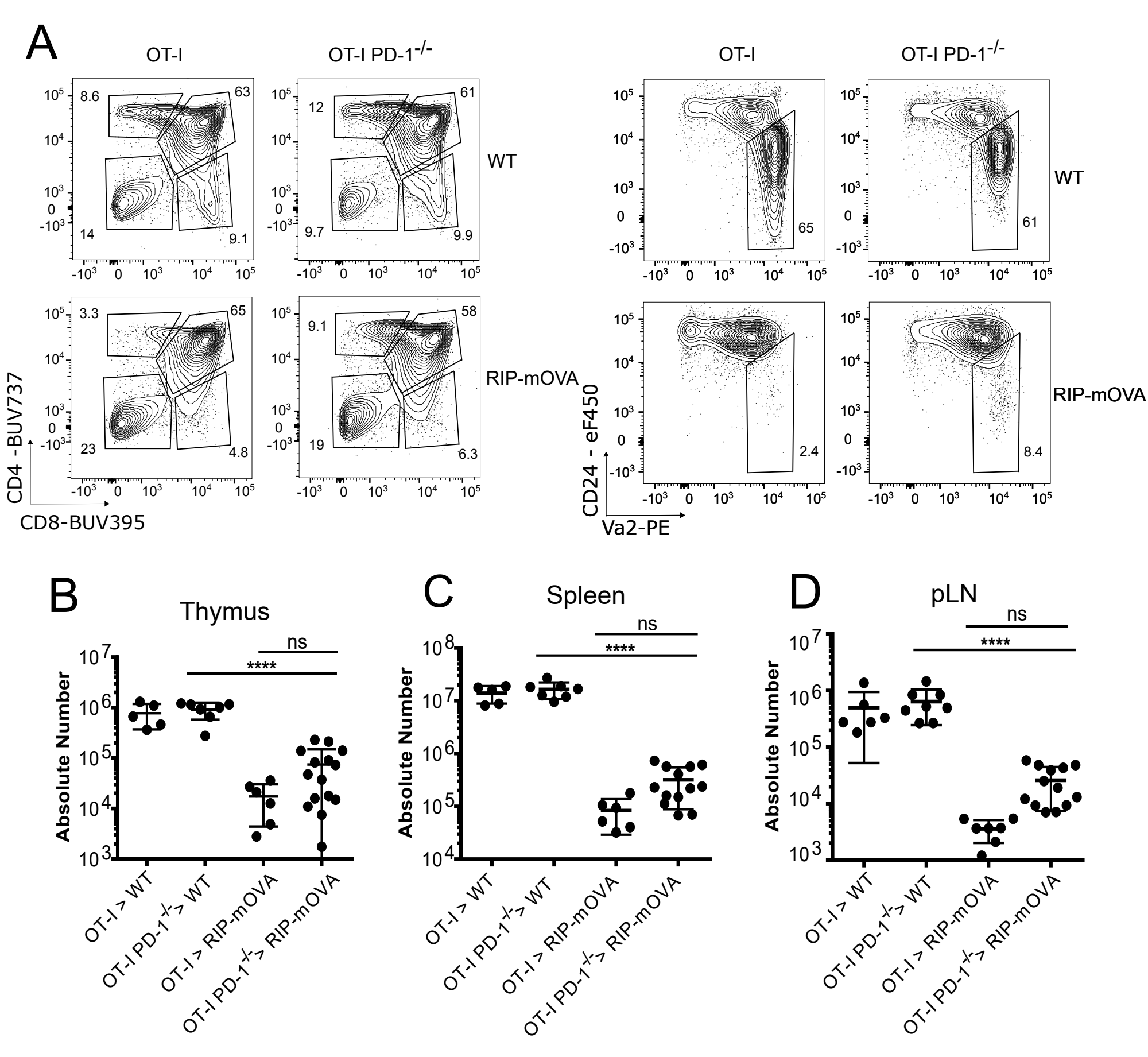

A
OT-I
OT-I Bim-/- > Rip-mOVA
(-)
(+) unmixed
(+) mixed
CD25 - PE
CD69 - BV650
B
Single culture
Co-culture
CD25+ CD69+ (%)
OT-I WT
OT-I Bim-/- >RIP
C
(-)
OT-I WT (+)
OT-I WT mixed (+)
mode
CFSE
D
Single culture
Co-culture
Proliferation Index
OT-I WT
OT-I Bim-/- >RIP
E
% Diabetes Free
Weeks post-adoptive transfer
F
p = 0.0583
Absolute number
OT-I WT
OT-I Bim-/- > RIP-mOVA
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
***

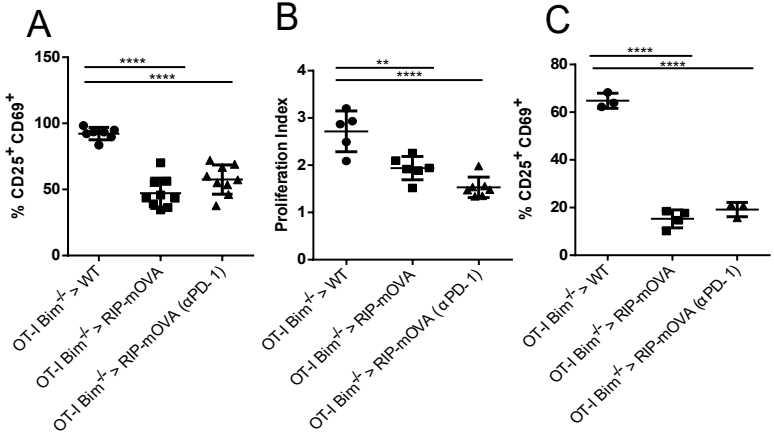

A
Culture with OVA-pulsed stimulators
Thymus
AT
OT-I Bim-/-
CD45.2+
RIP-mOVA
or
WT
Spleen
RIP-mOVA
B
Unstimulated
Stimulated
****
ns
***
CD69+ CD25+ (%)
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
C
(-)
OVA 100 nM
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
Mode
Cell Trace Violet
D
*
Proliferation Index
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
E
OT-I Bim-/- > WT
OT-I Bim-/- > RIP-mOVA
24,5
25,3
48,7
4,93
12,8
81,6
CD69 BV650
H-2Kb eFluor 450
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)
*


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)
****

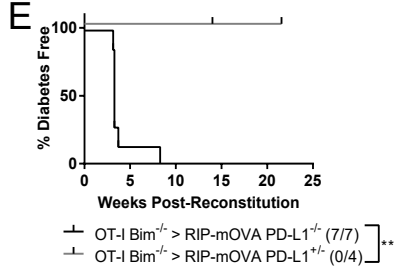

C
% Diabetes Free
Weeks Post-Reconstitution
CD8-cre OT-I PD-1fl/ko > WT (0/9)
CD8-cre OT-I PD-1fl/ko > RIP-mOVA (11/11)
CD8-cre OT-I PD-1fl/wt > WT (0/3)
CD8-cre OT-I PD-1fl/wt > RIP-mOVA (0/5)
****
ns

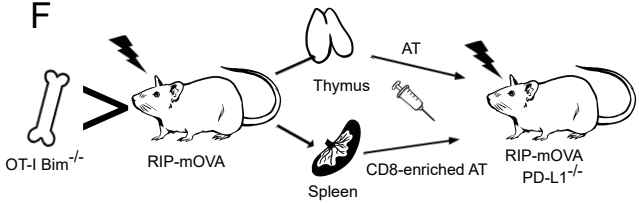

A
OT-I
OT-I PD-1-/-
OT-I
OT-I PD-1-/-
WT
RIP-mOVA
WT
RIP-mOVA
8.6
63
14
9.1
12
61
9.7
9.9
65
61
3.3
65
23
4.8
9.1
58
19
6.3
2.4
8.4
CD4 -BUV737
CD8-BUV395
CD24 - eF450
Va2-PE
B
Thymus
C
Spleen
D
pLN
Absolute Number
ns
****
OT-I > WT
OT-I PD-1-/- > WT
OT-I > RIP-mOVA
OT-I PD-1-/- > RIP-mOVA

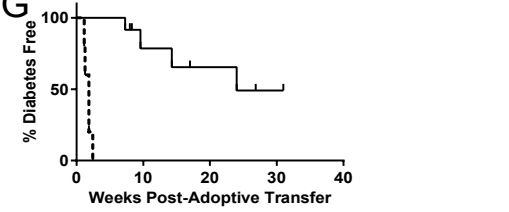


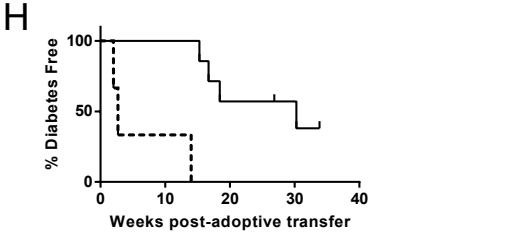
D

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

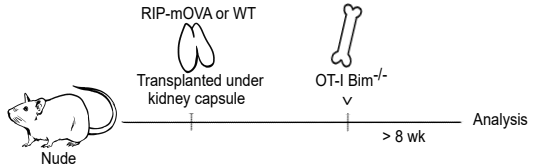






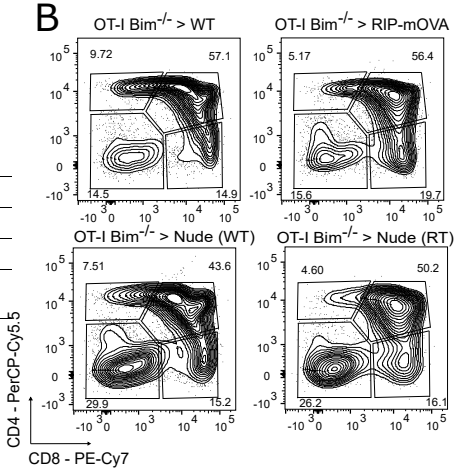


A



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



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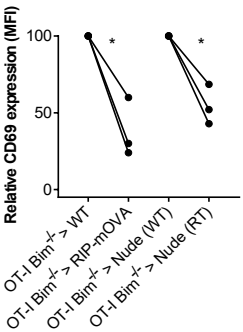

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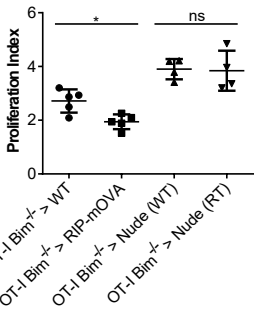

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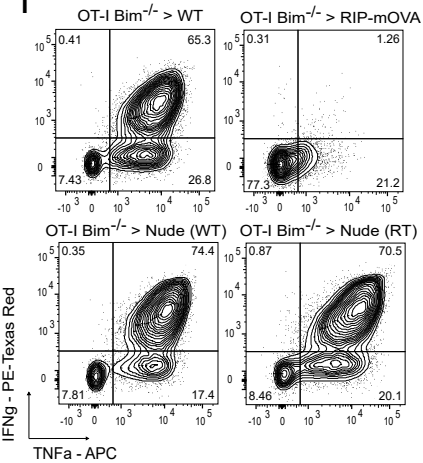

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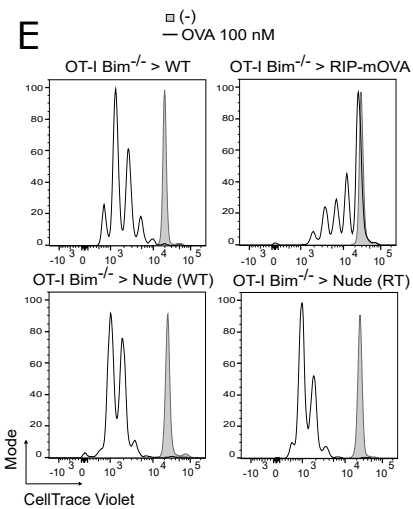
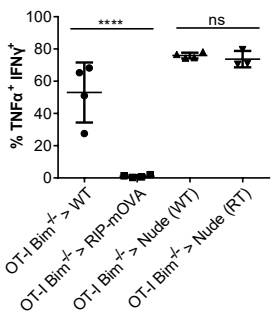
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