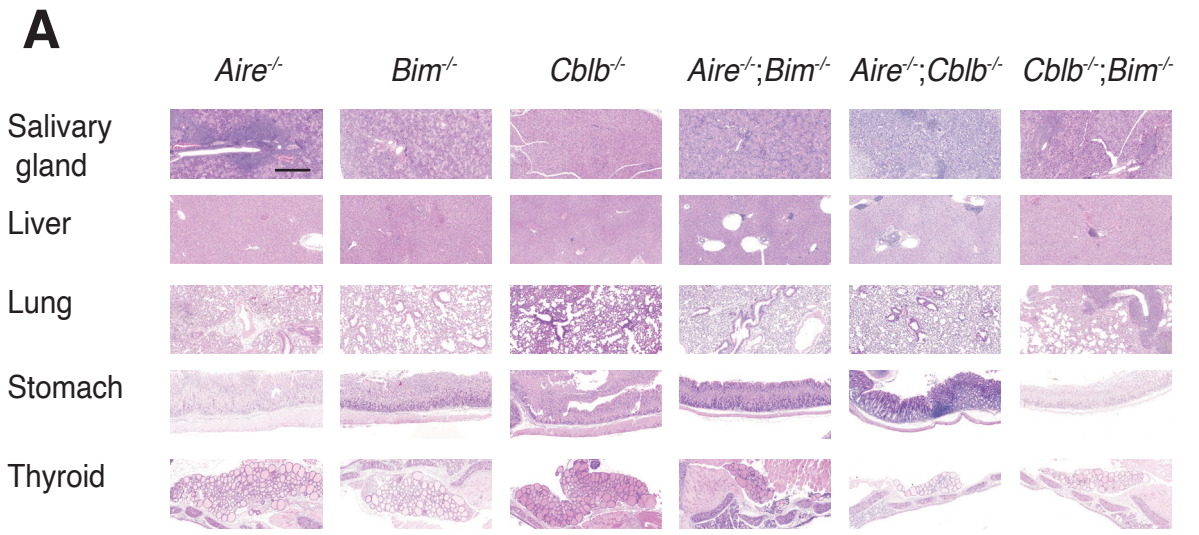
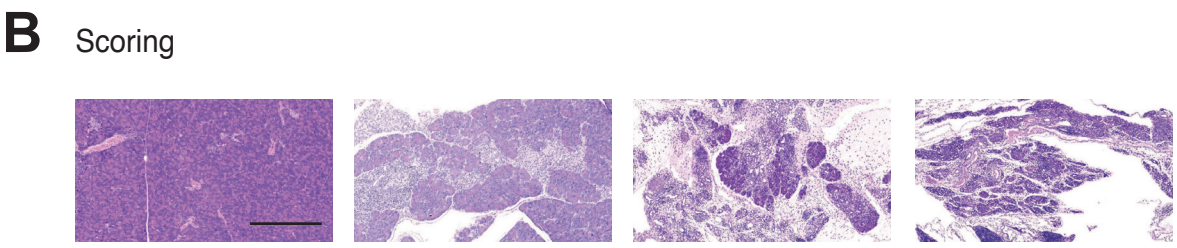
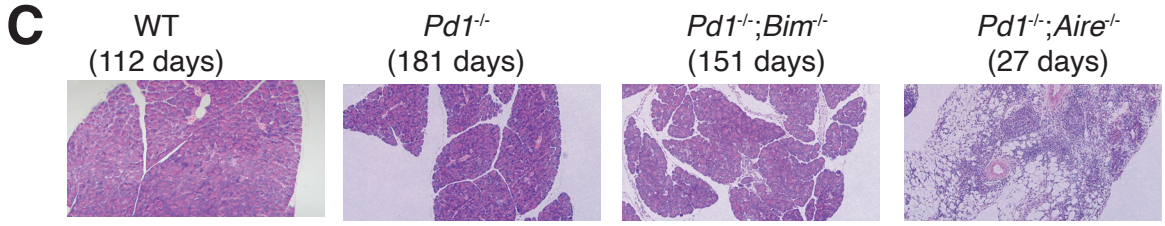
Supplementary figure 1





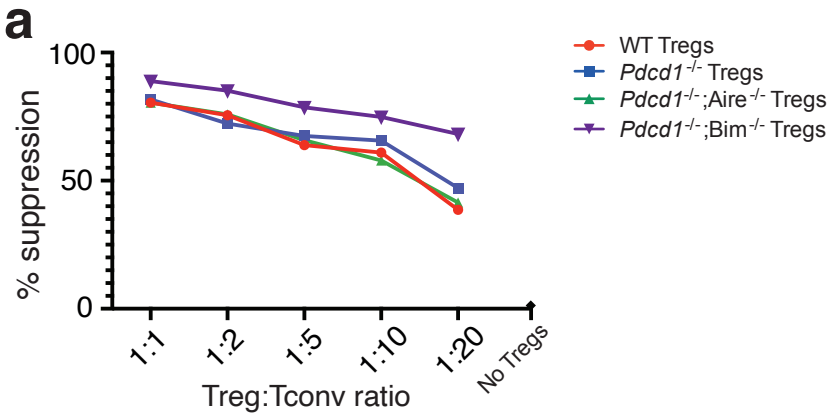


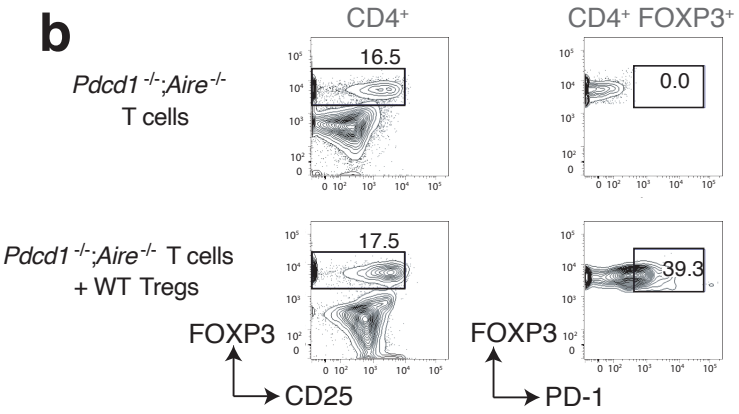
Supplementary Figure 1

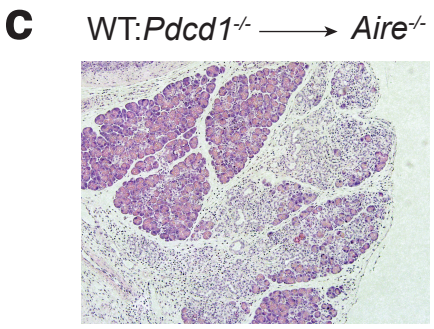
Autoimmune pathology resulting from combined defects in peripheral and central tolerance mechanisms.

a. Representative images of H&E-stained sections of the salivary gland, liver, lung, stomach and thyroid gland from mice of the indicated genotypes taken at 150 days. Scale bar = 500 μm.**b.** Representative images of H&E stained sections of pancreas showing examples of the scoring system used to quantify lymphocytic infiltration and tissue damage. Sections of pancreas are shown for scores of 0 (no infiltration or damage), 1 (mild lymphocytic infiltration, 50% or less), 2 (florid lymphocytic infiltration over 50% with/without some tissue damage) or 3 (complete tissue destruction). **c.** Representative images of H&E stained sections of pancreas from mice of the indicated genotype and age. Scale bar = 500 μm.

Supplementary figure 2

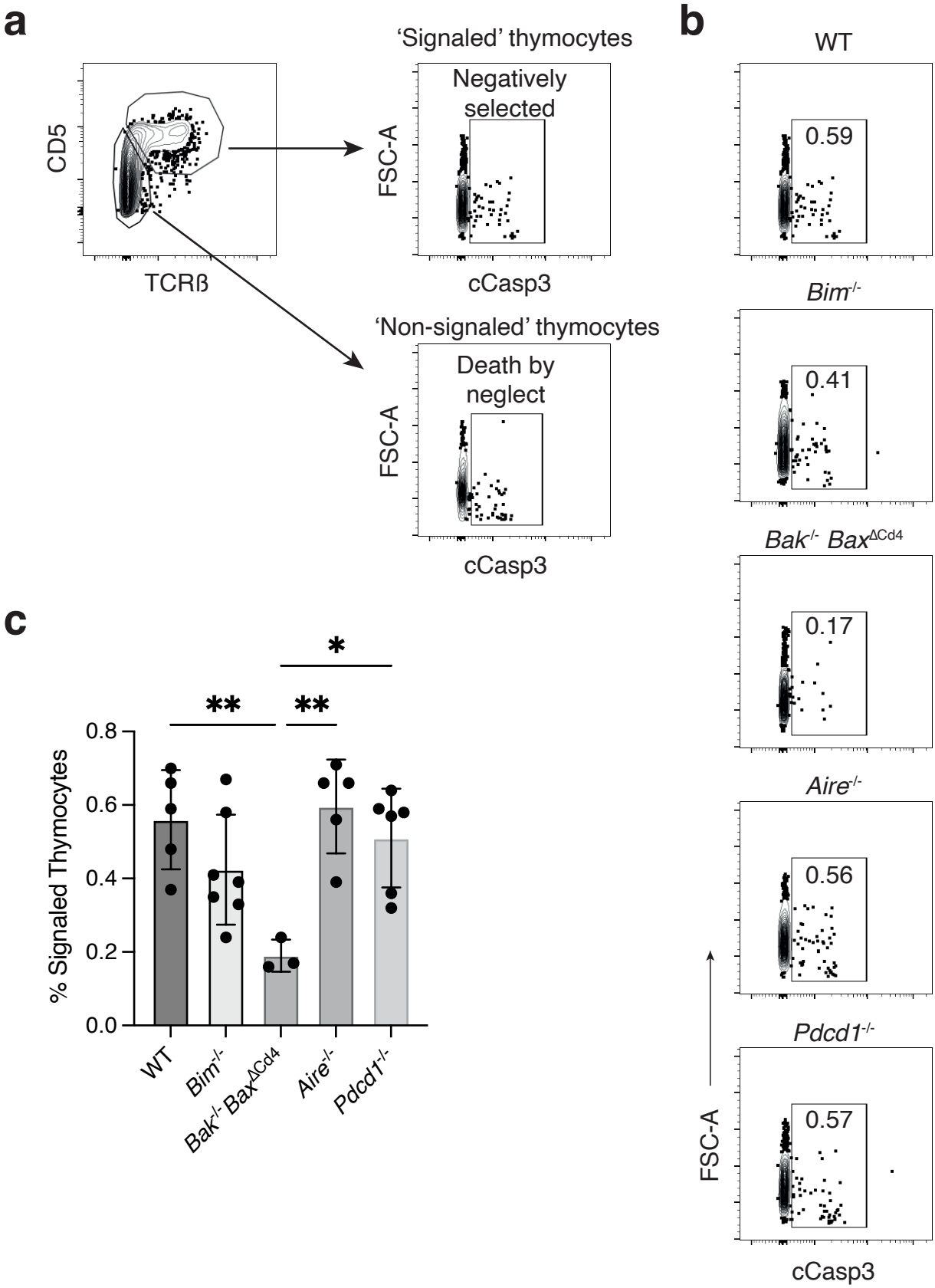






Supplementary Figure 2. Treg cell function in *Pdcd1*$^{-/-}$;*Aire*$^{-/-}$ mice. a. Purified CD4^{+} CD24^{+} Treg cells from the indicated genotypes were co-cultured with CTV-labeled, activated CD4^{+} CD25^{-} conventional T cells from WT mice. The percentage suppression was plotted across a range of Treg:Tconv ratios. **b.** Representative contour plots of FOXP3 versus CD25 (gated on CD4^{+} splenocytes) and FOXP3 *vs* PD-1 (gated on CD4^{+}FOXP3^{+} splenocytes) from *Rag1*$^{-/-}$ mice adoptively transferred with T cells from *Pdcd-1*$^{-/-}$;*Aire*$^{-/-}$ mice with or without co-administration of CD4^{+}Foxp3^{+} Treg cells from WT mice. **c.** Representative image of H&E-stained section of pancreas from *Aire*$^{-/-}$ haematopoietic chimera reconstituted with 50:50 WT:*Pdcd1*$^{-/-}$ bone marrow cells.

Supplementary figure 3



Supplementary Figure 3. Quantification of thymocyte deletion. a. Flow cytometry gating strategy for the identification of thymocytes undergoing clonal deletion or death by neglect by active (cleaved) caspase 3 expression. 'Signalled' and 'non-signalled' cells were distinguished on the basis of CD5 and TCRβ expression following the exclusion of $CD25^+$, $CD19^+$, $NK1.1^+$ and $TCR\gamma\delta^+$ cells. **b.** Representative contour plots of cells undergoing clonal deletion based on active Caspase 3 expression in signalled ($CD5^+$ $TCR\beta^+$) thymocytes from WT, *Bim*$^{-/-}$, *Bak*$^{-/-}$ *Bax*$^{\Delta Cd4}$, *Aire*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice. Numbers indicate the percentage of active Caspase 3^+ cells within the gate. **c.** Quantification of the percentages of active Caspase 3^+ 'signalled' thymocytes from WT, *Bim*$^{-/-}$, *Bak*$^{-/-}$ *Bax*$^{\Delta Cd4}$, *Aire*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice. Plots in **b.** are representative of two independent experiments, totalling n=3-7 mice/genotype. Data in **c.** is presented as mean ± s.d. * and ** denotes $p<0.05$ and $p<0.01$, respectively, by one-way ANOVA with multiple comparisons test.

Supplementary Methods

Flow cytometry

Dissected lymphoid organs were collected in RPMI medium with 25.96 mM HEPES, mashed between frosted glass slides and filtered through 100 μM mesh. Cells ($3x10^6$) were stained with antibody conjugates to detect cell surface proteins for 15 min at 4˚C. Cells were then washed in FACS buffer and resuspended in a final volume of 200 μL of FACS buffer containing Propidium Iodide at a final concentration of 2.5 μg/mL prior to acquiring the data to enable exclusion of dead cells.

Intra-cellular staining was performed with the FOXP3 staining kit (Invitrogen eBiosciences, California). Cell pellets were resuspended in 100 μL of fixation/permeabilization diluent solution (1:4) for 1 h at 4ºC and cells were then stained with antibodies for 30 min at 4ºC. Cells were washed with permeabilization solution and then resuspended in 100 μL FACS buffer. The stained cells were acquired on a Fortessa flow cytometer (BD Biosciences), calibrated with single-color stains for electronic compensation of spectral overlap between fluorochromes. Data were analyzed using Flowjo software (TreeStar).

Antibodies and immunoconjugates

Surface labeling of lymphocyte populations was performed using the following antibodies that were made in the WEHI Monoclonal Antibody Facility, unless otherwise stated: unconjugated anti-mouse CD16/32 FcγR block, anti-CD4 PerCP/Cy5.5 (clone 30-F11, Biolegend, California), anti-CD8 APC/Cy7 or BV650 (clone 53-6.7, Biolegend, California), anti-TCRβ PE-CY7 (clone H57.59.1, Biolegend) or anti-TCRβ BUV395 (clone H57.59.1, BD Biosciences, California), anti-CD25 BV510 (clone PC61, Biolegend, California) or anti-CD25 FITC (clone PC61), anti-CD44 FITC (clone IM781) or anti-CD44 Alexa Fluor 700 (clone 1M7, Biolegend, California), anti-CD19 FITC (clone 1D3) ,anti-CD62L APC/Cy7 (clone MEL-14, Biolegend, California), anti-NK1.1 biotin (clone PK136, Biolegend, California), anti-TER119 biotin (TER119), anti-GR1 biotin (clone RB6-8C5), anti-MAC-1 biotin (clone M1-170), anti-B220 biotin (RA3-6B2), anti-TCRγδ biotin (clone GL3, Biolegend, California), anti-CD5 APC (clone 53-7.3, Biolegend,

California), anti-PD-1 PE (clone 29F.1AI2, Biolegend, California) and anti-CTLA4 APC (clone UC10-4B9, Biolegend, California). Second step labeling to detect biotinylated antibodies was performed with streptavidin BV786 or BV711 (Biolegend, California). Intracellular staining was performed with anti-FOXP3 e450 (clone FJK-165, Invitrogen eBioscience, California), anti-active Caspase 3 PE (clone C92-605, BD Pharmingen, California) and anti-human Ki67 FITC (clone MOPC-21, BD Pharmingen) after fixation and permeabilization with eBioscience FOXP3 staining kit.

***In vitro* Treg cell suppression assay**

Single cell suspensions of spleen and lymph nodes (pooled axial and brachial) were mashed and filtered. Splenocyte suspensions were incubated with 2 mL red cell removal buffer (RCRB) for 2 min, the cell suspension underlayed with 1 mL of FCS and washed in KDS/BSS medium. Spleen and lymph node cell suspensions were combined and stained with antibody conjugates labeling CD4, CD8, CD25 for 30 min at 4°C and washed. Cells were resuspended with 2.5 μg/mL PI in KDS/BSS medium and then viable $CD4^+$ conventional T cells or $CD4^+$ Treg cells were purified using an Aria II FACS sorter (BD Biosciences). Cells were washed in KDS/BSS medium and re-filtered into polypropylene tubes. Conventional T cells were washed with PBS, resuspended in Cell Trace Violet (CTV) solution in 0.1% BSA/PBS at a final concentration of 5 μM for 10 min at 37°C. RPMI complete medium (5 mL) was added to the cells and incubated for 10 min at 4°C to quench the reaction. Cells were then washed at 4°C and resuspended in RPMI complete medium for plating at $1x10^4$/well. Sorted Treg cells were recovered by centrifugation and resuspended in RPMI complete medium with anti-CD3 antibodies (1 μg/mL) and plated at varying cell numbers. Irradiated splenocytes (30 Gy) were used as APCs and plated at $4x10^4$/well in RPMI complete medium. Cells were cultured for 72 h in an incubator set at 37°C with 10% CO_2-in-air. Flow cytometry was performed on a Fortessa (BD Biosciences). The percentage suppression was calculated as (% divided cells in no Treg well/% divided cells in test well)/% divided cells in no Treg well $\times$ 100.