Inventory of Supplemental Information

Main text figure 1 is supported by Supplemental Figure 1.

Main text figure 2 is supported by Supplemental Figure 2.

Main text figure 3 is supported by Supplemental Figure 3.

Main text figure 4 is supported by Supplemental Figure 4.

Main text figure 5 is supported by Supplemental Figure 5.

Main text figure 6 is supported by Supplemental Figure 6.

A

Chimera	Foxp3⁻ n mice (n expts)	Foxp3⁻ Sorted # x10³ Mean (SE)	Foxp3⁻ # of reads	Foxp3⁻ Unique # of TCRs	Foxp3⁺ n mice (n expts)	Foxp3⁺ Sorted # x10³ Mean (SE)	Foxp3⁺ # of reads	Foxp3⁺ Unique # of TCRs
WT → WT	7 (3)	420 (35)	19762	4558	7 (3)	35 (4)	21545	1451
MHC II def. → WT	7 (3)	613 (55)	18276	5021	7 (3)	41 (4)	17480	1488
WT → WT	6 (3)	540 (40)	10960	1978	6 (3)	36 (1)	6694	768
WT → C2TAkd	7 (3)	345 (22)	13029	2173	7 (3)	22 (3)	9941	734

B

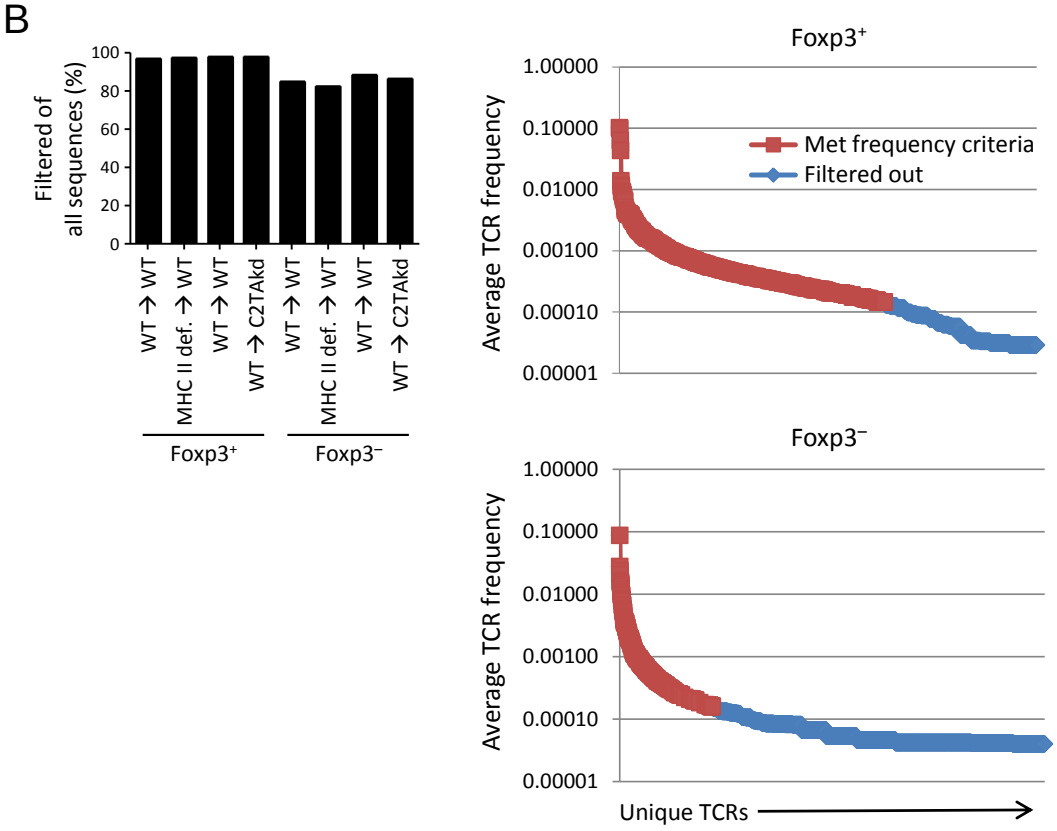


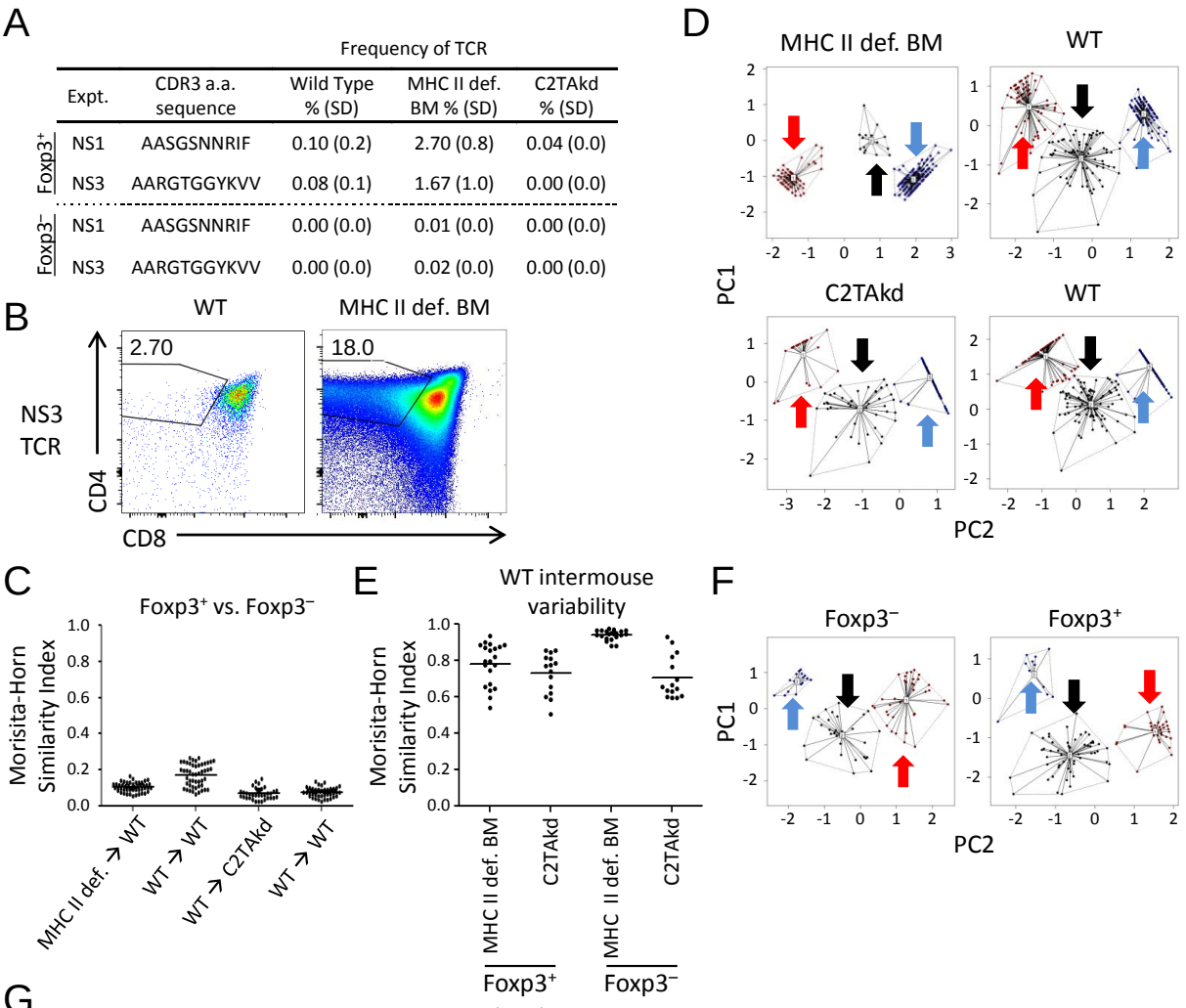
Figure S1, related to Figure 1. Both BM and mTEC APCs mediate negative selection.

(A) 454 pyrosequencing of TCRs from MHC II deficient (def.) BM and C2TAkd chimeras and the corresponding controls which are wild-type (WT) for MHC expression. Chimeras were generated using TCliβ *Tcra*$^{+/-}$ (MHC II def. or WT) TCR transgenic donors. After 6 weeks, Foxp3^{+} (CD4^{+}CD8^{-}Foxp3^{gfp+}) and Foxp3^{-} (CD4^{+}CD8^{-}CD62L^{hi}CD24^{lo}Foxp3^{gfp-}) cells were sorted and the TCRs sequenced by 454. (B) Effect of filtering TCRs that are found > 0.1% in at least one mouse in a given condition. Shown are plots of the total frequency captured by the filtered TCRs (left) and the average frequency in a given

experimental condition of the TCRs lost by this filtering criteria in the WT → WT Treg and Tconv repertoires (right)**.**

A

	Expt.	CDR3 a.a. sequence	Frequency of TCR: Wild Type % (SD)	MHC II def. BM % (SD)	C2TAkd % (SD)
Foxp3+	NS1	AASGSNNRIF	0.10 (0.2)	2.70 (0.8)	0.04 (0.0)
	NS3	AARGTGGYKVV	0.08 (0.1)	1.67 (1.0)	0.00 (0.0)
Foxp3−	NS1	AASGSNNRIF	0.00 (0.0)	0.01 (0.0)	0.00 (0.0)
	NS3	AARGTGGYKVV	0.00 (0.0)	0.02 (0.0)	0.00 (0.0)



G

Frequency of Top 15 WT Foxp3+ CD4+ TCR in indicated subsets

		Wild Type		MHC II def. BM		C2TAkd	
TCR Name	CDR3 a.a. sequence	Foxp3+ Mean (SD)	Foxp3− Mean (SD)	Foxp3+ Mean (SD)	Foxp3− Mean (SD)	Foxp3+ Mean (SD)	Foxp3− Mean (SD)
G25	AASADYSNNRLT	9.49 (2.4)	0.00 (0.0)	1.28 (1.6)	0.52 (0.2)	10.81 (4.8)	0.01 (0.0)
G69	AARNYNQGKLI	8.19 (3.0)	0.01 (0.0)	0.48 (0.3)	0.02 (0.0)	10.08 (2.2)	0.01 (0.0)
G41	AASAGYSNNRLT	7.44 (2.5)	0.00 (0.0)	0.02 (0.0)	0.98 (0.3)	8.38 (2.7)	0.01 (0.0)
G126	AANSGTYQR	3.24 (2.4)	0.00 (0.0)	2.33 (1.1)	0.00 (0.0)	0.88 (1.0)	0.00 (0.0)
R19	AASSGTYQR	1.24 (0.8)	0.10 (0.0)	0.13 (0.2)	0.50 (0.1)	0.54 (0.6)	0.07 (0.1)
R25	AASFYNQGKLI	0.99 (0.8)	0.00 (0.0)	1.34 (0.6)	0.03 (0.0)	0.43 (0.5)	0.00 (0.0)
JP2	AAKGNNNAPR	0.77 (0.6)	0.00 (0.0)	0.22 (0.2)	0.13 (0.0)	0.44 (0.4)	0.00 (0.0)
R35	AASSDYSNNRLT	0.76 (0.5)	0.02 (0.0)	0.16 (0.2)	0.02 (0.0)	0.96 (1.6)	0.00 (0.0)
G32	AASAGNYAQGLT	0.70 (0.7)	0.31 (0.2)	2.51 (1.3)	0.05 (0.0)	0.01 (0.0)	0.47 (0.3)
JP5	AAKSGSFNKLT	0.70 (0.7)	0.00 (0.0)	0.89 (0.4)	0.00 (0.0)	0.89 (1.7)	0.00 (0.0)
JP13	AASVSGSFNKLT	0.44 (0.5)	0.04 (0.0)	0.98 (0.9)	0.30 (0.1)	1.21 (2.1)	0.05 (0.0)
JP3	AAKDNNNAPR	0.43 (0.2)	0.01 (0.0)	0.09 (0.2)	0.08 (0.0)	0.84 (1.6)	0.00 (0.0)
JP8	AARGTNAYKVI	0.40 (0.4)	0.00 (0.0)	0.17 (0.3)	0.14 (0.0)	1.08 (2.2)	0.01 (0.0)
G103	AASAQTGGYKVV	0.38 (0.4)	0.02 (0.0)	0.09 (0.2)	0.02 (0.0)	0.59 (0.7)	0.05 (0.0)
R117	AASLDYSNNRLT	0.33 (0.3)	0.00 (0.0)	0.00 (0.0)	0.15 (0.0)	0.34 (0.7)	0.00 (0.0)

Figure S2, related to Figure 2. Role of BM APCs and mTECs in thymic Treg cell selection.

(A) Distribution of NS1 and NS3 in the TCR repertoires of WT, MHC II def. BM and C2TAkd conditions. (B) NS3 is negatively selected by BM APCs. Representative FACS plots of *Rag1*$^{-/-}$ thymocytes retrovirally-transduced with TCR NS3 and transferred into the thymi of WT, MHC II def. BM and C2TAkd chimeric mice. Plots are representative of 2 independent experiments with 1-2 replicates. (C) Assessment of similarity between Treg and Tconv TCR repertoires using Morisita-Horn analysis. Each Treg cell data set was compared to each Tconv data set for the indicated BM chimeras. (D) Principal component analysis comparing Foxp3^{+} versus Foxp3^{-} CD4SP TCR data sets from the MHC II def. BM and C2TAkd conditions (left panels, Tconv variances: MHC II def. BM = 13.6%, C2TAkd = 7.6%; Treg variances: MHC II def. BM = 60.0%, C2TAkd = 39.2%), and the corresponding WT condition (right panels, Tconv variances: MHC II def. BM = 20.2%, C2TAkd = 9.1%; Treg variances: MHC II def. BM = 47.7%, C2TAkd = 30.0%). Red and blue dots/arrows indicate TCRs enriched in Tconv and Treg subsets respectively, whereas black dots/arrow indicate TCRs that overlap both subsets. Centroids represent the middle most point of a given cluster and the shorter the line the more similar a given TCR is to that centroid. (E) Assessment of intermouse variability using Morisita-Horn similarity analysis. TCR data sets from each WT mouse was compared with every other mouse within a given APC condition and T cell subset. (F) Principal component analysis of MHC II def. BM and C2TAkd TCR repertoires (Tconv variances: PC1 = 10.3%, PC2 = 54.2%; Treg variances: PC1 = 12.0%, PC2 = 25.1%). Dots/arrows are as described in (D). (G) Amino acid sequence of the top 15 WT TCRs and their frequencies in the WT, MHC II def. BM, and C2TAkd Treg and Tconv cell data sets.

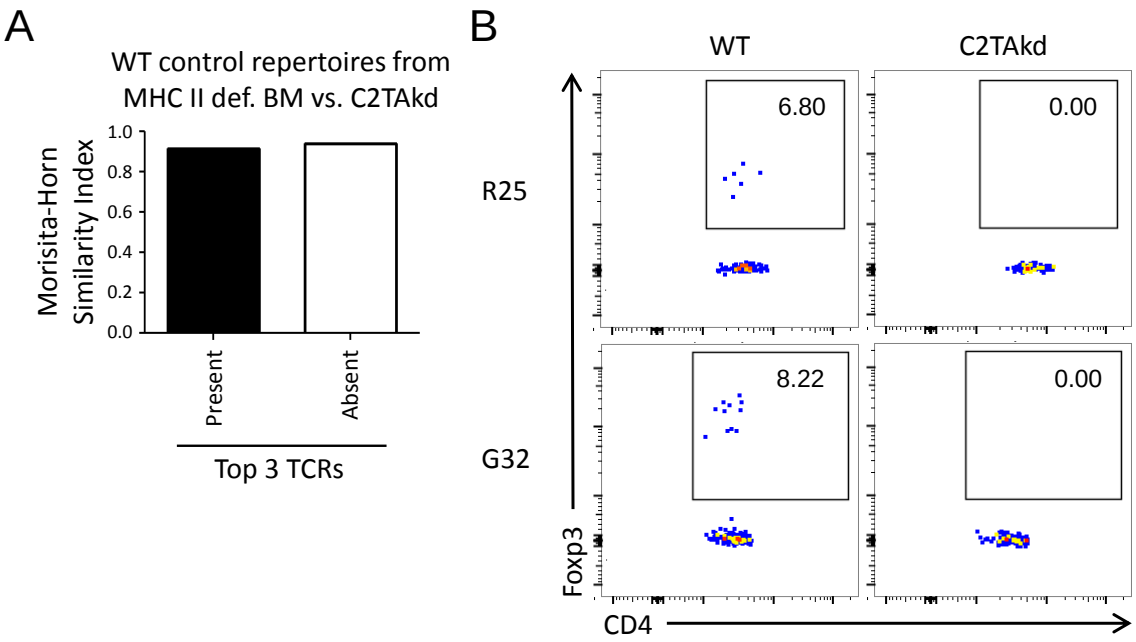


Figure S3, related to Figure 3. mTEC antigen presentation is required for in vivo Treg cell selection of certain TCRs.

(A) Morisita-Horn similarity analyses between TCR repertoires of control WT → WT BM chimeras in the MHC II def. → WT and WT → C2TAkd chimera experiments in Figure 3B. (B) As summarized in Figure 3C, TCRs dependent on autologous mTEC presentation for Treg cell selection (R25, G32) were retrovirally expressed in *Rag1*$^{-/-}$ thymocytes and intrathymically injected into WT or C2TAkd hosts. After 2.5 weeks, thymi were analyzed by flow cytometry. Plots shown are gated on CD45 congenic markers, Vα2^{+} and CD4SP for TCR expressing cells, and are representative of 3-4 replicates.

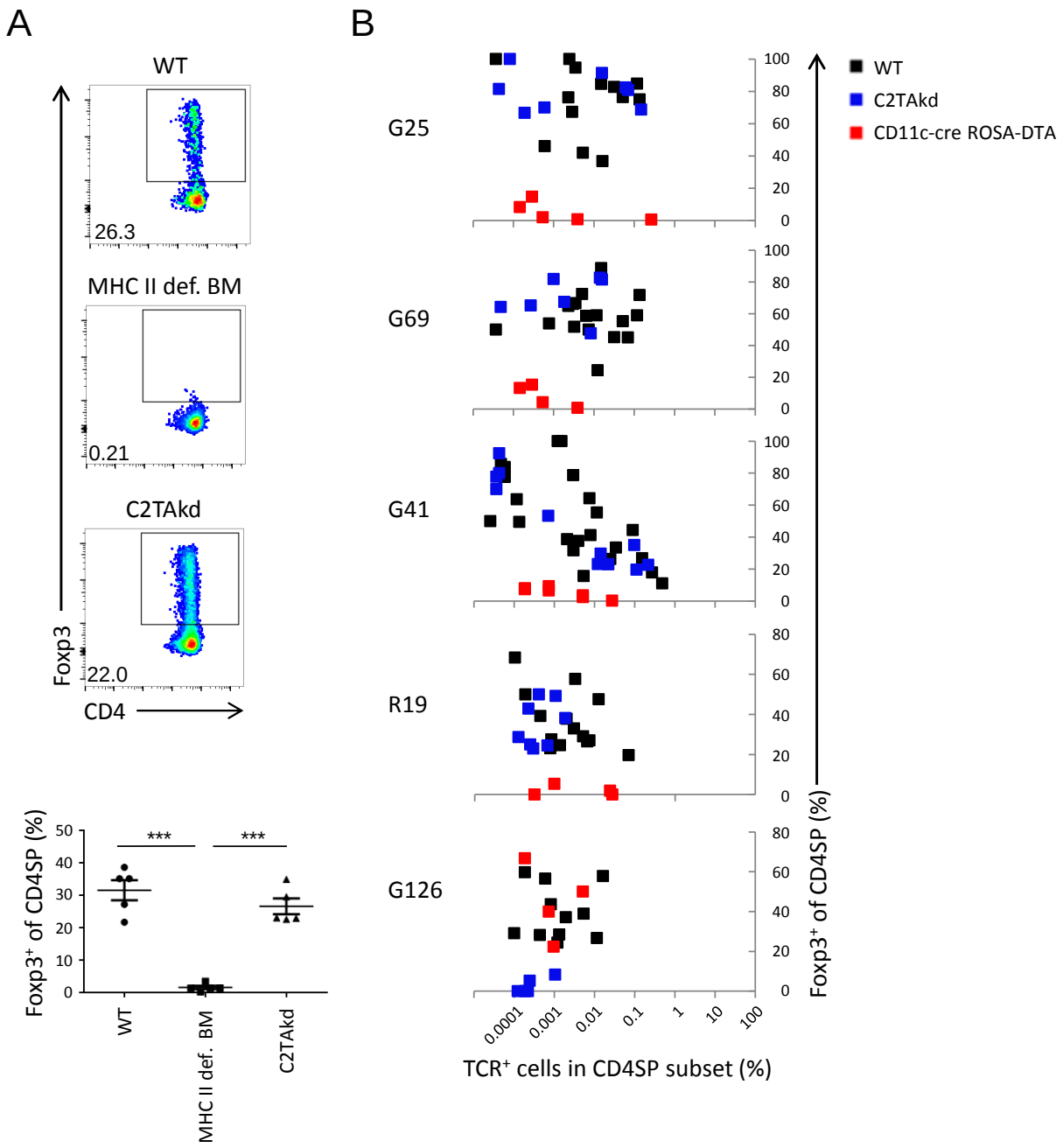


Figure S4, related to Figure 4. DCs are the primary BM APC subset involved in Treg cell selection.

(A) Analysis of BM APC-dependent Treg TCR G41 in vivo. Data shown are FACS plots of G41 expressing *Rag1*$^{-/-}$ thymocytes after intrathymic injection into WT, MHC II def. BM, and C2TAkd chimeric mice. Plots are representative of 2-3 replicates from 2 independent experiments. ***p < .001, Mann-Whitney U. (B) Clonal frequency plots after intrathymic injection of *Rag1*$^{-/-}$ thymocytes retrovirally-transduced with the indicated

Treg TCRs for the experiments in Figure 4. Data shown represent the percentage of transferred compared to the total CD4SP population. Black dots represent WT hosts, blue dots represent C2TAkd hosts, and red dots represent CD11c-Cre ROSA-DTA hosts.

A

Population	*Aire*$^{+/+}$ n mice (n expts)	*Aire*$^{+/+}$ Sorted # x10^3 Mean (SE)	*Aire*$^{+/+}$ # of reads	*Aire*$^{+/+}$ Unique # of TCRs	*Aire*$^{-/-}$ n mice (n expts)	*Aire*$^{-/-}$ Sorted # x10^3 Mean (SE)	*Aire*$^{-/-}$ # of reads	*Aire*$^{-/-}$ Unique # of TCRs
Foxp3$^-$	6 (2)	540 (40)	18015	3130	4 (2)	270 (49)	7688	1691
Foxp3$^+$	6 (2)	36 (1)	17204	1280	4 (2)	21 (3)	7244	615

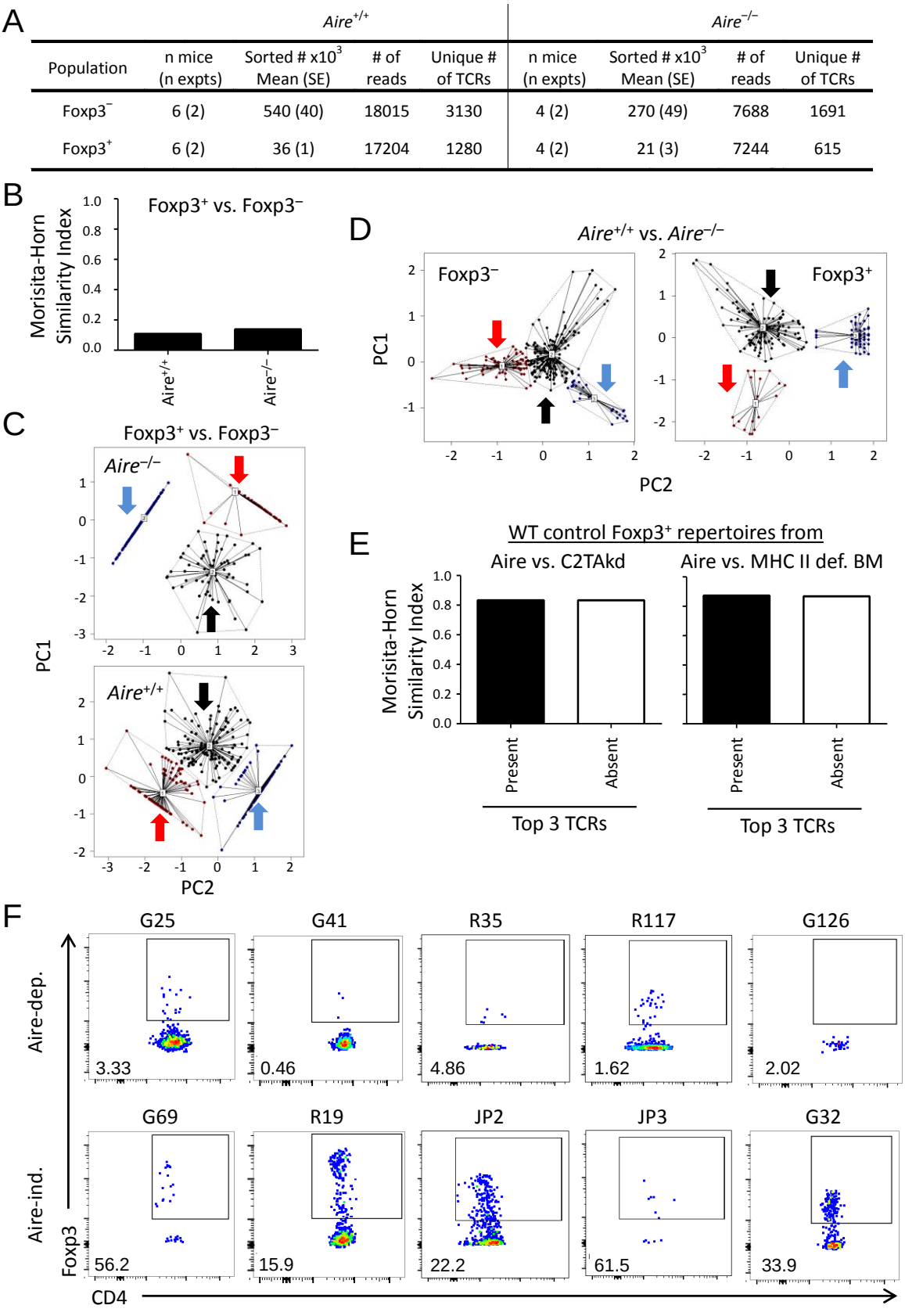


Figure S5, related to Figure 5. Aire selects a subset of thymic Treg cells.

(A) Summary of TCR sequences obtained from WT versus *Aire*$^{-/-}$ fixed TCRβ mice. (B) Morisita-Horn similarity analysis between Treg and Tconv TCRs from WT and *Aire*$^{-/-}$ mice. (C) Principal component analysis of TCR frequencies between WT and *Aire*$^{-/-}$ mice (WT variances: PC1 = 15.2%, PC2 = 26.8%; *Aire*$^{-/-}$ variances: PC1 = 13.2%, PC2 = 44.9%) (D) Principal component analysis of the TCR frequencies between *Aire*$^{-/-}$ and WT mice for the indicated subset (Tconv variances: PC1 = 15.2%, PC2 = 26.8%; Treg variances: PC1 = 13.2%, PC2 = 44.9%). (E) Morisita-Horn similarity analysis between control WT → WT BM chimera Treg TCR repertoires from the *Aire*$^{-/-}$ and MHC II def. → WT as well as WT → C2TAkd chimeric experiments in Figure 5D. (F) Representative FACS plots of *Rag1*$^{-/-}$ thymocytes retrovirally-transduced with indicated Treg TCRs and injected into *Aire*$^{-/-}$ hosts for the experiments summarized in Figure 5F. Data are representative of at least 2 independent experiments with 1-3 replicates per experiment.

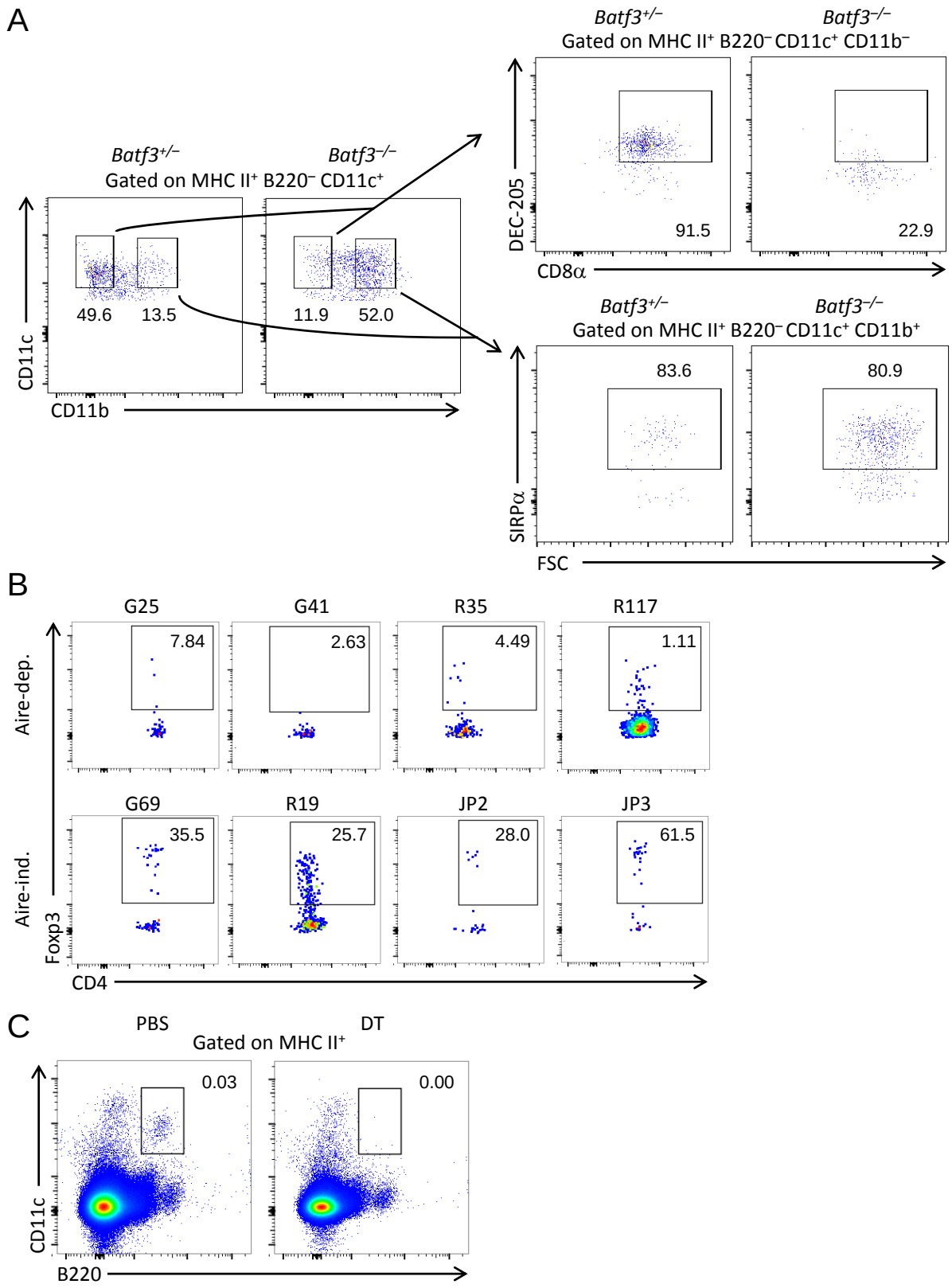


Figure S6, related to Figure 6. CD8α$^+$ DCs preferentially acquire and present Aire-dependent antigens to developing Treg cells.

(A) FACS plots of CD8α^+ and SIRPα^+ DCs from the thymi of *Batf3*$^{+/-}$ or *Batf3*$^{-/-}$ mice. Plots are representative of 4 replicates. (B) FACS plots after intrathymic injection of *Rag1*$^{-/-}$ thymocytes retrovirally-transduced with the indicated TCRs into *Batf3*$^{-/-}$ mice for the experiments summarized in Figure 6A. Plots are representative of at least 2 experiments with 1-3 replicates per experiment. (C) FACS plots of B220$^+$ CD11c$^+$ MHC II$^+$ plasmacytoid DCs from *CLEC4C*-HBEGF mice treated with PBS or DT. Plots are representative of 3 replicates.