Supplemental Figures for

"Peripherally induced tolerance depends on pTreg cells that require Hopx to inhibit intrinsic IL-2 expression"

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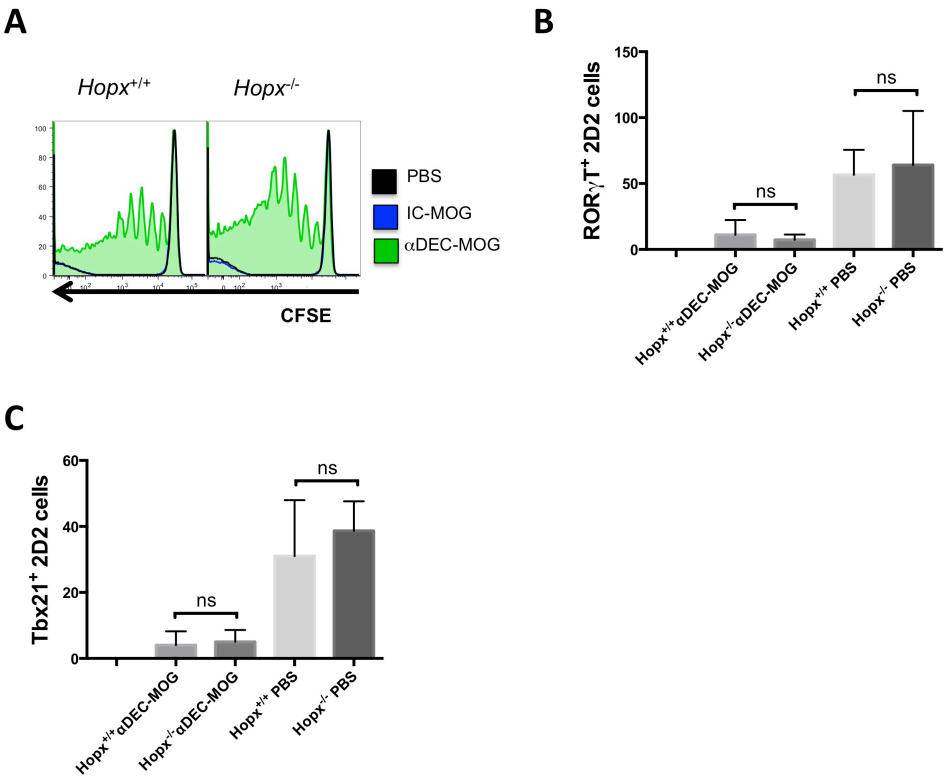
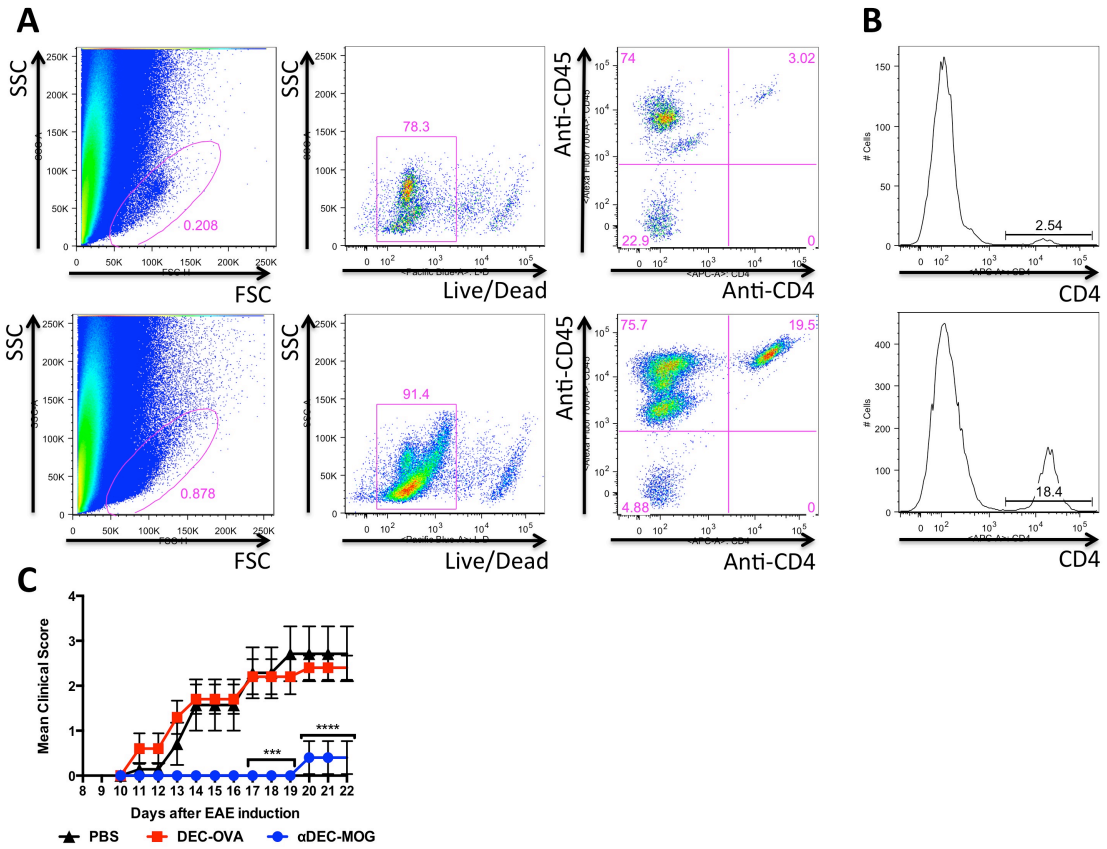


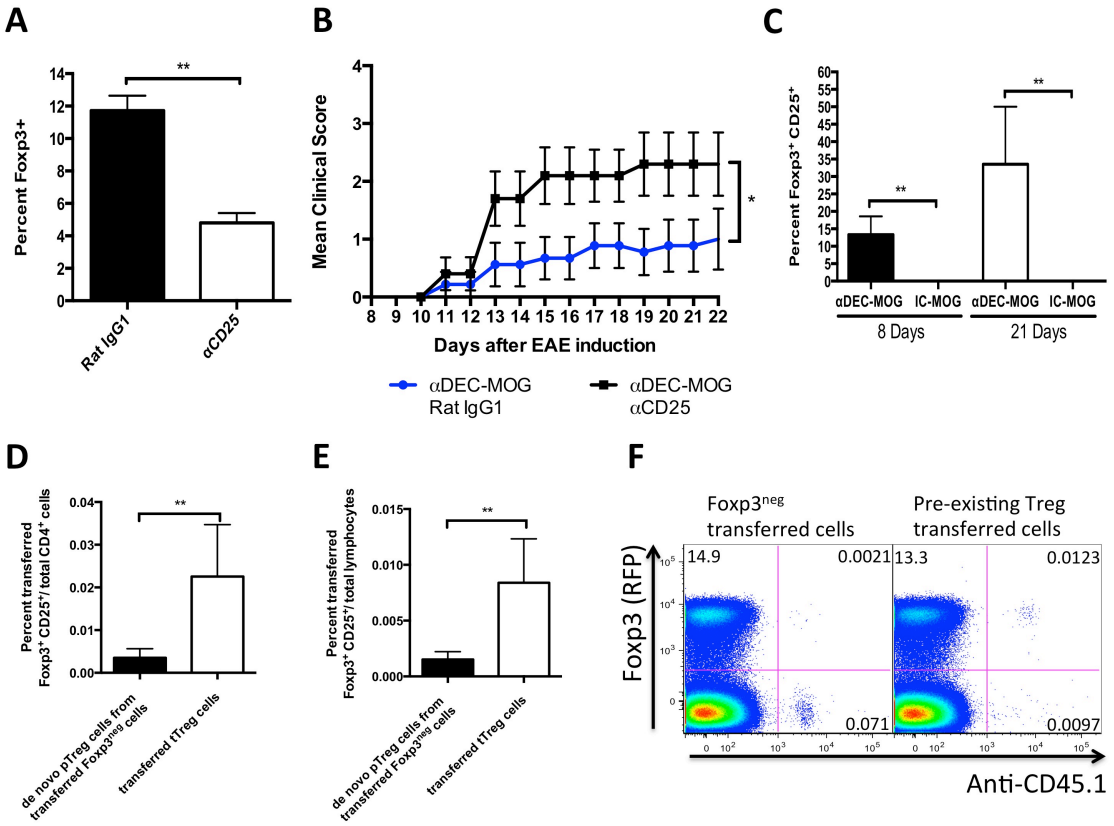
Supplemental Figure 1. (A) Histograms show CFSE dye dilution of gated populations of $CD4^+$ $CD45.1^+$ 2D2 TCR tg T cells adoptively transferred into $Hopx^{+/+}$ and $Hopx^{-/-}$ mice that were treated with either (■) αDEC-MOG, (■) IC-MOG or (■) PBS as indicated. The results represent one of two similar experiments. **(B-C)** $Hopx^{+/+}$ or $Hopx^{-/-}$ $CD4^+$ $Foxp3^{neg}$ $CD25^{neg}$ 2D2 TCR tg T cells were adoptively transferred into $CD45.1^+$ recipient mice that were then treated with αDEC-MOG. 8 days later recipient mice were immunized with MOG/CFA and *PTX*. Graphs show mean number of transferred cells that stained positive for (B) RORγT or (C) Tbx21 7 days after MOG/CFA and *PTX* treatment +/- SD.

Results shown in (A-C) are from lymph nodes and similar results were obtained from spleens.



Supplemental Figure 2.

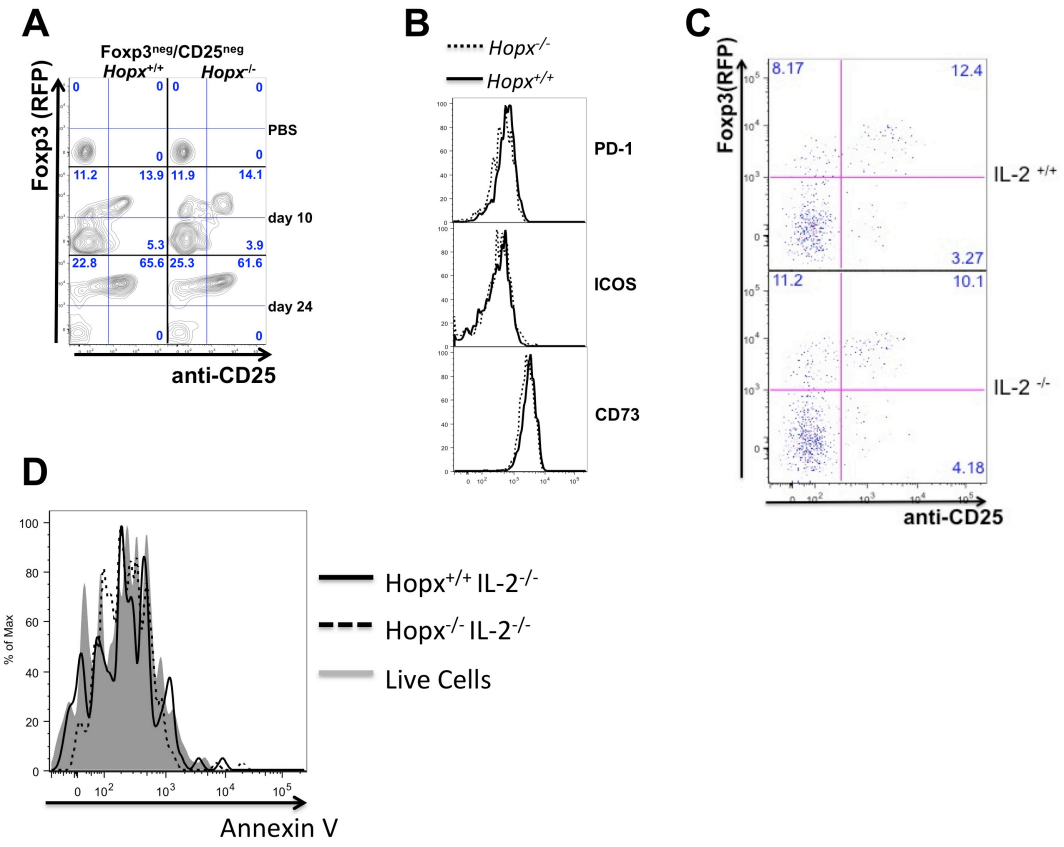
(A) Gating strategy starting with an initial "lymphoid gate" for spinal cords analyzed by FACS from mice with an EAE score of 0 (top panels) or an EAE score of 3 (bottom panels). **(B)** FACS analysis of the same spinal cord samples of as in (A) and as employed in figures 1D, 1E, 3C, 3E and 5D, based on initial "lymphoid gate". **(C)** Multiple groups of mice were treated with αDEC-MOG, αDEC-OVA or PBS 5 weeks before immunization with MOG_{35-55} in CFA + *PT*. Graphs show mean disease scores (n=10 per group from 2 experiments). Results are mean +/- SEM, *** P< 0.001 and **** P< 0.0001 determined by two-way ANOVA.



Supplemental Figure 3.

(A) Foxp3^{RFP} mice were treated with either αCD25 or the same dose of Rat IgG1. 3 weeks after treatment lymph nodes were analyzed by FACS. Graphs show the percent of Foxp3^{+} cells among CD4^{+} cells (n=3-4 per group). Results show mean +/- SEM, ** P<0.01 determined by Students T test. Similar results were obtained from spleens. **(B)** Two groups of C57BL/6 mice were treated with αDEC-MOG. 3 weeks after treatment with αDEC-MOG, individual groups of mice were injected with either αCD25 or the same dose of Rat IgG1. After another 3 weeks EAE was induced. Graph shows mean disease scores (n=9-10 per group from 2 experiments). Results show mean +/- SEM, * P<0.05 determined by two-way ANOVA. **(C)** GFP^{neg}/RFP^{neg}/CD25^{neg} CD4^{+} T cells were purified by sorting from 2D2 TCR tg Hopx$^{Flag\text{-}viral2A\text{-}GFP}$/Foxp3^{RFP} double-reporter mice and then adoptively transferred into CD45.1 recipient mice. Spleens from recipient mice were

analyzed at multiple days after treatment with αDEC-MOG or IC-MOG as indicated. Graphs show the average percentage of transferred cells that were foxp3^{+} CD25^{+} +/- SD, ** P<0.01 determined by Students T test. **(D-F)** CD4^{+} CD45.1^{+} Foxp3^{neg} or Foxp3^{+} T cells were adoptively transferred into multiple CD45.2 recipient mice that were then treated with αDEC-MOG. Lymph nodes from recipient mice were analyzed by FACS 6 weeks after treatment with αDEC-MOG. **(D)** Graphs show the frequency of transferred cells that were Foxp3^{+}CD25^{+} among total CD4^{+} cells. **(E)** Graphs show the frequency of transferred cells that were Foxp3^{+} CD25^{+} among total lymphocytes. **D** and **E** (n=3-7) +/- SD, ** P<0.01 determined by Students T test. **(F)** Representative plots show Foxp3 (RFP) expression (Y-axis) and staining intensity with anti-CD45.1 (X-axis) among CD4^{+} cells. Similar results were obtained from spleens.



Supplemental Figure 4.

(A-B) Foxp3^{neg} CD25^{neg} CD4^{+} T cells were purified by sorting from either *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ 2D2 TCR tg Foxp3^{RFP} mice and then adoptively transferred into CD45.1^{+} recipient mice that were then treated with αDEC-MOG or PBS. (A) Plots show Foxp3 (RFP) expression (Y-axis) and staining intensity with anti-CD25 (X-axis) of gated populations of adoptively transferred cells from recipient mice 10 days or 24 days after αDEC-MOG treatment or PBS treatment. The results represent one of three similar experiments. (B) Histograms show staining intensity with anti-PD-1, anti-ICOS and anti-CD73 of induced *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ Foxp3^{+}/CD25^{+} cells 24 days after αDEC-MOG treatment. Results represent one of two similar experiments. **(C)** Foxp3^{neg} CD25^{neg} CD4^{+} T cells were purified by sorting from either *Hopx*$^{-/-}$ IL-2$^{+/+}$ or *Hopx*$^{-/-}$ IL-2$^{-/-}$/2D2 TCR tg Foxp3^{RFP} mice and then adoptively transferred into CD45.1^{+} recipient mice that were then treated with

αDEC-MOG. Plots show Foxp3 (RFP) expression (Y-axis) and staining intensity with anti-CD25 (X-axis) of gated populations of adoptively transferred cells from lymph nodes of recipient mice after treatment with αDEC-MOG. Results represent one of two similar experiments. **(D)** $Foxp3^{neg}$ $CD25^{neg}$ $CD4^{+}$ T cells were purified by sorting from either *Hopx*$^{+/+}$ IL-$2^{-/-}$ or *Hopx*$^{-/-}$ IL-$2^{-/-}$ /2D2 TCR tg $Foxp3^{RFP}$ mice and then adoptively transferred into $CD45.1^{+}$ recipient mice that were then treated with αDEC-MOG. After 9 days recipient mice were treated with MOG/CFA and PTX. Overlaid histograms show staining intensity with Annexin V in $Foxp3^{+}CD25^{+}$ pTreg cells 3 days after MOG/CFA and *PTX*. Results represent one of two similar experiments. Results shown in (A-D) are from lymph nodes and similar results were obtained from spleens.