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Supplementary Information for

Unregulated antigen-presenting cell activation by T cells breaks self tolerance

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Supplementary text
Figs. S1 to S6

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Materials and Methods

Mice. Various strains of mice were maintained under specific pathogenic-free condition at Postech Biotech Center, South Korea. Foxp3-GFP mice were a gift from Talal Chatila (Boston Children's hospital) and bred onto CD90.1 or CD45.1 B6 background. C57BL/6, CD90.1 B6, CD45.1 B6, *Rag1*$^{-/-}$, CD4 DN TGFβRII, *H2M*$^{-/-}$ mice were also obtained from Jackson laboratory. SPF *Rag1*$^{-/-}$ mice for examining donor T cell proliferation were obtained from conventionalized GF *Rag1*$^{-/-}$ mice and were maintained under SPF condition. To obtain naïve CD4 T cells lacking TGFβ receptors, CD4 DN TGFβRII mice were bred onto a CD45.1 Foxp3-GFP background. In order to examine CLIP/MHCII selected T cell responses upon elimination of Tregs, *H2M*$^{-/-}$ mice were bred onto a CD90.1 Foxp3-GFP background for preparation of donor T cells, or bred onto a Foxp3-DTR background for use as host mice. Nur77-GFP mice (1) were kindly provided by Kristin Hogquist (Univ. of Minnesota). Foxp3-RFP mice were kindly provided by Richard Flavell (Yale Univ.) To obtain donor naïve T cells from Nur77-GFP mice, these mice were bred onto a CD45.1 Foxp3-RFP background.

Cell isolation from tissues. Single cell suspensions of T cells were prepared from LN and spleen. For analysing DC in LN and spleen, tissues were digested with collagenase D and Dnase I. T cells in the intestine were prepared as described previously (2). Colon tissue was cut into small pieces and incubated for 30 min at 37'C in PBS buffer containing fetal bovine serum (FBS, 3% vol/vol), EDTA (10mM), HEPES (20mM), polymyxin B (10mg/ml), penicillin (100U/ml), streptomycin (100mg/ml) and sodium pyruvate (1mM), to remove epithelial cells. Tissues were extensively washed with PBS, cut with tissue chopper, and digested with collagenase D and Dnase I in RPMI medium containing FBS (3% vol/vol), HEPES (20mM), penicillin (100U/ml), streptomycin (100mg/ml), sodium pyruvate (1mM) and non-essential amino acids (1mM). Further enrichment of lymphocytes was achieved by 40:75% Percoll density gradient centrifugation.

Antibodies and staining reagents for flow cytometry and sorting. Isolated cells were washed with PBS containing 1% FBS and 0.02% sodium azide (Sigma), stained with propidium iodide (Sigma) or Ghost viability dye (Tonbo) to eliminate dead cells. For surface staining, cells were stained with the following fluorochrome-conjugated

antibodies (eBioscience, Biolegend, and Tonbo): anti-CD4 (RM4-5), anti-TCRβ (H57-597), anti-CD8a (53-6.7), anti-Thy1.1 (HIS51 or OX7), anti-Thy1.2 (53-2.1 or 30H12), anti-CD5 (53-7.3), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-NK1.1 (PK136), anti-B220 (RA3-6B2), anti-MHCII (M5/114.15.2), anti-CD11c (N418), anti-CD11b (M1/70), anti-CD103 (2E7), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CX3CR1 (SA011F11), anti-Ly6C (HK1.4). For intracellular staining, surface stained cells were fixed and permeabilized with a Foxp3 staining kit (eBioscience) according to manufacturer's instruction and were stained with the following antibodies: anti-Foxp3 (FJK-16s), anti-CTLA-4 (UC10-4B9), anti-IFNγ (XMG1.2), anti-IL-17A (eBio17/37), anti-IL-13 (eBio13A). For intracellular staining of cytokines, isolated cells were cultured for 4 hr in RPMI-1640 medium containing 10% FBS, penicillin (100U/ml), streptomycin (100mg/ml) and 55mM β-mercaptoethanol in the presence of PMA/Ionomycin with protein transport inhibitors (eBioscience). Data from the stained cells were collected with FACS Forteassa or FACS Canto-II flow cytometry with DIVA software (BD Bioscience) and were analysed by FlowJo (TreeStar). Stained cells were also sorted to obtain specific populations with Moflo-XDP or Astrios (Beckman Coulter) for *in vitro* assays, adoptive transfer experiments, or RNAseq analysis.

In vivo DT and mab treatment. DT was purchased from Sigma, and 50ug/kg of DT suspended in PBS was injected IP every other day. Neutralizing mabs against TNFa, CD40L, ICOSL, TGFβ were purchased from Bio X cell and 300ug of mabs were used to treat mice every other day by IP injection until analysis. The S4B6.1 and JES6-1A12 hybridomas were obtained from the American Type Culture Collection (ATCC). IL-2 blocking mabs (S4B6 and JES6-1) were produced from hybridomas and 200ug/mouse of each mab was used to treat mice by IP injection every other day. In vivo neutralization mabs against CD80 (16-10A1) and CD86 (GL1) were gifts from Stephen Schoenberger (La Jolla Institute For Allergy and Immunology, CA) and were injected IP at 200ug/mouse every other day.

Quantification of precursor frequency. Precursor frequencies of responding donor T cells at d3 were measured based on numbers of cell division as described previously (3). Precursor frequency was defined as the fraction of P_{div}/P_{tot} (i.e., the precursor cells that divided as a proportion of total cells). P_{div} was calculated by using the CTV-dilution

profile at d3 after initial DT-treatment and extrapolating to derive the number of T cells that entered the dividing pool. P_{tot} was calculated to derive the number of total T cell number prior to cell division. P_{div}: precursor of divided T cells. P_{tot}: precursor of total donor T cells

H&E staining of tissues. Liver and colon specimens were fixed with 4% paraformaldehyde solution (Sigma) and paraffin-embedded sections were prepared. Routine hematoxylin and eosin (H&E) staining was performed by using hematoxylin for nucleus staining, and eosin for cytoplasm and muscle layer.

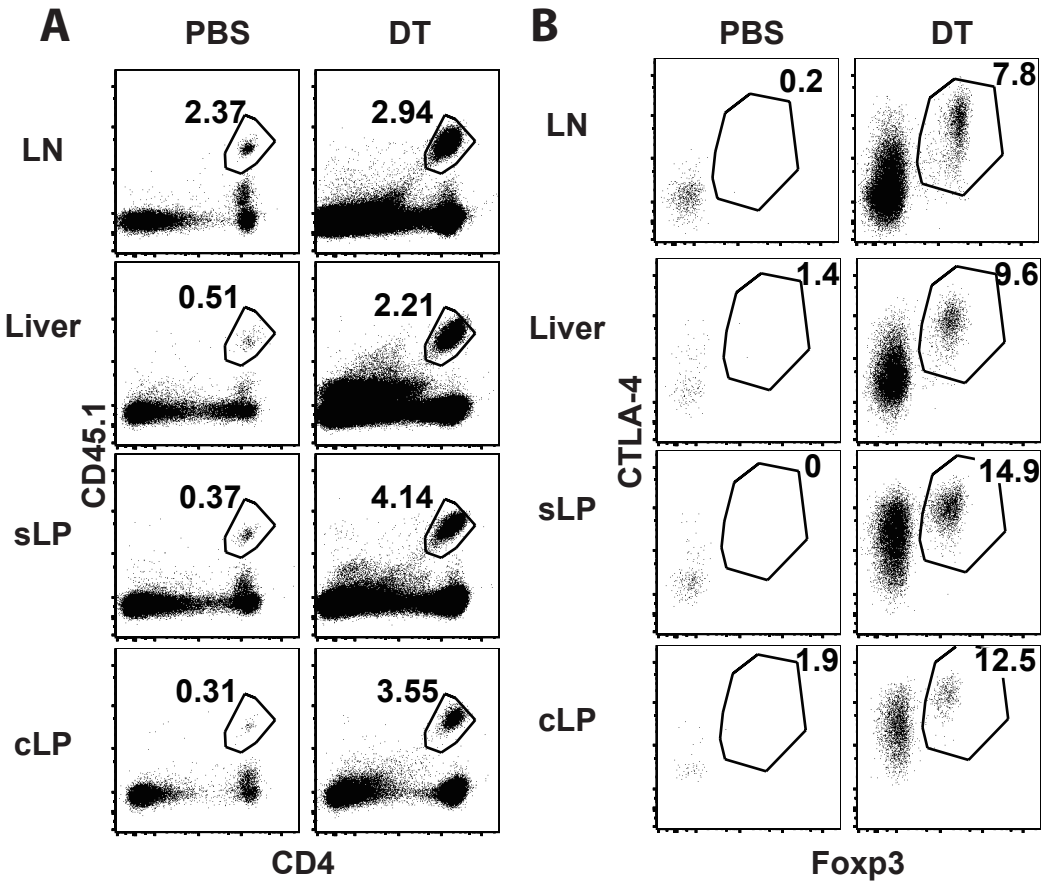


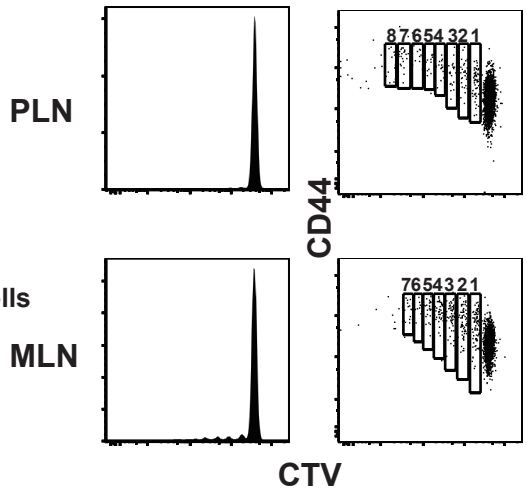
Fig. S1. Expanded donor T cells following Treg-depletion migrate into non-lymphoid tissues. (**A** and **B**) Phenotype of donor T cells at d7 post-injection in Foxp3-DTR hosts adoptively transferred with 1 x 10^7 $CD44^{lo}$ $CD62L^{hi}$ naïve CD4 T cells from CD45.1 Foxp3-GFP mice followed 1d later by PBS (n=2) or DT treatment (n=3). (A) Representative FACS plots for percentages of donor T cells (gated from total lymphocytes) from LN, liver, small intestinal lamina propria (LP), or colonic LP. (B) Representative FACS plots for Foxp3 and CTLA-4 expression on donor T cells.

A

$$P_{div} = \sum_{n=1}^{8} \frac{\boldsymbol{Cell\ number\ on\ each\ peak}}{2^n}$$

$$Precursor\ frequnecy = \frac{\boldsymbol{P_{div}}}{\boldsymbol{P_{total}}}$$

P_{div}: precursor of divided cells, P_{total}: precursor of total cells
Precursor frequency of donor cells in PLN= 0.89 %
Precursor frequency of donor T cells in MLN= 2.88 %



B

Precursor frequency (%) in the AF DT-Foxp3-DTR hosts

	PLN	MLN	SPL
Precursor frequency	**0.92**	**1.58**	**1.43**

Fig. S2. Precursor frequency of responding CD4 T cells following Treg-depletion. Precursor frequency of responding T cells was calculated (see Materials and Methods) based on number of cell divisions of donor T cells at d4 post-transfer in Foxp3-DTR hosts adoptively transferred with 2 x 10^6 CTV-labeled $CD44^{lo}$ $CD62L^{hi}$ naïve CD4 T cells followed 1d later by DT-injection. (A) Precursor frequency calculation for donor cells in PLN or MLN in SPF Foxp3-DTR host. Representative calculation is shown from two independent experiments (n=3 per each experiment). (B) Calculated precursor frequency as in A for donor cells in PLN, MLN, or SPL of AF Foxp3-DTR hosts. Representative calculation from two mice.

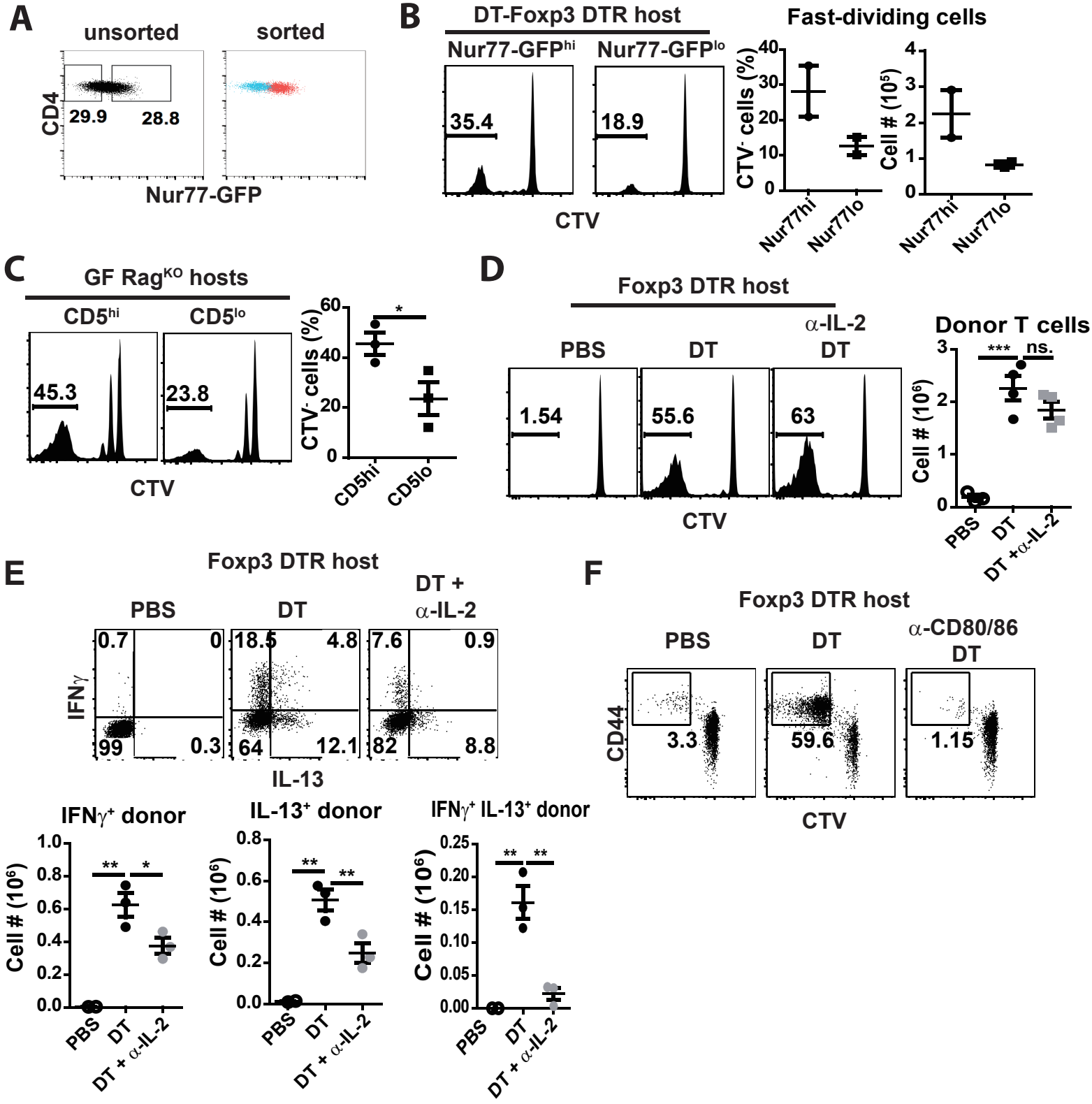

A
unsorted
sorted
CD4
29.9
28.8
Nur77-GFP
B
DT-Foxp3 DTR host
Nur77-GFPhi
Nur77-GFPlo
35.4
18.9
CTV
Fast-dividing cells
CTV- cells (%)
Cell # (10^5)
Nur77hi
Nur77lo
C
GF RagKO hosts
CD5hi
CD5lo
45.3
23.8
CTV
CTV- cells (%)
CD5hi
CD5lo
D
Foxp3 DTR host
α-IL-2
PBS
DT
DT
1.54
55.6
63
CTV
Donor T cells
Cell # (10^6)
***
ns.
PBS
DT
DT +α-IL-2
E
Foxp3 DTR host
PBS
DT
DT +
α-IL-2
IFNγ
IL-13
0.7
0
99
0.3
18.5
4.8
64
12.1
7.6
0.9
82
8.8
IFNγ+ donor
IL-13+ donor
IFNγ+ IL-13+ donor
Cell # (10^6)
PBS
DT
DT + α-IL-2
F
Foxp3 DTR host
PBS
DT
α-CD80/86
DT
CD44
3.3
59.6
1.15
CTV

Fig. S3. Factors inducing strong T cell proliferation in the absence of Tregs. (**A** and **B**) $CD44^{lo}$ $CD62L^{hi}$ naïve CD4 T cells from CD45.1 Nur77-GFP x Foxp3-RFP mice were separated based on Nur77 expressions (upper 30 percent or lower 30 percent). (A) Sorting purity of $Nur77^{hi}$ or $Nur77^{lo}$ naïve CD4 T cells. (B) Representative FACS plots for proliferation of donor T cells at d6 post-transfer in Foxp3-DTR hosts adoptively transferred with 2 x 10^6 $Nur77^{hi}$ or $Nur77^{lo}$ $CD44^{lo}$ $CD62L^{hi}$ naïve CD4 T cells followed 1d later by DT-injection (left) and percent or absolute number graph of rapidly dividing cells (right) (n=2 group). (**C**) Comparison of donor T cell proliferation at d7 post-injection in GF $Rag1^{-/-}$ hosts adoptively transferred with 1 x 10^6 CTV-labeled $CD5^{hi}$ or $CD5^{lo}$ naïve CD4 T cells from Foxp3-GFP mice (n=3 per group), showing representative CTV-histogram (left), and graph of CTV-cells/total donor T cells (right). (**D** and **E**) Comparison of donor T cell proliferation and expansion at d7 post-injection in Foxp3-DTR hosts adoptively transferred with 2 x 10^6 purified CTV-labeled $CD44^{lo}$ $CD62L^{hi}$ naïve CD4 T cells from CD45.1 Foxp3-GFP mice followed 1d later by PBS-, DT-, or DT + IL-2 neutralization mabs (S4B6 and JES6-1)-injection. (D) Shown are representative CTV-histogram on donor T cells (left), and graph of total donor cell numbers (right). (PBS: n=3, DT: n=4, DT + anti-IL2 mabs: n=4). (E) Shown are representative FACS plots for IFNγ and IL-13 expression by donor T cells after 4 hr of ex vivo re-stimulation by PMA/Ionomycin in the presence of protein transport inhibitors (PBS: n=2, DT: n=3, DT + anti-IL-2: n=3) (upper), and graphs of total donor T cell numbers synthesizing IFNγ, IL-13, or IFNγ and IL-13 (lower). (**F**) Representative FACS plots for proliferation & CD44 expression of donor T cells at d7 in Foxp3-DTR hosts adoptively transferred with 2 x 10^6 CTV-labeled $CD44^{lo}$ $CD62L^{hi}$ naïve CD4 T cells followed 1d later by treatment with PBS, DT or DT + mAbs against CD80 and CD86 (n=2 per each). P values were determined by Student's t test or by one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test. Error bars show mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001

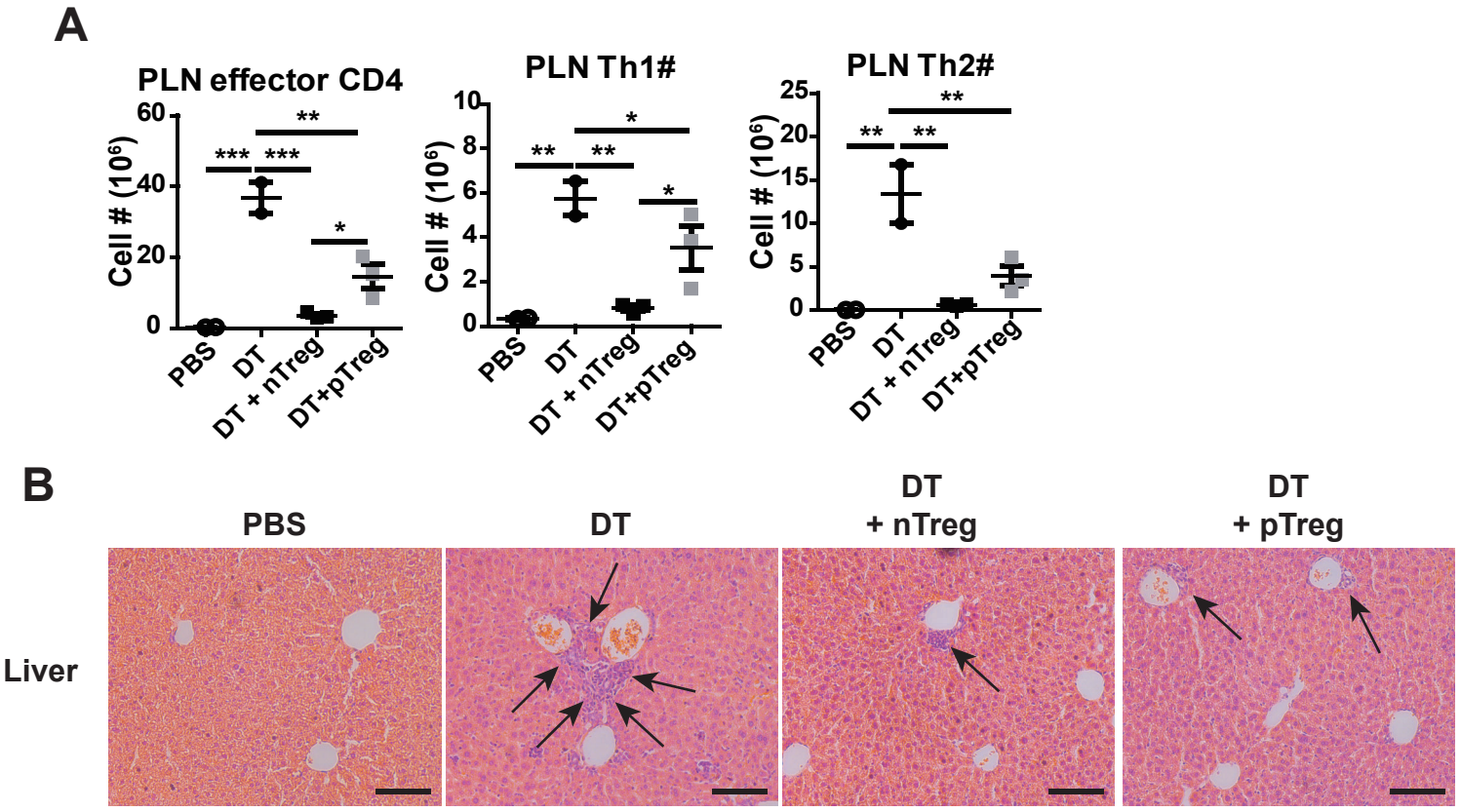


Fig. S4. pTregs suppress lymphoproliferation following transfer to secondary DT-Foxp3-DTR hosts. Comparison of lymphoproliferative disease induction at 13d after transfer of 3 x 10^5 pTreg or nTreg populations into Foxp3-DTR mice, followed 1d later by PBS or DT-treatment (n=3 per group). pTregs were generated in primary Foxp3-DTR hosts adoptively transferred with 1 x 10^7 $CD44^{lo}$ $CD62L^{hi}$ naïve CD4 T cells from CD45.1 Foxp3-GFP mice followed 1d later by DT-injection; at d7 post-injection, pTregs were resorted from LN for adoptive transfer to secondary Foxp3-DTR hosts. nTregs were prepared from LN of Foxp3-GFP mice. (**A**) Shown are total numbers of CD44hi effector CD4 T cells, IFNγ-producing CD4 T cells, or IL-13-producing CD4 T cells from PLN. (**B**) H&E staining for sections of liver. Bars, 100um. Arrows indicate lymphocyte infiltrations. P values were determined by one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test. Error bars show mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001

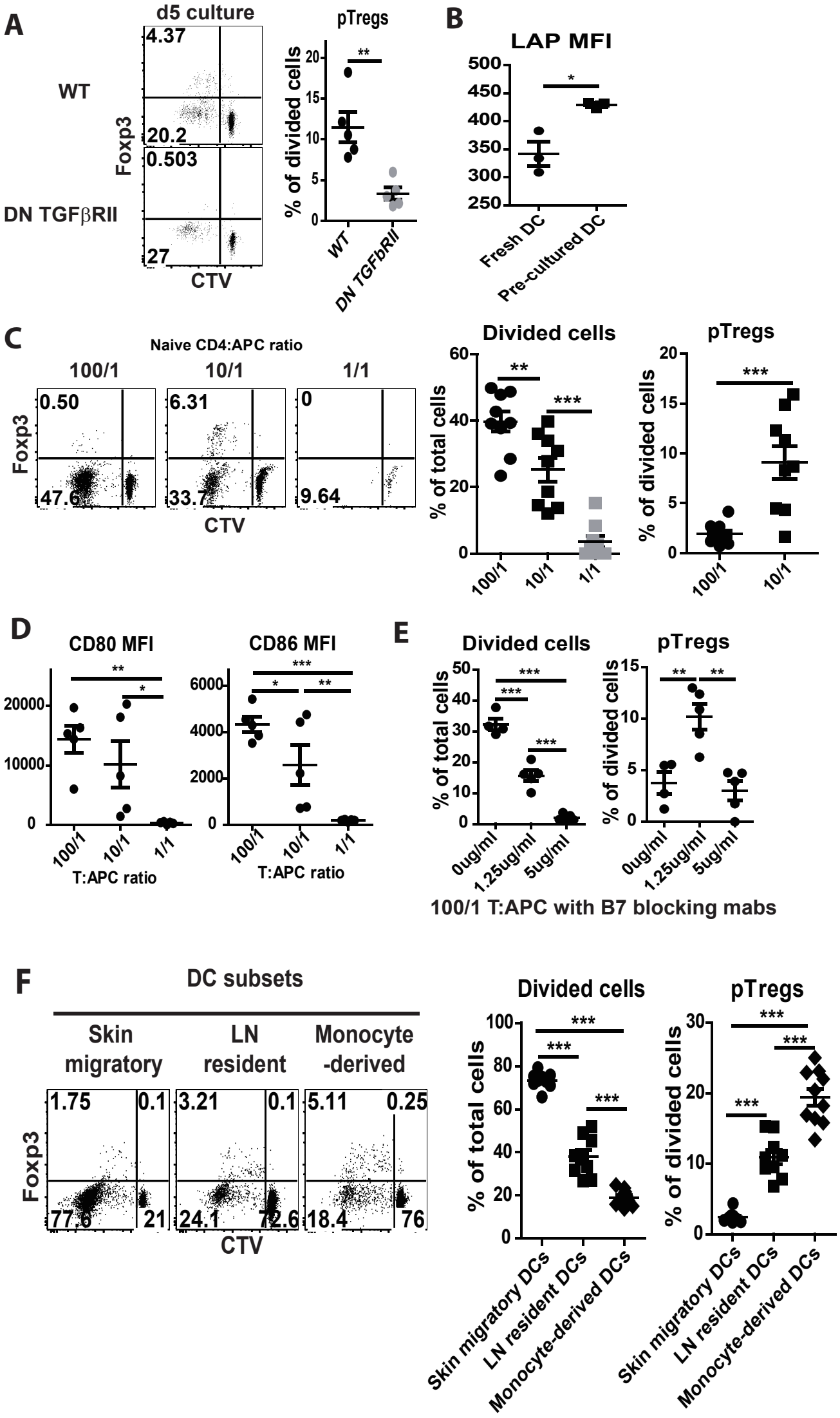

A
d5 culture
WT
DN TGFβRII
Foxp3
CTV
4.37
20.2
0.503
27
pTregs
% of divided cells
WT
DN TGFbRII
B
LAP MFI
Fresh DC
Pre-cultured DC
C
Naive CD4:APC ratio
100/1
10/1
1/1
0.50
47.6
6.31
33.7
0
9.64
Divided cells
% of total cells
pTregs
% of divided cells
D
CD80 MFI
CD86 MFI
T:APC ratio
E
Divided cells
pTregs
0ug/ml
1.25ug/ml
5ug/ml
100/1 T:APC with B7 blocking mabs
F
DC subsets
Skin migratory
LN resident
Monocyte -derived
1.75
0.1
77.6
21
3.21
0.1
24.1
72.6
5.11
0.25
18.4
76
Skin migratory DCs
LN resident DCs
Monocyte-derived DCs

Fig. S5. pTreg induction during auto-MLR. (**A**) Auto-MLR with 1 x 10^4 GF B6 Rag1$^{-/-}$ PLN cells (B6 PLN APC) cultured with 1 x 10^5 CTV-labeled CD44^{lo} CD62L^{hi} B6 naïve CD4 T cells prepared from WT Foxp3-GFP mice or DN TGFβRII mice for 5d (n=5 per each group). Shown are representative FACS plots for CTV dilution and Foxp3-GFP expression (left), and percent Foxp3-GFP$^+$ cells among divided T cells (right) (**B**) Mean fluorescence intensity (MFI) of LAP expression on purified B6 PLN DC before and after culture for 24 hr alone in vitro. (**C** to **E**) Effect of different T:APC ratios on auto-MLR. 1 x 10^6, 1 x 10^5, or 1 x 10^4 of B6 CD44^{lo} CD62L^{hi} naïve CD4 T cells were co-cultured with 1 x 10^4 B6 PLN APC (100/1~1/1 T:APC ratio). Shown are representative FACS plots of CTV-dilution and Foxp3-GFP expression on co-cultured T cells (left), and percent graph of CTV- divided cells and of Foxp3-GFP$^+$ cells among divided T cells (right) at d7 (100/1~1/1, n=9 per each group) (C), MFI on co-cultured DC at d5 (D), and percent of divided total T cells and percent of Foxp3-GFP$^+$ cells among divided T cells when 100/1 T:APC cultures were supplemented with titrated concentrations of anti-CD80 and anti-CD86 mabs blocking mabs for 7d (n=4-5)(E). (**F**) Capacity of PLN DC subsets from DT-Foxp3-DTR mice to induce auto-MLR. Populations of skin migratory DC, resident DC, or monocyte-derived DC were FACS-purified from DT-Foxp3-DTR mice at d7 post-DT injection; doses of 1 x 10^4 of each DC subset were co-cultured with 1 x 10^5 CTV-labeled CD44^{lo} CD62L^{hi} B6 naïve CD4 T cells from Foxp3-GFP mice for 7d. Shown are representative FACS plots of CTV-dilution and Foxp3-GFP expression on co-cultured T cells (left), and percent of divided cells among total T cells and percent of Foxp3-GFP$^+$ cells among divided T cells (right) (n=9-10 per group). DC subsets were defined as TCRβ$^-$ B220$^-$ Thy1.2$^-$ NK1.1$^-$ PLN cells that were MHCII^{hi} CD11c^{int} cells for skin migratory DC, MHCII^{int} CD11c^{hi} cells for LN resident DC, and CD11b$^+$ Ly6C$^+$ cells for monocyte-derived DC. P values were determined by one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test. Error bars show mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001

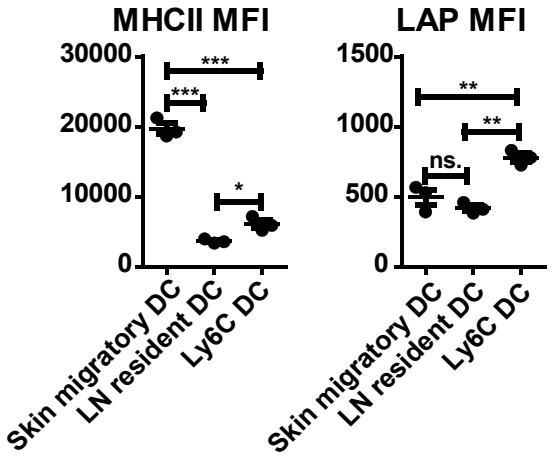


Fig. S6. Inflammatory Ly6C DC express lower MHCII and higher LAP. Shown are comparisons of MHCII and LAP expression on PLN DC populations at d7 post-DT-injection to Foxp3-DTR mice. DC subsets were defined as TCRβ$^-$ B220$^-$ Thy1.2$^-$ NK1.1$^-$ PLN cells that were MHCII^{hi} CD11c^{int} cells for skin migratory DC, MHCII^{int} CD11c^{hi} cells for LN resident DC, and CD11b$^+$ Ly6C$^+$ cells for monocyte-derived DC. P values were determined by one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test. Error bars show mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001

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