Supplementary Information for

IL-27 confers a protumorigenic activity of regulatory T cells via CD39

Young-Jun Park, Heeju Ryu, Garam Choi, Byung-Seok Kim, Eun Sook Hwang, Hun Sik Kim, and Yeonseok Chung

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Supplementary Materials and Methods

Cell line and Reagents

B16F10 (ATCC) and EL4 (Korean Cell Line Bank) were cultured in DMEM or RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. *In vitro* T cell cultures were performed in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and 55 μmol/L 2-mercaptoethanol. ATP, busulfan, ZM241385, and ARL67165 were obtained from Sigma-Aldrich. The microbeads for lymphocyte selection were purchased from Miltenyi Biotec (Bergish Galdbach, Germany).

In vivo tumor models

To analyze the characteristics of tumor-infiltrating Tregs (Ti-Tregs), the mice were inoculated subcutaneously (s.c.) with 2×10^5 B16F10 cells or 5×10^5 EL4 cells. To block IFN-γ, 100 μg of anti-IFN-γ (XMG1.2) were injected i.p. 7 days after tumor implantation (total 4 times/every other day). For the Treg transfer tumor model, Foxp3$^{YFP\text{-}Cre}$ and *Il27ra*$^{-/-}$ × Foxp3$^{YFP\text{-}Cre}$ naïve CD4 T cells were differentiated into iTregs in the presence of 12.5 ng/ml TGF-β for iTreg transfer, and Foxp3$^{YFP\text{-}Cre}$ and *Stat1*$^{-/-}$ × Foxp3$^{YFP\text{-}Cre}$ nTregs were isolated for nTreg transfer. FACS-sorted YFP$^+$ cells were mixed with CD25$^-$GITR$^-$CD4$^+$ and CD8$^+$ T cells in a ratios of physiological relevance, and transferred into *Rag1*$^{-/-}$ mice. B16F10 cells were implanted s.c. 2 days later. To identify the effect of CD39 on tumor growth, CD39 inhibitor ARL67156 (5 mg/kg) was treated for 5 consecutive days starting from 1 day after tumor inoculation. In some experiments, the mixture of FACS-sorted YFP$^+$ Tregs (CD45.2$^+$) from Foxp3-YFP and *Il27ra*$^{-/-}$ × Foxp3-YFP and B16F10 tumor cells were subcutaneously injected into *Rag1*$^{-/-}$ mice reconstituted with naïve CD4$^+$ T and CD8$^+$ T cells (CD45.1$^+$). Tumor size was monitored every 2-3 days by caliper in 3 dimensions. TILs were obtained and analyzed at 15 days post tumor inoculation unless otherwise indicated. In some experiments, the amount of IL-27 in tumor lysates was measured by ELISA

(eBioscience), according to the manufacturer's protocol.

In vitro suppression assay

To identify the effect of IL-27-Tregs on T cell functions, naïve CD4 T cells from Foxp3$^{YFP\text{-}Cre}$ mice were differentiated under iTreg–skewing conditions in the presence of IL-2 (20 ng/ml) and TGFβ (5 ng/ml) with or without IL-27 (10 ng/ml). YFP^{+} iTregs were sorted and plated with CFSE-labeled naïve CD8 T cells for 3 days (2 μg/ml anti-CD3/CD28 antibodies, plate-coated). CFSE dilution and cytokine productions (IFN-γ, TNF-α, and IL-2) were analyzed by FACS. To identify the effect of CD39 and adenosine on T cell function, CD39 inhibitor ARL67156 (150 μM) and A_{2A} receptor antagonist ZM241385 (1 μM) were added to Treg-CD8 T cell coculture.

FACS antibodies and recombinant proteins

The following antibodies were used for cell surface and intracellular staining: APC/Cy7-labeled anti-CD45.2 (104), PerCP/Cy5.5, PE/Cy7 or PacificBlue-labeled anti-CD45.1 (A20), APC-labeled anti-CD44 (IM7), Alexa 488-labeled anti-CD62L (MEL-14), PE-labeled anti-CD25 (PC61), Brilliant Violet 510, PE/Cy7 or PerCP/Cy5.5-labeled anti-CD4 (GK1.5), PE/Cy7-labeled anti-NK1.1 (PK136), PacificBlue-labled anti-TCRβ (H57-597), Alexa 488-labeled anti-PD-1 (RMP1-14), APC-labeled anti-CD73 (TY/11.8), PE-labeled anti-CD39 (24DMS1), PE-labeled anti-TIM-3 (RMT3-23), APC-labeled anti-CTLA4 (UC10-4B9), PE/Cy7 or PerCPCy5.5-labeled anti-ICOS (C398.4A), PacificBlue or PE/Cy7-labeled anti-granzyme B (GB11), PE/Cy7 or PerCP/Cy5.5-labeled anti-CD8 (53-6.7), APC or eFluor 450-labeled anti-TNF-α (MP6-XT22), Alexa 488 or eFluor 450-labeled anti-Foxp3 (FJK-16s), Alexa 488 or PerCP/Cy5.5 or APC-labeled anti-IFN-γ (XMG1.2), PE-labeled anti-IL-2 (JES6-5H4), PerCP/Cy5.5-labeled anti-CXCR3 (CXCR3-173), PE/Cy7-labeled anti-CCR6 (29-2L17), PE-labeled anti-CCR4

(2G12), biotin-labeled anti-CXCR5 (L138D7), APC-labeled anti-RORγt (AFKJS-9), PE-labeled anti-BCL6 (IG191E/A8), BV421-labeled anti-GATA3 (L50-823), PE/Cy7-labeled anti-T-bet (eBio4B10), PacificBlue-labeled anti-pSTAT3 (4/P-STAT3), APC-labeled anti-pSTAT1 (4a), PE-labeled anti-Ki67 (16A8), APC-labeled Annexin V, and strepavidin-labeled APC from BD Biosciences, Biolegend, or eBioscience. Recombinant mouse IL-2, TGF-β, IL-27, IFN-γ, and IL-35-Fc fusion proteins were obtained from PeproTech, eBioscience, R&D Systems, and Chimerigen Laboratories.

Antibody staining and flow cytometry analysis

For intracellular cytokine analysis, cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/mL; Sigma-Aldrich) and ionomycin (1 μmol/L; Sigma-Aldrich) plus Brefeldin A and monensin (both from eBioscience) before staining. IC fixation buffer and permeabilization buffer from eBioscience were used for intracellular cytokine staining, and the Foxp3 staining kit (eBioscience) was used for transcription factor staining, according to the manufacturer's instructions. For phospho-STAT staining, cells were fixed with IC fixation buffer and permeabilized with ice-cold methanol for 30 min. Samples were analyzed with the FACSVerse and LSR Fortessa X-20 flow cytometer (BD Biosciences), and acquired data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

Analysis of tumor-infiltrating lymphocytes

To separate tumor-infiltrating lymphocytes (TILs), tumors were collected and single-cell suspensions were prepared by using the gentle MACS dissociator (Miltenyi Biotec). Then, tumor cells were incubated at 37 °C for 30 min in RPMI-1640 containing 1 mg/mL collagenase IV (Roche), 50 μg/mL DNase I (Sigma). The cells were filtered through a 100-μm nylon mesh, and TILs were isolated by density gradient separation. In some experiments, macrophages, Tregs, non-Tregs T cells in TILs were FACS-sorted, and

the relative expression of *Il27* and *Ebi3* was determined as described previously[23].

Isolation and stimulation of Tregs

Single cell suspension of $CD4^+$ cells from $Foxp3^{YFP\text{-}Cre}$ mice was positively selected with CD4 microbeads (L3T4; Miltenyi Biotec). Subsequently, $Foxp3^+YFP^+$ Tregs were sorted with the FACSAria III cell sorter (BD Biosciences) and stimulated with plate-coated anti-CD3 and anti-CD28 (145-2C11 and 37.51 respectively, 2 μg/mL: Bio X Cell) in the presence of IL-2 (10 ng/ml) ± IL-27 (10 ng/ml) for 3 days. For iTreg differentiation, naïve $CD4^+$ T cells ($CD62L^{high}CD44^{low}$) were isolated from $Foxp3^{YFP\text{-}Cre}$ or C57BL/6 mice. The cells were stimulated with plate-coated anti-CD3 and anti-CD28 (1 μg/ml) in the presence of IL-2 (20 ng/ml) and TGF-β (5 ng/ml or 12.5 ng/ml) ± IL-27, IFN-γ, or IL-35-Fc (10 ng/ml) for 3 days.

ATP and adenosine quantification assay

To measure enzymatic activity of CD39 induced by IL-27 stimulation, $Foxp3^+YFP^+$ nTregs or iTregs, incubated as described above, were cultured with 100 μM ATP for 6 hrs. The remaining ATP was quantified by the luminescence ATP detection assay kit (ATPlite, Perkin-Elmer) according to the manufacturer's instructions. Adenosine was also measured by colorimetric assay (BioVision) using adenosine deaminase to convert adenosine to xanthine. For ATP hydrolysis inhibition, iTregs or IL-27-iTregs were preincubated for 3 hrs in the presence or absence of 200 μM ARL67156. ATP (50 μM) was added in the last 1 hr. The supernatant was analyzed for ATP and adenosine with the detection kit above mentioned.

Statistics

Data were analyzed with Prism 6.0 software (GraphPad Software). Statistical significance between 2 groups was determined by using the unpaired 2-tailed Student *t*-test. For comparison of multiple

conditions within a group or between more than 2 groups, 1-way or 2-way ANOVA with Bonferroni post-tests was used, respectively. *P* values of less than 0.05 were considered statistically significant.

Supplementary Figure Legends

Fig. S1. Non-Tregs CD4$^+$ T cells marginally acquire inhibitory phenotype in tumor tissue compared to Ti-Tregs.

Naïve and B16F10 s.c. tumor-bearing mice were analyzed for the phenotype of CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ T cells 15 days after tumor inoculation. The geometric MFI of indicated proteins expression in tumor-distal, tumor-draining lymph node, tumor tissue, and lymph node from naïve mice was shown. The graphs show means ± SEM. ***, $p<0.001$. The data is representative of two independent experiments (n=6-7).

Fig. S2. Ti-Tregs preferentially express T-bet and CXCR3 in EL4 s.c. tumor model.

EL4 s.c. tumor-bearing mice were analyzed for the phenotype of CD4$^+$Foxp3$^+$ T cells in tumor-draining lymph node and tumor tissue at 15 days after tumor inoculation. The geometric MFI of (*A*) indicated transcription factors and (*B*) CXCR3 was shown. The representative plots and graphs are shown. The graphs show means ± SEM. *, $p<0.05$; ***, $p<0.001$.

Fig. S3. Immune cell accumulation in tumor tissues of IL-27R-deficient mice.

WT, *Il27ra*$^{-/-}$ mice (*A* and *B*), or WT:*Il27ra*$^{-/-}$ mixed BM chimeric mice(*C*) were inoculated s.c. with B16F10 tumor cells. The frequencies and numbers of Foxp3$^+$ and Foxp3$^-$CD4$^+$T cells (*A* and *C*) as well as the number of total TILs were determined 15 days post tumor inoculation. The graphs show means ± SEM.

Fig. S4. A role of IL-27 signal on tumor-infiltrating NK and NKT cells.

WT or *Il27ra*$^{-/-}$ mice were inoculated s.c. with B16F10 and were analyzed 15 days post inoculation. (*A*-

C) The frequencies and numbers of NK ($NK1.1^+TCR\beta^-$) and NKT ($NK1.1^+\alpha GC$ tetramer$^+$) cells in tumor tissue were determined. (*D-F*) The frequency and number of IFN-γ-producing NK and NKT cells were determined. The graphs show means ± SEM. *, $p<0.05$; **, $p<0.01$ (n=3-5).

Fig. S5. Growth of subcutaneous B16F10 tumor *Ebi3*$^{-/-}$ and *Il27ra*$^{-/-}$ mice.

(*A, B*) WT, *Ebi3*$^{-/-}$, and *Il27ra*$^{-/-}$ mice were inoculated s.c. with B16F10, and tumor growth was monitored. The graphs show means ± SEM. ***, $p<0.001$. The data are representative of three independent experiments (n=4-5).

Fig. S6. Expression of IL-27 by tumor-infiltrating cells.

Foxp3$^{YFP\text{-}Cre}$ mice were injected s.c. with B16F10. Tumor-bearing mice were sacrificed 12 and 15 days after tumor inoculation. Tregs (Foxp3-YFP$^+$), Foxp3$^-$CD4$^+$ T cells ($TCR\beta^+$Foxp3-YFP$^-$), and macrophages ($F4/80^+CD11c^{+/-}$) cells were sorted from tumor and spleen, and the relative gene expression of *Il27* (*A*) and *Ebi3* (*B*) were examined by qPCR. The tumor growth (*C*), IL-27 level in tumor lysates (*D*), Foxp3$^+$ frequency among CD4$^+$ T cells (*E*), CD39 geometric MFI on Ti-Tregs (*F*), and CD11c$^+$ frequency among $CD11b^+F4/80^+$ macrophages (*G*) were analyzed at indicated time points. The graphs show means ± SEM. *, $p<0.05$ (n=3-5).

Fig. S7. IL-27-STAT1 signal induces CD39 upregulation on Tregs in steady-state.

(*A*) Schematic outline of mixed BM chimeric mouse experiments. (*B*) $CD4^+Foxp3^+$ T cells in the thymus and the spleen from indicated BM chimeric mice were analyzed for CD39 expression. Representative plots and geometric MFI of CD39 are shown. The graphs show means ± SEM. *, $p<0.05$; **, $p<0.01$ (n=3-4).

Fig. S8. CD39 expression on Tregs stimulated with IL-35.

Naïve $CD4^+$ T cells were differentiated under iTreg-skewing condition in the presence or absence of IL-27 or IL-35-Fc protein. The geometric MFI of CD39 expression on $CD4^+Foxp3^+$ T cells was determined. The graphs show means ± SEM. ***, $p<0.001$.

Fig. S9. Putative STAT1 binding sites in *Entpd1* locus.

(*A*) The *Entpd1* conservation alignment plot for mouse was depicted by using ECR Browser. The vertical position corresponds to the level of nucleotide identity in this alignment. Corresponding *Entpd1* loci were depicted below (*B*) Five highly conserved regions (Red squares, 1-5) were selected to predict STAT1, Smad3, and Foxp3 binding affinity. % Dissimilarity presented by PROMO was shown. nd, no binding detected.

Fig. S10. A role for IFN-γ on CD39 expression on Tregs.

(*A*) WT and *Il27ra*$^{-/-}$ mice were inoculated s.c. with B16F10 tumor cells. The mice were treated with either IFN-γ neutralizing or isotype-matched control antibodies starting from 7 days after tumor inoculation (n=3-4). The geometric MFI of CD39 expression on Ti-$Foxp3^+$ cells was analyzed. (*B*) Naïve $CD4^+$ T cells from WT or *Stat1*$^{-/-}$ mice were differentiated under iTreg-skewing condition in the presence or absence of IL-27 or IFN-γ. The geometric MFI of CD39 expression on $Foxp3^+$ cells was analyzed. The graphs show means ± SEM. **, $p<0.01$; ***, $p<0.001$.

Fig. S11. Adenosine-A_{2A} adenosine receptor interaction is crucial for IL-27-stimulated Treg-mediated suppression.

(*A*) CFSE-labeled naïve $CD8^+$ T cells were incubated with iTregs or IL-27-iTregs for 3 days. % suppression on T cell proliferation is shown. (*B*) The effect of ARL67156, CD39 blockade, on adenosine production, and (*C*) the effect of ZM241385, A_{2A} receptor blockade, on IL-2-producing $CD8^+$ T cells. The graphs show means ± SEM. **, $p<0.01$; ***, $p<0.001$.

Fig. S12. IL-27 signal on Tregs is required for its protumorigenic activity in tumor.

(*A-C*) WT and *Il27ra*$^{-/-}$ Tregs ($CD45.2^+$) were mixed with B16f10 tumor cells, and subcutaneously injected into *Rag1*$^{-/-}$ mice reconstituted with naïve $CD4^+$ T and $CD8^+$ T cells ($CD45.1^+$). The tumor growth was monitored (*A*). The frequency of indicated cytokine-producing $CD8^+$ T cells (*B)* and NK cells (*C*) were analyzed 15 days after tumor inoculation. The graphs show means ± SEM. *, $p<0.05$ (n=3-5).

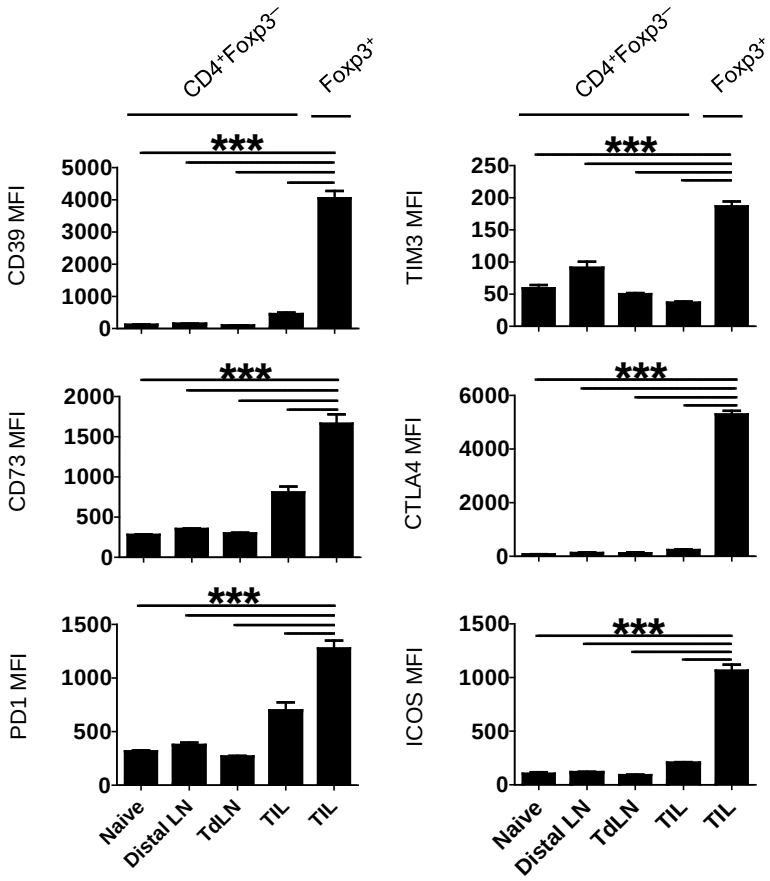
(*D-G*) Separately, WT and *Il27ra*$^{-/-}$ Tregs or *(H-K*) WT and *Stat1*$^{-/-}$ Tregs ($CD45.2^+$) were mixed with B16F10 tumor cells, and subcutaneously injected into *Rag1*$^{-/-}$ mice reconstituted with T cells as indicated above. Some mice were treated i.p. with ARL67156 (*D-G*). Tumor weight, TIL number (*D* and *H*), Treg frequency/number (*E* and *I*), Annexin V (*F* and *J*), and Ki67 levels (*G* and *K*) were analyzed 12 days after tumor inoculation. The graphs show means ± SEM. *, $p<0.05$ (n=4-5).

Fig. S13. Schematic model of the mechanisms by which IL-27 tunes Tregs to inhibit effector $CD8^+$ T cells.

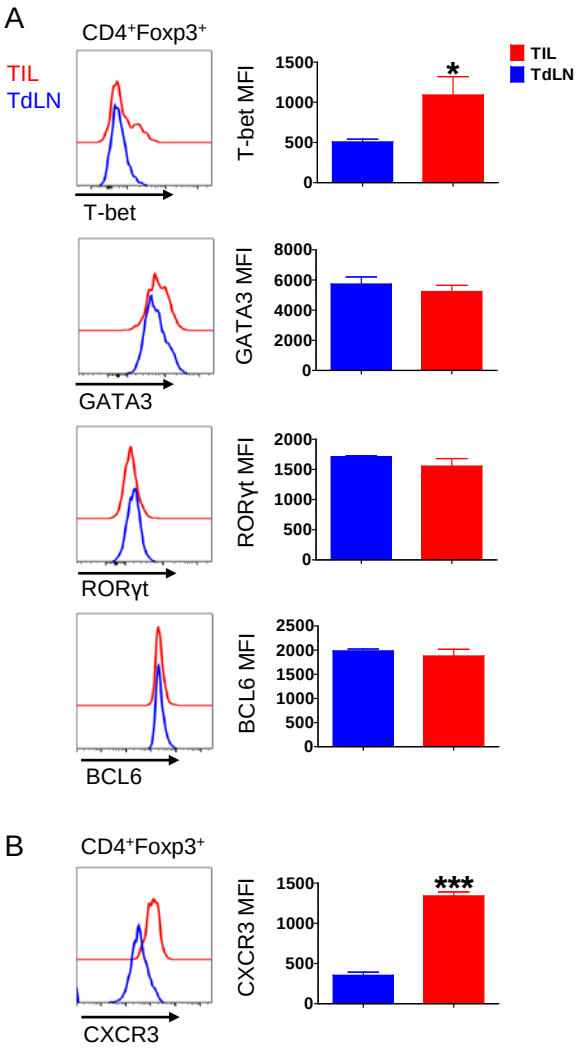
Upon encounter of IL-27 with its hetero-dimeric receptor (gp130-IL-27Rα) on Tregs in tumor tissue, the phosphorylation of STAT1 induces the expression of ectonucleotidase CD39 by interplay with unknown molecular partners. The increased CD39 on Tregs vigorously hydrolyzes ATP, leading to an increase in adenosine which, in turn, suppresses effector function of tumor-infiltrating $CD8^+$ T cells. Blockade of IL-27 signaling thus prevents Tregs from STAT1 phosphorylation and CD39 upregulation, which relives

$CD8^+$ T cells from Treg-mediated suppression in tumor tissue.

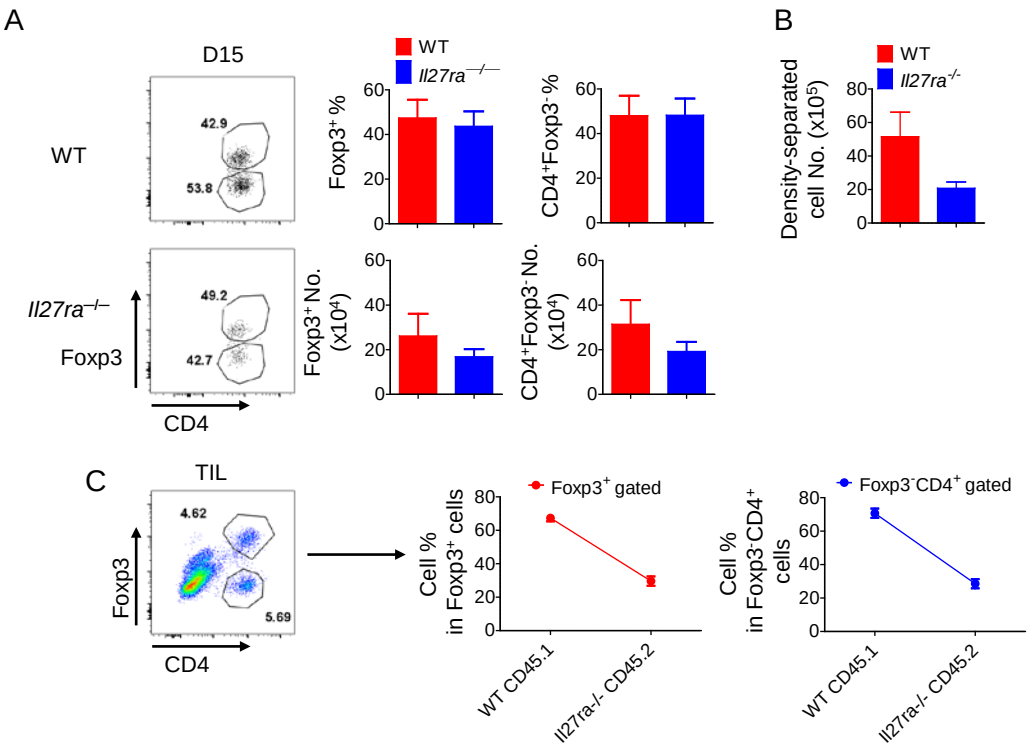
Supplementary Figure 1



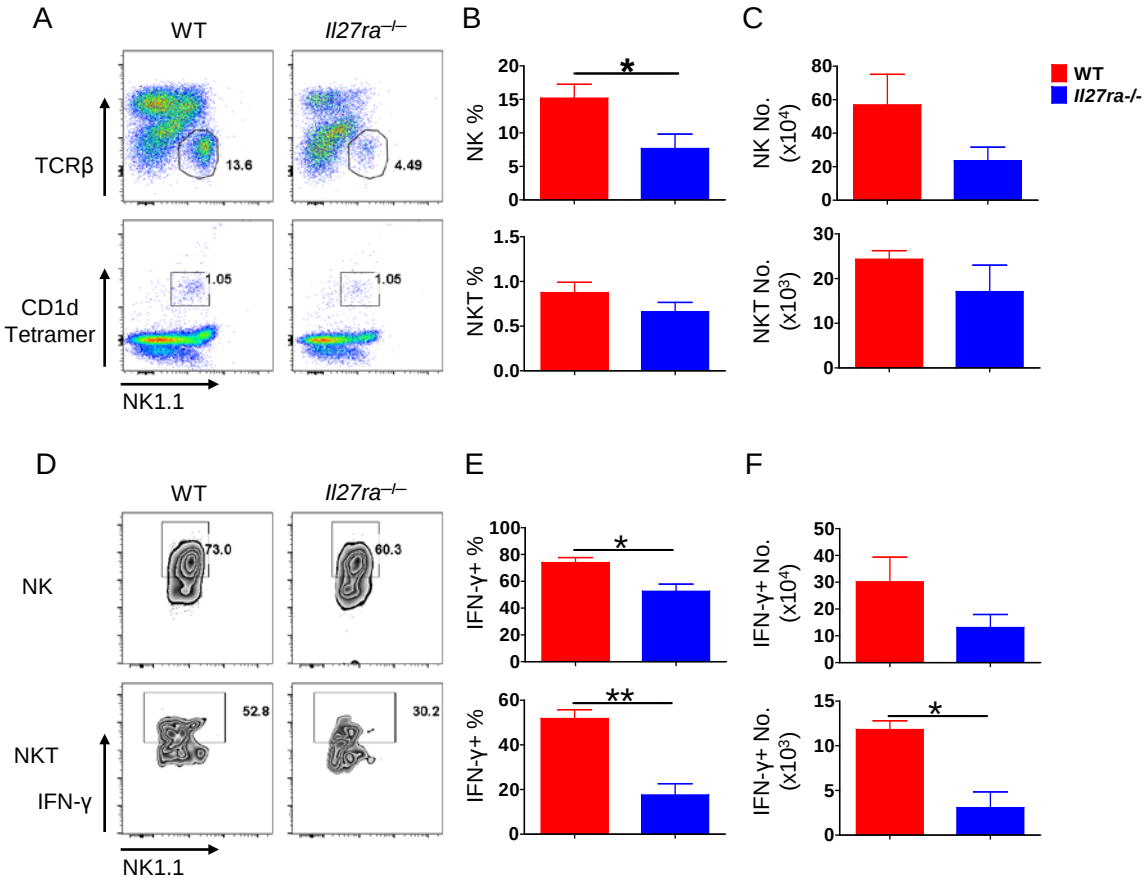
Supplementary Figure 2



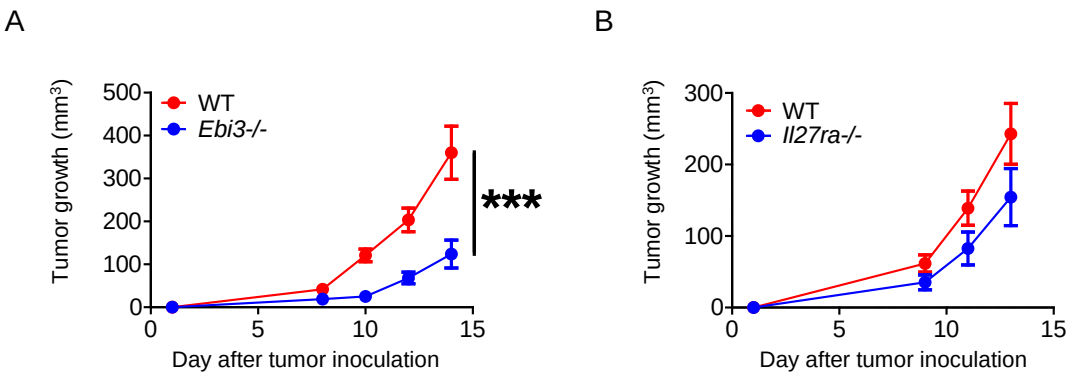
Supplementary Figure 3



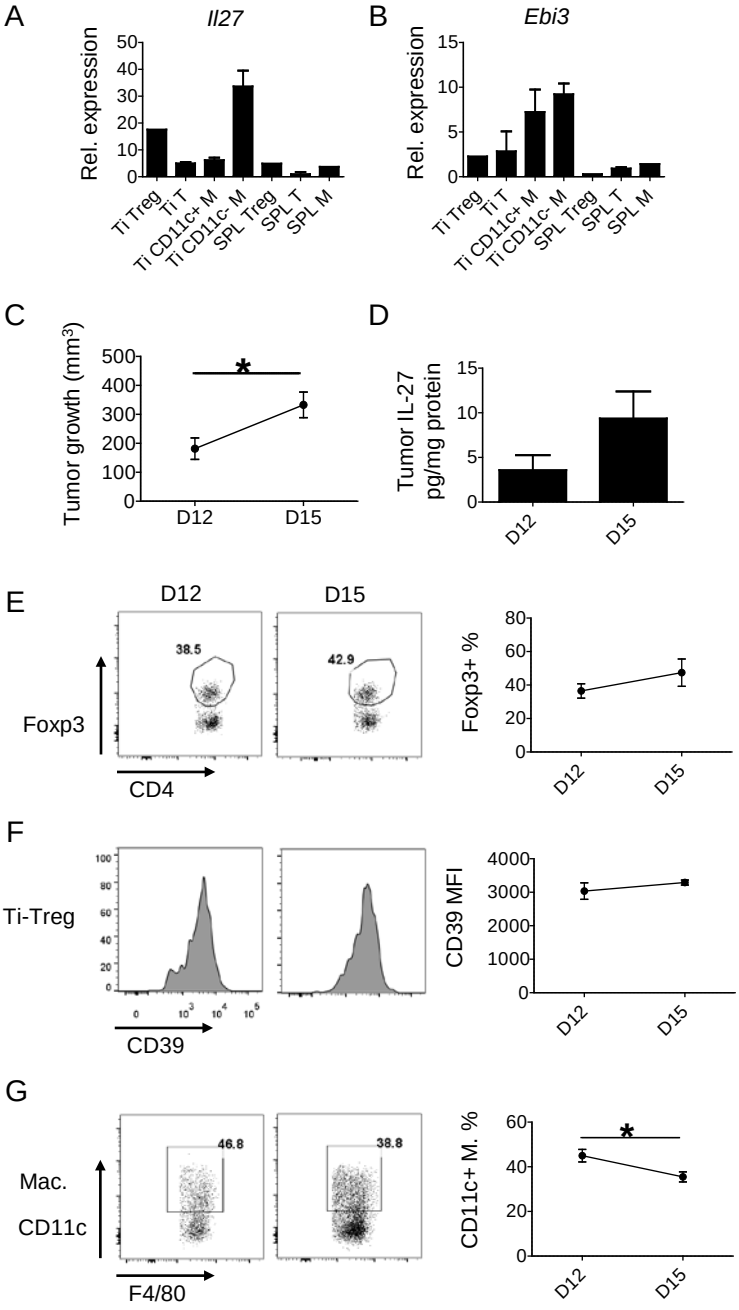
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



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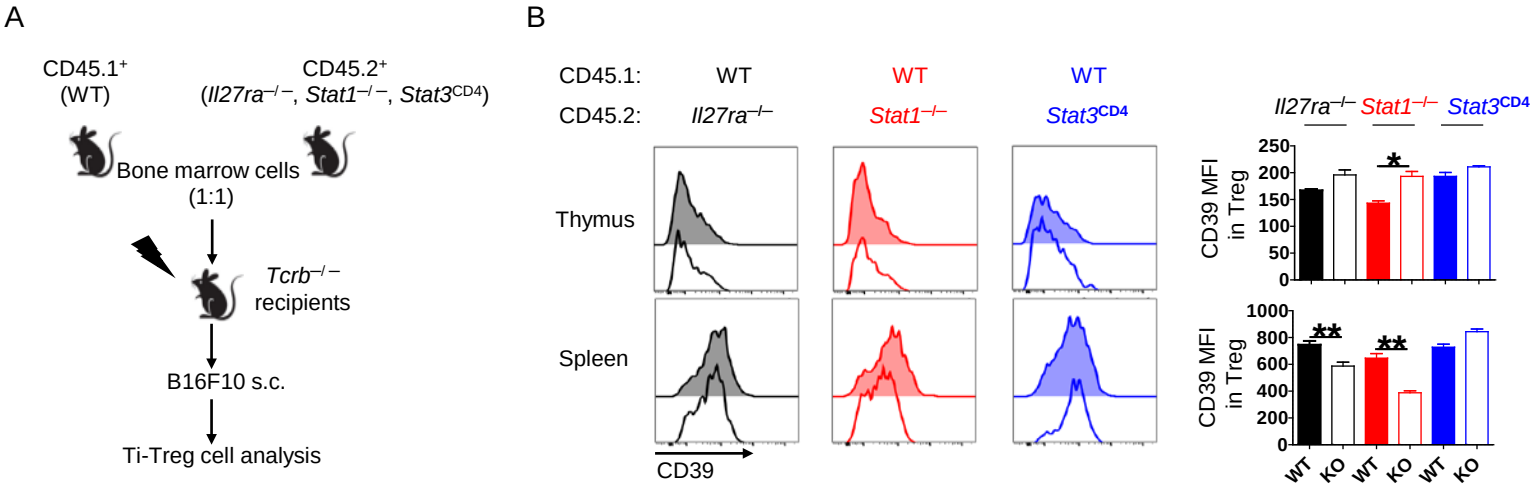
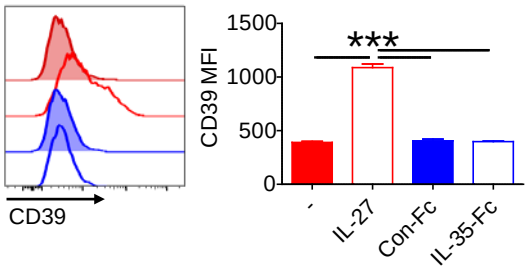


Supplementary Figure 8



Supplementary Figure 9

A

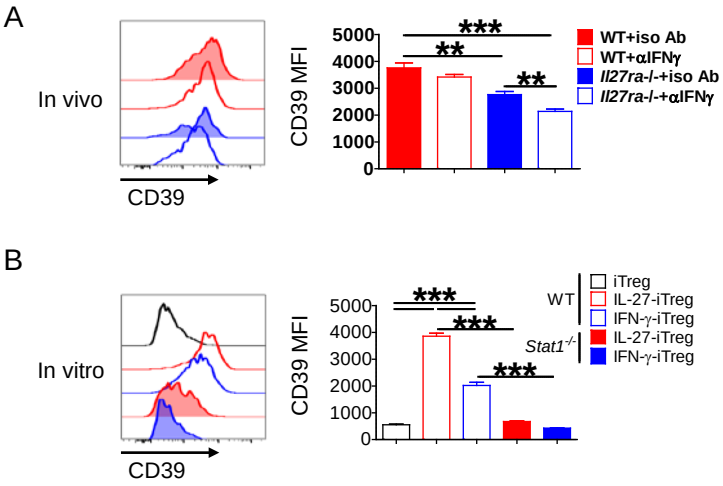
Conservation plot for *Entpd1*

B

Dissimilarity %

	1	2	3	4	5
STAT1	5.8	5.8	2.9	5.8	5.8
Foxp3	3.08	4.26	0	0	3.08
Smad3	3.2	1.94	0.94	0	nd

Supplementary Figure 10



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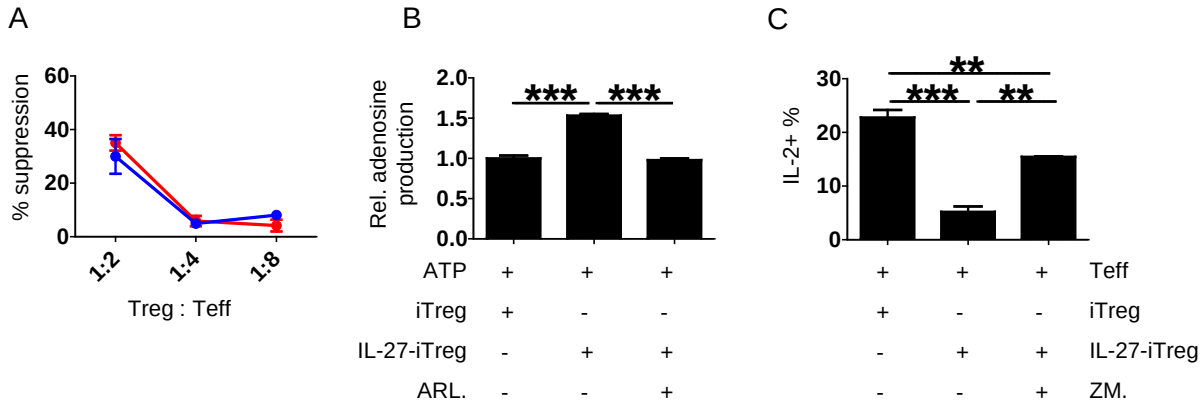
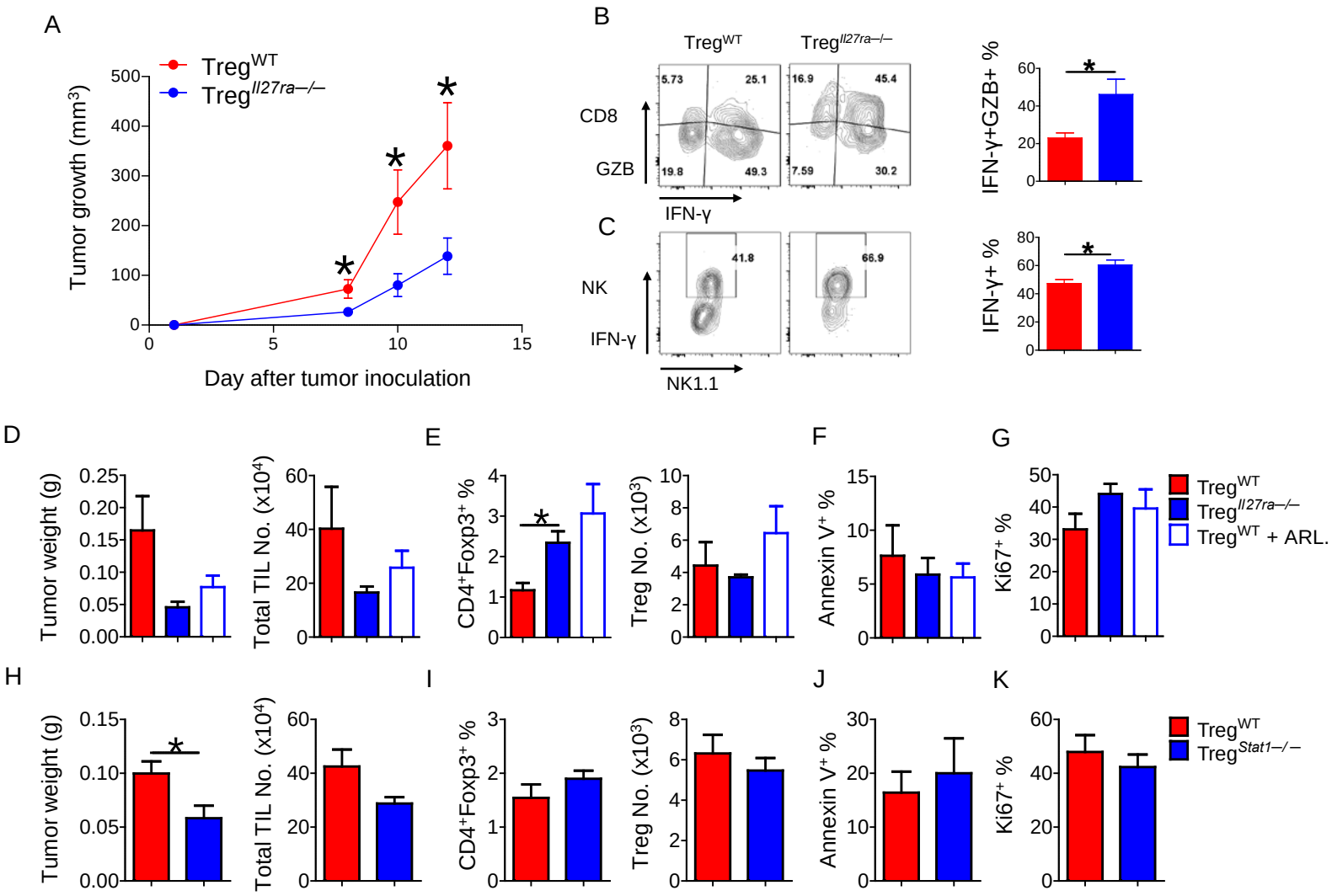


Supplementary Figure 12



Supplementary Figure 13