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Dendritic cells (DCs) are important for adaptive immune responses through the activation of T cells. The molecular interplay between DCs and T cells determines the magnitude of T cell responses or outcomes of functional differentiation of T cells. In this study, we demonstrated that DCs in mice that are *Rbpj* deficient in CD11c$^+$ cells (Rbpj$^{-/-}$ mice) promoted the differentiation of IL-17A–producing Th17 cells. *Rbpj*-deficient DCs expressed little Aldh1a2 protein that is required for generating retinoic acid. Those DCs exhibited a reduced ability for differentiating regulatory T cells induced by TGF-β. Rbpj protein directly regulated *Aldh1a2* transcription by binding to its promoter region. The overexpression of *Aldh1a2* in Rbpj-deficient DCs negated their Th17-promoting ability. Transfer of naive CD4$^+$ T cells into *Rag1*-deficient Rbpj$^{-/-}$ mice enhanced colitis with increased Th17 and reduced induced regulatory T cells (iTreg) compared with control *Rag1*-deficient mice. The cotransfer of iTreg and naive CD4$^+$ T cells into *Rag1*-deficient Rbpj$^{-/-}$ mice improved colitis compared with transfer of naive CD4$^+$ T cell alone. Furthermore, cotransfer of DCs from Rbpj$^{-/-}$ mice that overexpressed *Aldh1a2* or Notch-stimulated DCs together with naive CD4$^+$ T cells into Rbpj$^{-/-}$ *Rag1*-deficient mice led to reduced colitis with increased iTreg numbers. Therefore, our studies identify Notch signaling in DCs as a crucial balancer of Th17/iTreg, which depends on the direct regulation of *Aldh1a2* transcription in DCs. ***The Journal of Immunology*****, 2017, 199: 1989–1997.**

Dendritic cells (DCs) are specialized APCs for T cell activation and differentiation in lymphoid organs (1). Immature DCs are activated through pattern recognition receptors, including TLR. Activated mature DCs express high levels of coreceptor molecules, including CD80 and CD86, required for T cell activation (2, 3). Mature DCs also secrete various types of cytokines to enhance or dampen T cell differentiation in a context-dependent manner (3).

Notch is an evolutionarily conserved molecule, and mammals have four Notch receptors and five Notch ligands. The interaction between Notch and its ligands induces the cleavage of Notch receptors, and the intracellular domain of Notch translocates into the nucleus (4, 5). The intracellular domain of Notch interacts with recombinant binding protein suppressor of hairless (Rbpj), which further recruits mastermind-like 1 and p300 and controls gene expressions. We and other groups have revealed that Notch signaling has diverse roles in immune cell activation and survival. For instance, Notch regulates the effector function of CD4 and CD8 T cells. Notch also controls the survival of memory CD4 T cells (4–8). Notch is known to control the survival of CD8$^-$ DCs (9), and Notch2 deletion leads to the loss of CD11b$^+$CD103$^+$ DCs in the intestinal lamina propria with decreases of IL-17–producing CD4$^+$ T cells in the intestine (10). Furthermore, Notch2-dependent intestinal CD11b$^+$ DCs secrete IL-23 required for survival post-infection with *Citrobacter rodentium* (11).

Retinoic acid (RA) is a natural metabolite of vitamin A and contributes to the differentiation of regulatory T cells (Tregs) by enhancing TGF-β signaling through activation of Smad3 (12–14) while simultaneously inhibiting proinflammatory Th17 differentiation (14). RA cannot be synthesized de novo by animals. Instead, it must be obtained from retinol. Retinol is oxidized to retinaldehyde, which in turn is converted to RA by retinaldehyde dehydrogenases, including Aldh1a2.

In this study, we demonstrate that *Rbpj*-deficient DCs strongly induce IL-17A–producing CD4$^+$ T (Th17) cells. The *Rbpj*-deficient DCs express lower amounts of Aldh1a2 than control DCs and thus have a reduced capacity to promote induced Treg (iTreg) differentiation. Using a naive T cell transfer model, we found that mice that lack *Rbpj* in CD11c-positive cells exhibited high sensitivity to colitis compared with wild-type mice. Those data demonstrate that Notch signaling in DCs acts as a suppressor for Th17 cells by controlling iTreg differentiation through RA synthesis. Furthermore, the strong regulation by Notch-treated DCs, as well as by direct Notch agonists in vivo, suggests potential clinical implications for autoimmune diseases.

Materials and Methods

Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). OT-II TCR transgenic and Rag1$^{-/-}$ mice were purchased from Taconic. All mice were maintained under specific pathogen-free conditions in the

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The sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE100593.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; DC, dendritic cell; iTreg, induced Treg; qPCR, quantitative PCR; RA, retinoic acid; Th17, IL-17A–producing CD4$^+$ T; Treg, regulatory T cell; tTreg, thymus-derived Treg.

animal research center of Tokushima University, and all animal experiments were approved by the animal research committee of Tokushima University and performed in accordance with our institution's guidelines for animal care and use.

T cell proliferation assay in vitro and in vivo

Naive $CD4^+CD62L^+CD25^-CD44^{low}$ T cells were isolated from the spleens of C57BL/6 mice or OT-II TCR transgenic mice with a naive $CD4^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA). T cells (1×10^6/well) were stimulated with DCs (5×10^5/well) in 48-well plates in the presence of anti-CD3 mAb (2C11; 1 μg/ml) or OVA peptide (323–339; 1 μg/ml). In some experiments, anti–IL-6 mAb (MAB406; R&D Systems) (10 μg/ml), RA (10 nM; Sigma-Aldrich), or RA inhibitor (LE540, 1 μM; Wako) was added to the culture. CFSE-labeled naive $CD4^+$ T cells (3×10^6) from OT-II TCR transgenic mice were transferred into nonirradiated recipient mice, and OVA peptide emulsified with CFA was immunized 2 d after T cell transfer.

Culture of bone marrow–derived DCs

Bone marrow cells were harvested from femurs and tibias of C57BL/6 mice and cultured with 20 ng/ml GM-CSF for 5 d. $CD8\alpha^-CD11c^+$ DCs were then purified by flow cytometric sorting. Total RNA from DCs from $Rbpj^{+/+}$ or $Rbpj^{-/-}$ was isolated with Reliaprep RNA cell miniprep system (Promega), and their expressions were evaluated with Whole Mouse Genome Microarray 4 × 44K v2 (Agilent). The raw data and normalized results were deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession GSE100593. Retrovirus vector (pKE004) (6) carrying mouse *Aldh1a2* was constructed. Retrovirus was obtained by transfecting the vector in Plat-E cells (15). Retrovirus was spin infected in DCs twice (days 0 and 1). In some experiments, $CD8\alpha^-CD11c^+$ DCs were stimulated with Delta1-Fc (10 μg/ml) or control IgG (6) for 24 h.

The generation of iTreg

Naive $CD4^+CD62L^+CD25^-CD44^{low}$ T cells were isolated from spleen cells of C57BL/6 mice with a naive $CD4^+$ T cell isolation kit (Miltenyi Biotec). Cells were cultured in 48-well plates and stimulated with anti-CD3 mAb (2C11; 1 μg/ml) and DCs in the presence of TGF-β (2 ng/ml; R&D Systems) for 3 d.

T cell suppression assay

Graded numbers of thymus-derived Tregs (tTregs) or iTregs or naive $CD4^+$ T cells (2×10^5) were cultured with DCs (1×10^5) and anti-CD3 mAb (2C11, 1 μg/ml). The tTregs were isolated with a Treg isolation kit (Miltenyi Biotec). Cells were cultured for 3 d, and 1 μCi/well [^{3}H] thymidine was added for the last 8 h of culture. The proliferation was analyzed by assessing thymidine incorporation.

Splenic DC isolation

Using a 26G needle, spleens were infused with collagenase D (Roche) and DNase I (2 mg/ml) (Roche) enzymes dissolved in prewarmed RPMI 1640 media containing 10% FBS. The spleens were shredded and incubated at 37°C for 1 h. Small pieces were mashed with a glass slide, and RBC were lysed by NH_4Cl. $CD11c^+CD8\alpha^-$ and $CD11c^+CD8\alpha^+$ DCs were sorted with a BD FACS Aria III (BD Biosciences).

T cell–mediated colitis

Naive $CD4^+$ T cells were isolated from C57BL/6 mice using a naive $CD4^+$ T cell isolation kit (Miltenyi Biotec), and cells were transferred to recipient mice (4×10^5 cells per mouse) by i.p. injection. In some experiments, DCs (1×10^6 cells per mouse) or in vitro generated iTreg (1×10^5 cells per mouse) were transferred into recipient mice at 5-d intervals that began when naive $CD4^+$ T cells were transferred.

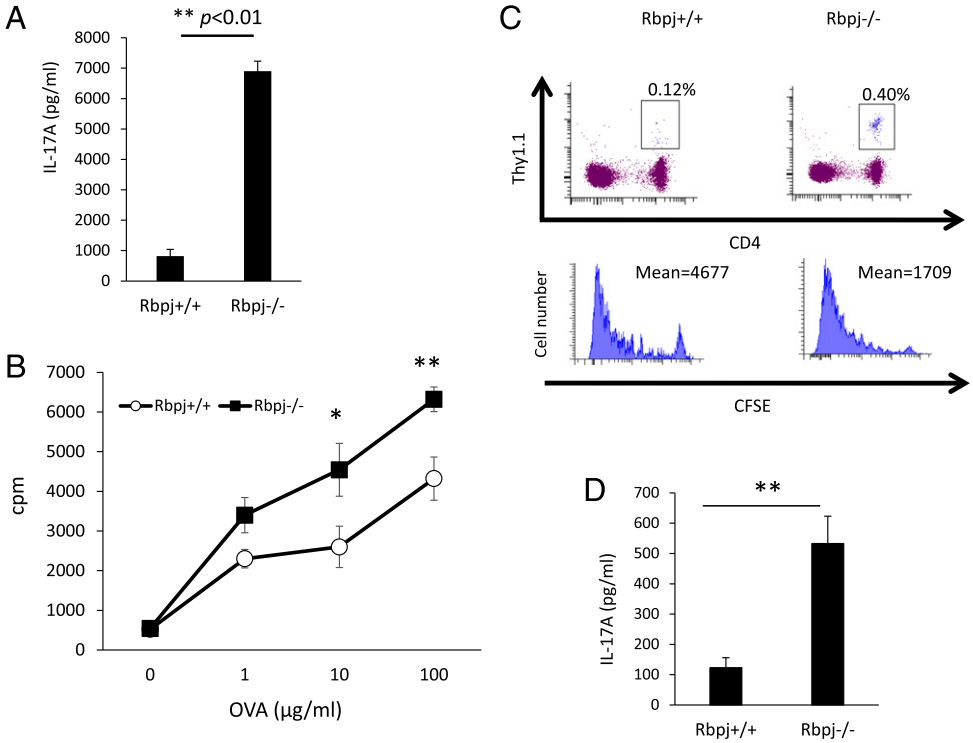

FIGURE 1. Enhanced Th17 differentiation in $Rbpj^{-/-}$ mice. (**A**) Bone marrow–derived $CD8\alpha^-CD11c^+$ cells from $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice were cultured with $CD4^+$ T cells from OT-II TCR transgenic mice in the presence of OVA peptide for 3 d. The concentration of IL-17A in the supernatant was evaluated by ELISA. Data are mean ± SD. $^{**}p < 0.01$. (**B**) The proliferation of T cells was evaluated by [^{3}H]thymidine incorporation during the final 6 h. Data are mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$. (**C**) CFSE-labeled $CD4^+$ T cells from OT-II TCR transgenic mice (Thy1.1) were transferred into $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice ($n = 5$ in each group). OVA peptide emulsified with CFA was immunized 2 d after T cell transfer. T cell proliferation was evaluated 4 d after OVA immunization by CFSE intensity. The number in the upper panel shows the percentage of $CD4^+Thy1.1^+$ cells in the total $CD4^+$ cell population. The number in the lower panel shows mean fluorescence intensity of $CD4^+Thy1.1^+$ cells. (**D**) $CD4^+$ T cells from OT-II TCR transgenic mice were transferred into $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice ($n = 5$ in each group). OVA peptide emulsified with CFA was immunized 2 d after T cell transfer. $Thy1.1^+$ cells were sorted 7 d after OVA immunization and stimulated with OVA peptide for 3 d. The concentration of IL-17A in the supernatant was evaluated by ELISA. Data are mean ± SD. These data are representative of three independent experiments. $^{**}p < 0.01$.

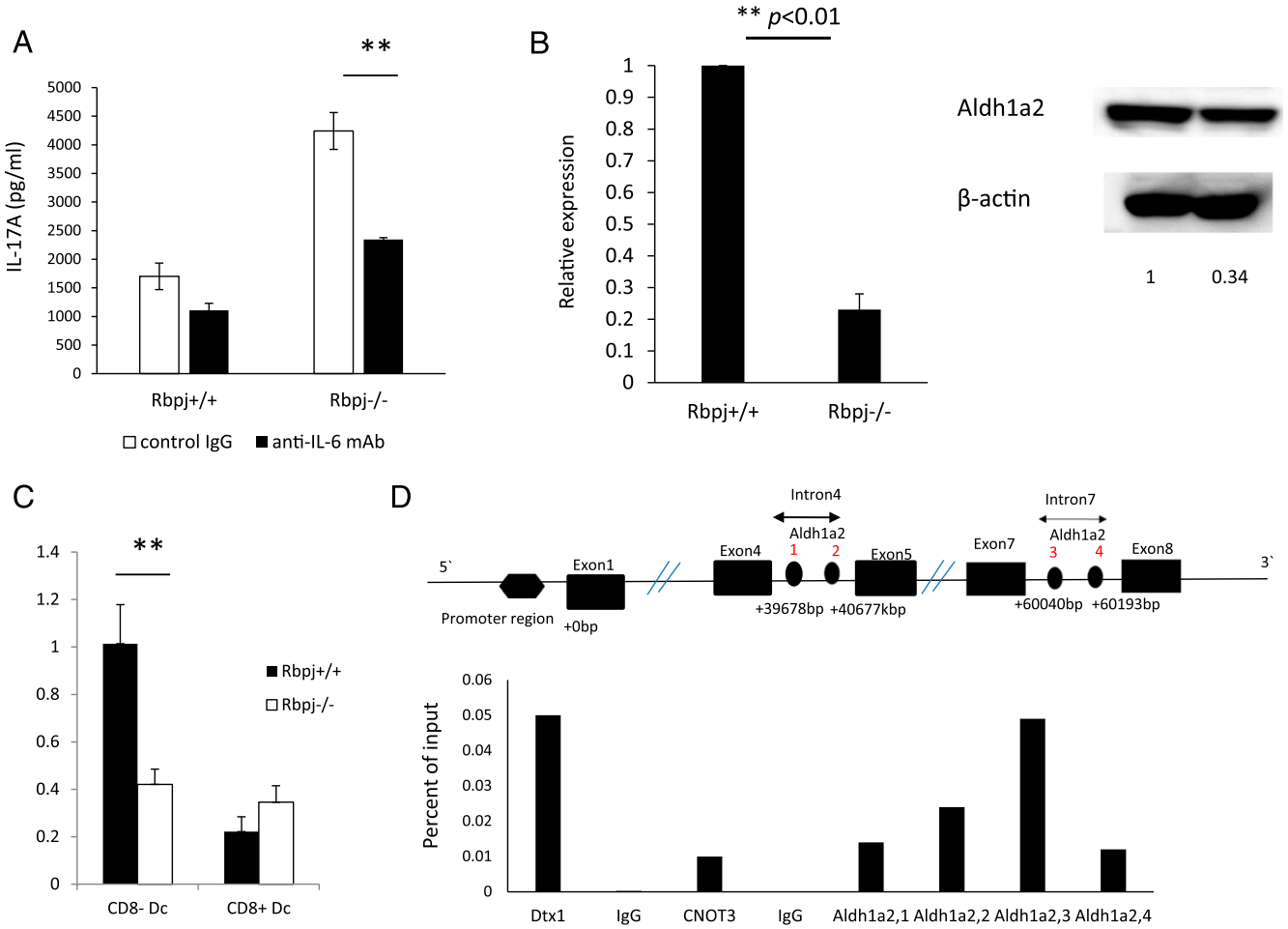

FIGURE 2. Direct regulation of *Aldh1a2* by Notch. (**A**) $CD4^+$ T cells from OT-II TCR transgenic mice were stimulated with OVA protein and bone marrow–derived $CD8\alpha^-CD11c^+$ cells from $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice in the presence of anti–IL-6 mAb (closed) or control IgG (open) (10 μg/ml) for 3 d. The concentration of IL-17A in the supernatant was evaluated by ELISA. Data are mean ± SD. $^{**}p < 0.01$. (**B**) The expression of *Aldh1a2* in bone marrow–derived $CD8\alpha^-CD11c^+$ cells from $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice was assessed by real-time PCR (left) and Western blot (right). Data are mean ± SD. $^{**}p < 0.01$. (**C**) The expression of *Aldh1a2* in sorted $CD11c^+CD8\alpha^-$ or $CD11c^+CD8\alpha^+$ DCs from the spleens of $Rbpj^{-/-}$ (open) or $Rbpj^{+/+}$ (closed) mice. Data are mean ± SD. $^{**}p < 0.01$. (**D**) Configuration of the *Aldh1a2* gene. Numbers (1–4) in red indicate the putative Rbpj binding regions. Chromatin samples from $CD8\alpha^-CD11c^+$ DCs were immunoprecipitated with anti-Rbpj Ab. Enrichment of Rbpj binding sites was assessed by real-time PCR. Data are shown as percent values against input DNA. The data shown in this figure are representative of three independent experiments.

Flow cytometric analysis

Cells were first incubated with 2.4G2 for 10 min on ice. Then multicolor staining was conducted using combinations of the indicated mAbs. The following Abs were used: CD11b (Tonbo Biosciences), CD11c (Tonbo Biosciences), MHCII (BD Biosciences), and CD8α (Tonbo Biosciences). Foxp3 staining was performed according to the BD Biosciences protocol. After staining, cells were incubated for 15–20 min and samples were assessed using a flow cytometer (BD FACS CANTO II).

Real-time PCR

Total RNAs were isolated with the Reliaprep RNA cell miniprep system (Promega), transcribed to cDNA, and assayed by quantitative PCR (qPCR) on a Step-One RT-PCR system (Applied Systems) using SBYR green incorporation. All gene expression levels were normalized to *Hprt*, and relative expression was calculated using the ΔΔ cycle threshold method. All primer pairs were validated for amplification efficiency. The following primers were used: *Aldh1a2*, forward, 5′-ATGGGTGAGTTTGGCTTACG-3′ and reverse, 5′-GGTTCATTGGAAGGCAGAAA-3′; *Hprt*, forward, 5′-AGCCTAAGATGAGCGCAAGT-3′ and reverse, 5′-TTACTAGGCAGATGGCCACA-3′; and *Dtx1*, forward, 5′-CTGCACCCACCACCAGTAAG-3′ and reverse, 5′-TGTACCTCCGAACCACATCC-3′.

Chromatin immunoprecipitation

DCs (6×10^6) were fixed with 1% formaldehyde/PBS for 5 min and washed with ice-cold PBS. Cell nuclei were isolated by the addition of lysis buffer and centrifugation. Lysates were sonicated, and the DNA was sheared to an average length of 200 bp. Then anti-Rbpj (D10A4) rabbit mAb (Cell Signaling) or isotype control rabbit mAb IgG (Cell Signaling) was added. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K, and heat for decross-linking. Following immunoprecipitation with Dynabeads protein G (Invitrogen), complexes were washed and eluted from the beads with buffer elution (10% SDS, 1 M $NaHCO_3$) and subjected to RNase and proteinase K treatment. Cross-links were reversed by incubation overnight at 65°C, and chromatin immunoprecipitation (ChIP) DNA was purified by QIAquick PCR Purification/Gel Extraction Kit (Qiagen). Ethanol precipitation was used for the preparation of ChIP-qPCR analysis. qPCR reactions were carried out on specific genomic regions using SYBR Green SuperMix (Applied Biosystems). For the positive control and the negative control, *Dtx1* and *Cnot3*, respectively, were used. The following primers were used: *Aldh1a2*, forward, 5′-TTCAGGACAAAATGCCCACT-3′ and reverse, 5′-GATCTCAGCCCACCTTCTGA-3′; *Dtx1*, forward, 5′-CACACACCCTCCTGCAGTC-3′ and reverse, 5′-CAGGGAGAGAGTCTCGATGC-3′; and *Cnot3*, forward, 5′-CAAGACATGGGTAGCATCAA-3′ and reverse, 5′-TGGTTTCTAACCGTCTCAAT-3′. The relative expression was calculated by the ΔΔ cycle threshold method. All primer pairs were validated for amplification efficiency.

ELISA

The concentrations of IL-17A and TGF-β were assessed with ELISA kits (mouse IL-17A ELISA Ready-SET-Go! and TGF-β 1 ELISA Ready-SET-Go!; Affymetrix, eBioscience).

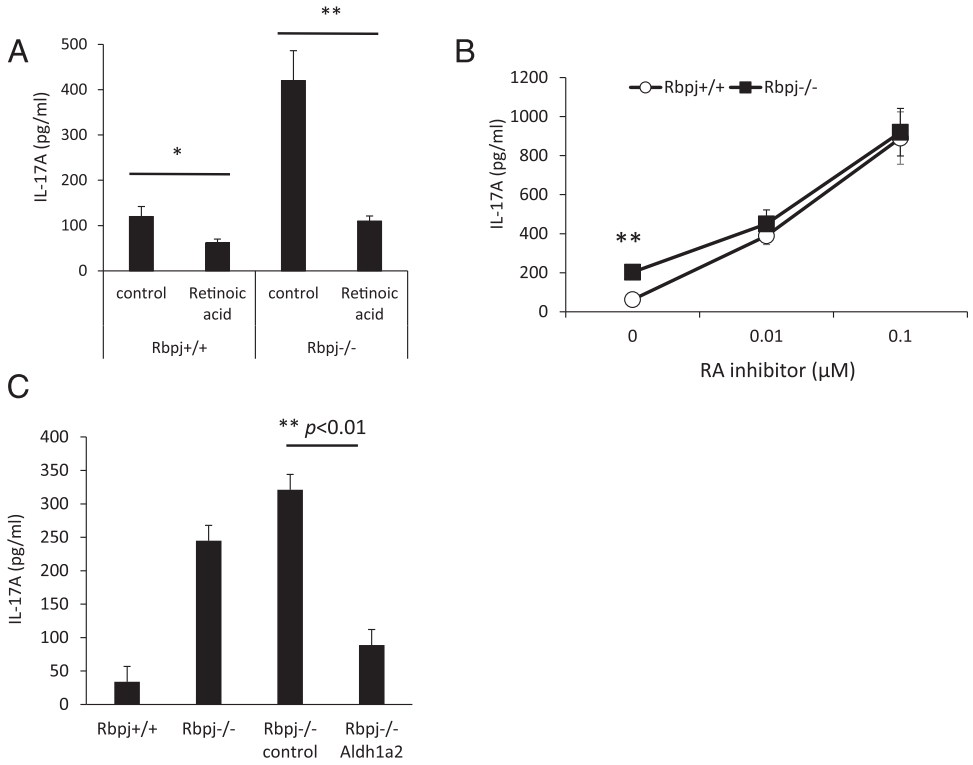

FIGURE 3. Notch-mediated regulation of RA controls Th17. (**A**) $CD4^+$ T cells from OT-II TCR transgenic mice were stimulated with OVA peptide and bone marrow–derived $CD8\alpha^-CD11c^+$ cells from $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice in the presence or absence of RA (0.5 nM) for 3 d. The concentration of IL-17A in the supernatant was evaluated by ELISA. Data are mean ± SD. $*p < 0.05$, $**p < 0.01$. (**B**) $CD4^+$ T cells from OT-II TCR transgenic mice were stimulated with OVA peptide and bone marrow–derived $CD8\alpha^-CD11c^+$ cells from $Rbpj^{-/-}$ (closed squares) or $Rbpj^{+/+}$ mice (open circles) in the presence of various concentration of RA inhibitor for 3 d. The concentration of IL-17A in the supernatant was evaluated by ELISA. Data are mean ± SD. $**p < 0.01$. (**C**) $CD4^+$ T cells from OT-II TCR transgenic mice were stimulated with OVA peptide and bone marrow–derived $CD8\alpha^-CD11c^+$ cells from $Rbpj^{-/-}$ mice infected with control retrovirus or retrovirus carrying the *Aldh1a2* gene for 3 d. As the control, $CD8\alpha^-CD11c^+$ cells from $Rbpj^{+/+}$ mice or uninfected $CD8\alpha^-CD11c^+$ cells from $Rbpj^{-/-}$ mice were used. The concentration of IL-17A in the supernatant was evaluated by ELISA. Data are mean ± SD. The data shown in this figure are representative of three independent experiments. $**p < 0.01$. ND, not detected.

Western blotting and blue native PAGE

Cell pellets were lysed in cold radioimmunoprecipitation assay buffer (Wako Pure Chemical Industries) and protease inhibitor (Roche), and the lysates were boiled in SDS loading dye. The samples were resolved by SDS-PAGE, and the blots were incubated with anti-Aldh1a2 (F1804; Sigma-Aldrich) Ab. They were next incubated with peroxidase-conjugated goat anti-mouse IgG (31430; Pierce Biotechnology) or goat anti-rabbit IgG (170-6515; Bio-Rad) Abs. As a control, membranes were probed with polyclonal anti-actin (A2066; Sigma-Aldrich) and HRP-conjugated goat anti-rabbit IgG Abs (170-6515; Bio-Rad). ECL prime Chemiluminescent Substrate (GE Healthcare) was added to the membranes, after which they were analyzed with the Lumino Image Analyzer (GE Healthcare).

Statistics

The statistical significance of between-group differences was evaluated by an unpaired, a two-tailed *t* test, or a parametric Dunnett test. A *p* value <0.05 was considered significant.

Results

DCs from $Rbpj^{-/-}$ mice promoted Th17 differentiation

We first sought to investigate whether Notch signaling in DCs affected T cell functions. As Rbpj is a crucial regulator of Notch signaling (4, 16), we crossed $Rbpj^{flox/flox}$ mice with CD11c-*Cre* transgenic ($Rbpj^{-/-}$) mice to delete *Rbpj* from DCs. $Rbpj^{+/+}$ mice with CD11c-*Cre* transgenic ($Rbpj^{+/+}$) mice were used as the controls. Bone marrow cells from $Rbpj^{-/-}$ or $Rbpj^{+/+}$ were cultured in the presence of GM-CSF for 7 d. The resultant purified $CD8\alpha^-CD11c^+$ cells were cocultured with $CD4^+$ T cells from OT-II TCR transgenic mice in the presence or the absence of OVA peptide. After 3 d of culture, we tested the production of IL-17A by $CD4^+$ T cells. The stimulation of $CD4^+$ T cells in the presence of DCs from $Rbpj^{-/-}$ mice enhanced the production of IL-17A compared with those from $Rbpj^{+/+}$ mice (Fig. 1A). The expression levels of coreceptor molecules, including MHC class II, were equivalent for DCs from wild-type and $Rbpj^{-/-}$ mice (Supplemental Fig. 1). The secretion levels of IFN-γ and IL-4 were equivalent for DCs from $Rbpj^{+/+}$ and $Rbpj^{-/-}$ mice (Supplemental Fig. 2). We compared the $CD4^+$ T cells' proliferation between those two groups in vitro and found increased $CD4^+$ T cell proliferation when stimulated with DCs from $Rbpj^{-/-}$ mice (Fig. 1B). We also evaluated the effect on T cell proliferation in vivo. CFSE-labeled OT-II T cells were transferred into $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice that were subsequently immunized with OVA protein emulsified with CFA. The donor (Thy1.1) and host (Thy1.2) T cells could be distinguished by Thy1.1 or Thy1.2 expression. The CFSE in $CD4^+$ T cells was diluted more in $Rbpj^{-/-}$ recipients than wild type (Fig. 1C), indicating that the absence of Notch signaling in DCs permitted increased $CD4^+$ T cell proliferation.

To evaluate the role of Notch-mediated DC functions in vivo, $CD4^+$ T cells from OT-II TCR transgenic mice were transferred into $Rbpj^{-/-}$ or control mice that were subsequently immunized with OVA peptide. The $CD4^+$ T cells were purified 7 d after immunization and stimulated with OVA peptide. The concentrations of IL-17A in the supernatant were measured. The $CD4^+$ T cells purified from $Rbpj^{-/-}$ mice produced more IL-17 than did cells from $Rbpj^{+/+}$ mice (Fig. 1D). Collectively, those data indicated that the deficiency of *Rbpj* in DCs augmented Th17 differentiation.

IL-6 was required for Th17 differentiation by DCs from Rbpj$^{-/-}$ mice

The differentiation of Th17 requires IL-6 signaling (17). Thus, we tested the contribution of IL-6 to the enhancement of Th17 differentiation by DCs from Rbpj$^{-/-}$ mice. CD4$^+$ T cells from OT-II TCR transgenic mice were stimulated with DCs from Rbpj$^{-/-}$ mice and OVA peptide in the presence or absence of anti–IL-6 blocking mAb. The addition of anti–IL-6 mAb inhibited the enhanced Th17 differentiation of DCs from Rbpj$^{-/-}$ mice (Fig. 2A).

To evaluate the molecular mechanisms by which DCs from Rbpj$^{-/-}$ mice enhanced Th17 differentiation, we compared the mRNA expression of bone marrow–derived DCs from Rbpj$^{-/-}$ and Rbpj$^{+/+}$ mice using DNA microarray (accession GSE100593 in Gene Expression Omnibus). We found that the expression of *Aldh1a2* that is essential for the synthesis for RA was less in DCs from Rbpj$^{-/-}$ than from control mice. We confirmed the reduced expression of *Aldh1a2* in DCs from Rbpj$^{-/-}$ mice by real-time PCR and Western blotting (Fig. 2B). We also evaluated the expression of *Aldh1a2* in CD8α$^-$ or CD8α$^+$ splenic DCs from Rbpj$^{-/-}$ or Rbpj$^{+/+}$ mice (Fig. 2C). The expression of *Aldh1a2* was reduced only in CD8α$^-$ DCs in Rbpj$^{-/-}$ mice.

We analyzed whether Notch signaling directly regulated the transcription of *Aldh1a2* by ChIP assay. As the positive control, the *Dtx1* gene (known as a Notch target gene) was used. There are four putative Rbpj binding regions in the *Aldh1a2* gene. The ChIP assay revealed that Rbpj bound only to number 3 at intron 7 of the *Aldh1a2* gene (Fig. 2D), which demonstrates that Rbpj directly controls *Aldh1a2* transcription.

RA was involved in suppressive functions of DCs from Rbpj$^{-/-}$ mice

RA inhibits IL-6–mediated Th17 differentiation and promotes anti-inflammatory Treg differentiation (13). The effect of RA in the suppression of T cells is attributable to direct T cell suppression as well as promotion of iTreg differentiation (12, 13, 18, 19). Thus, we tested the effect of RA or an inhibitor of RA on Th17 differentiation induced by DCs from Rbpj$^{-/-}$ mice. OVA peptide stimulation of OT-II T cells with DCs from Rbpj$^{+/+}$ mice in the presence of RA suppressed Th17 cells (Fig. 3A), indicating that RA suppressed Th17 cells in this culture system. RA could also suppress Th17 cells when DCs from Rbpj$^{-/-}$ mice were used. The addition of the RA inhibitor in cocultures of OT-II T cells with DCs from Rbpj$^{+/+}$ mice promoted Th17 differentiation to a level equivalent to Rbpj$^{-/-}$ DCs (Fig. 3B).

To directly analyze whether low expression of *Aldh1a2* in DCs from Rbpj$^{-/-}$ mice was attributable to the increase of Th17, *Aldh1a2* was transduced into DCs from Rbpj$^{-/-}$ mice. The overexpression of *Aldh1a2* in *Rbpj*-deficient DCs reduced Th17

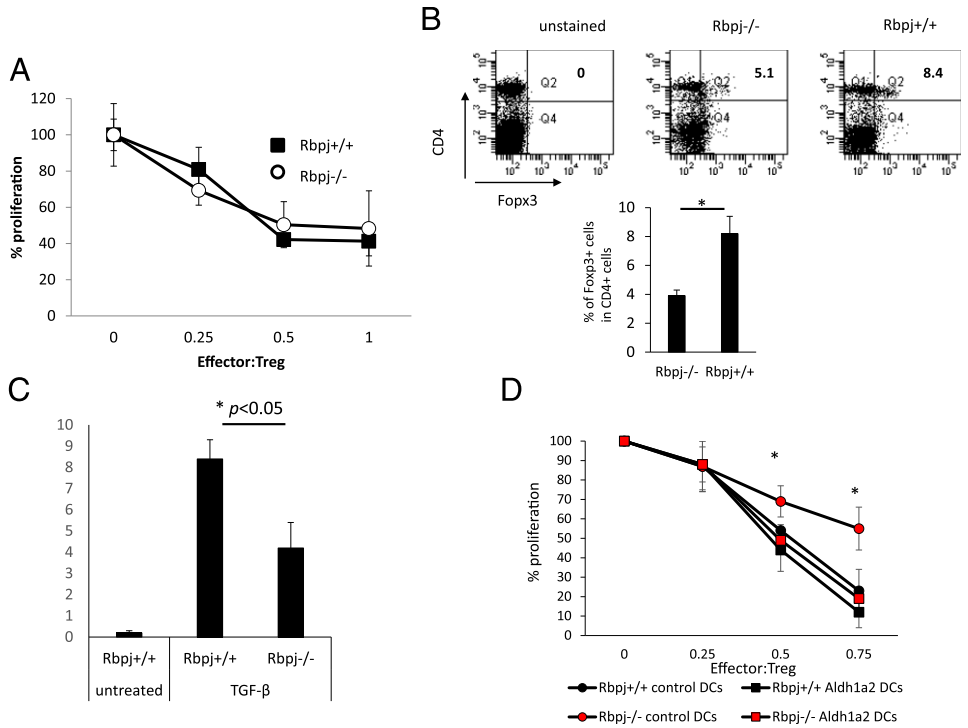

FIGURE 4. Aldh1a2 regulates iTreg differentiation. (**A**) CD4$^+$ T cells and CD4$^+$CD25$^+$ T cells from C57BL/6 mice were stimulated by anti-CD3 mAb (1 μg/ml) with bone marrow–derived CD8α$^-$CD11c$^+$ cells from Rbpj$^{-/-}$ (open circles) or Rbpj$^{+/+}$ (closed squares) mice for 3 d. T cell proliferation was evaluated by measuring incorporation of [^{3}H]thymidine during the final 8 h. Data show the ratio of the value of a particular culture/value without CD4$^+$CD25$^+$ T cells. Data are mean ± SD. (**B**) Naive CD4$^+$ T cells from C57BL/6 mice were stimulated by anti-CD3 mAb (1 μg/ml) and bone marrow–derived CD8α$^-$ CD11c$^+$ cells from Rbpj$^{-/-}$ or Rbpj$^{+/+}$ mice in the presence of TGF-β (2 ng/ml) for 3 d. Foxp3$^+$ cells in the CD4$^+$ T cell population were evaluated by flow cytometry. The number in the figure indicates the number of Foxp3$^+$ cells in CD4$^+$ T cells. Data from three independent experiments are shown as mean ± SD. *$p < 0.05$. (**C**) Naive CD4$^+$ T cells from C57BL/6 mice were stimulated by anti-CD3 mAb (1 μg/ml) and bone marrow–derived CD8α$^-$CD11c$^+$ cells from Rbpj$^{-/-}$ or Rbpj$^{+/+}$ mice in the presence of TGF-β (2 ng/ml) for 3 d. After washing the cells, T cells were stimulated with anti-CD3 mAb (1 μg/ml) for 48 h in the presence of irradiated spleen cells from C57BL/6 mice. The concentration of TGF-β in the culture supernatant was evaluated by ELISA. Data are mean ± SD. *$p < 0.05$. (**D**) iTregs were obtained by stimulation of naive CD4$^+$ T cells by anti-CD3 mAb (1 μg/ml) and bone marrow–derived CD8α$^-$CD11c$^+$ cells infected with control retrovirus (black) or Adh1a2-carrying retrovirus (red) from Rbpj$^{-/-}$ (circles) or Rbpj$^{+/+}$ (squares) mice in the presence of TGF-β (2 ng/ml) for 3 d. CD4$^+$ T cells and iTreg were stimulated by anti-CD3 mAb (1 μg/ml) with bone marrow–derived CD8α$^-$CD11c$^+$ cells from C57BL/6 mice for 3 d. T cell proliferation was evaluated by measuring incorporation of [^{3}H]thymidine during the final 8 h. Data show the ratio of the value of particular culture/value without CD4$^+$CD25$^+$ T cells. Data are mean ± SD. The data are representative of three independent experiments.

differentiation (Fig. 3C). Collectively, those data demonstrated that Notch signaling in DCs is required for upregulating *Aldh1a2*, which is important for balancing Th17 differentiation.

Lower production of RA from DCs from Rbpj$^{-/-}$ mice decreased iTreg differentiation

We investigated the capability of DCs from Rbpj$^{-/-}$ mice to suppress the function of tTregs. Different ratios of naive CD4$^+$ T cells and tTregs were stimulated with anti-CD3 mAb in the presence of DCs from Rbpj$^{-/-}$ or Rbpj$^{+/+}$ mice. The suppressive activity of tTregs was similar between DCs from Rbpj$^{-/-}$ and Rbpj$^{+/+}$ mice at all examined cell ratios (Fig. 4A).

We next evaluated how DCs from Rbpj$^{-/-}$ mice affected in vitro differentiation of iTregs. Naive CD4$^+$ T cells were stimulated with anti-CD3 mAb with DCs from Rbpj$^{-/-}$ or Rbpj$^{+/+}$ mice in the presence of TGF-β. The DCs from Rbpj$^{-/-}$ mice decreased Foxp3$^+$ cell numbers compared with those from Rbpj$^{+/+}$ mice (Fig. 4B). Three days after T cell stimulation, cells were washed and stimulated with anti-CD3 mAb for 2 d, and secretion of TGF-β was evaluated. The T cells stimulated by DCs from Rbpj$^{-/-}$ mice produced less TGF-β than those stimulated by DCs from control mice (Fig. 4C). We next evaluated the suppressive ability of CD4$^+$CD25$^+$ T cells after culture of naive CD4$^+$ T cells with DCs from Rbpj$^{-/-}$ mice in the presence of TGF-β. The CD4$^+$CD25$^+$ T cells after coculture with DCs from Rbpj$^{-/-}$ mice had a weaker suppressive ability with less Foxp3$^+$ cells (Supplemental Fig. 3) against effector CD4$^+$ T cells compared with CD4$^+$CD25$^+$ T cells after culture with DCs from Rbpj$^{+/+}$ mice (Fig. 4D). However, CD4$^+$CD25$^+$ T cells stimulated by DCs from Rbpj$^{-/-}$ mice that overexpressed Aldh1a2 had T cell suppressive activity that was equivalent to control DCs with increased Foxp3$^+$ cells (Fig. 4D, Supplemental Fig. 3). Taken together, those data demonstrated that the absence of Notch signaling in CD11$^+$ cells dampens the ability to promote iTreg differentiation, which is dependent on Aldh1a2 expression.

T cell transfer-mediated colitis was more evident in Rbpj$^{-/-}$;Rag1$^{-/-}$ than in Rbpj$^{+/+}$;Rag1$^{-/-}$ mice

We evaluated the roles of Notch signaling in DCs in vivo by transferring naive CD4$^+$ T cells into Rbpj$^{-/-}$ or Rbpj$^{+/+}$ mice that lacked the *Rag1* gene (Rbpj$^{-/-}$;Rag1$^{-/-}$ or Rbpj$^{+/+}$;Rag1$^{-/-}$, respectively). The transfer of naive CD4$^+$ T cells into Rbpj$^{+/+}$;Rag1$^{-/-}$ mice caused colitis with body weight loss that was first seen 3 wk after T cell transfer (Fig. 5A). The body weight loss was more evident in Rbpj$^{-/-}$;Rag1$^{-/-}$ mice than Rbpj$^{+/+}$;Rag1$^{-/-}$ mice. The concentration of serum IL-17A 27 d after T cell transfer was higher in Rbpj$^{-/-}$;Rag1$^{-/-}$ mice than in Rbpj$^{+/+}$;Rag1$^{-/-}$ mice (Fig. 5B). T cells were purified from mice that received naive CD4$^+$ T cells and stimulated with anti-CD3 mAb for 2 d, and IL-17A production was measured (Fig. 5C). Donor CD4$^+$ T cells recovered from Rbpj$^{-/-}$;Rag1$^{-/-}$ mice produced more IL-17A than did those from Rbpj$^{+/+}$;Rag1$^{-/-}$ mice, indicating that Th17 differentiation was enhanced in Rbpj$^{-/-}$;Rag1$^{-/-}$ recipients. The number of CD4$^+$Foxp3$^+$ cells in the spleen was lower in Rbpj$^{-/-}$;Rag1$^{-/-}$ mice than Rbpj$^{+/+}$;Rag1$^{-/-}$ mice that received naive CD4$^+$ T cells 27 d after T cell transfer (Fig. 5D). We then transferred iTregs with naive CD4$^+$ T cells into Rbpj$^{-/-}$;Rag1$^{-/-}$ mice. The cotransfer of iTreg ameliorated the colitis (Fig. 5E), demonstrating that the reduced number of iTregs was attributable to the enhanced colitis in Rbpj$^{-/-}$;Rag1$^{-/-}$ recipient mice.

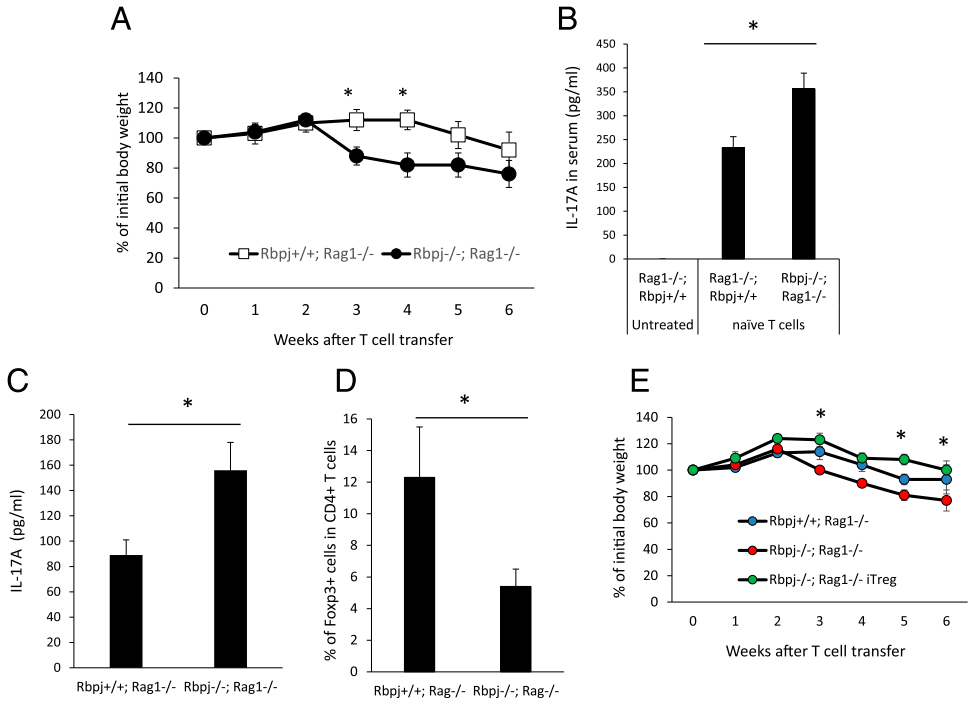

FIGURE 5. Aldh1a2 in DCs regulates colitis. (**A**) Mice ($n = 6$ in each group) were i.v. injected with splenic CD4$^+$CD25$^-$ T cells (4×10^5 cells). Body weight after T cell transfer in Rbpj$^{-/-}$;Rag1$^{-/-}$ (closed circles) or Rbpj$^{+/+}$;Rag1$^{-/-}$ mice (open squares). Data are mean ± SD. *$p < 0.05$. (**B**) Serum concentration of IL-17A 27 d after T cell transfer. Data are mean ± SD. *$p < 0.05$. (**C**) CD4$^+$ T cells were purified from spleens in Rbpj$^{-/-}$;Rag1$^{-/-}$ or Rbpj$^{+/+}$;Rag1$^{-/-}$ mice transplanted with naive CD4$^+$ T cells (27 d after T cell transfer), and stimulated with anti-CD3 mAb (1 μg/ml) for 2 d. (**D**) Percentage of Foxp3$^+$ cells in total splenic CD4$^+$ T cells in Rbpj$^{-/-}$;Rag1$^{-/-}$ or Rbpj$^{+/+}$;Rag1$^{-/-}$ mice 27 d after transplantation of naive CD4$^+$ T cells. (**E**) Mice ($n = 6$ in each group) were i.v. injected with splenic naive CD4$^+$ T cells (4×10^5 cells) into Rbpj$^{-/-}$;Rag1$^{-/-}$ (green) or Rbpj$^{+/+}$;Rag1$^{-/-}$ (yellow) mice. The iTregs (1×10^5 cells) (red) were transferred into Rbpj$^{-/-}$;Rag1$^{-/-}$ mice at 5-d intervals that began at T cell transfer. Data are mean ± SD. The data are representative of three independent experiments. *$p < 0.05$ (green and red).

Aldh1a2 overexpression or Notch stimulation of DCs could ameliorate colitis after T cell transfer

We evaluated whether decreased expression of Aldha12 in DCs from Rbpj$^{-/-}$;Rag1$^{-/-}$ mice was attributable to the colitis. Thus, DCs from Rbpj$^{-/-}$ mice transduced with *Aldh1a2* were cotransplanted with naive CD4$^+$ T cells into Rbpj$^{-/-}$;Rag1$^{-/-}$ mice. The transduction of *Aldh1a2* did not affect the DC survival itself (Supplemental Fig. 4). The body weight loss was much milder in Rbpj$^{-/-}$;Rag1$^{-/-}$ mice transplanted with Aldh1a2-expressing DCs than Rbpj$^{-/-}$;Rag1$^{-/-}$ mice transplanted with control DCs (Fig. 6A). CD4$^+$Foxp3$^+$ T cells were increased by transferring DCs overexpressing Aldh1a2 compared with control DCs (Fig. 6B).

Finally, we evaluated whether stimulation of DCs from C57BL/6 mice had a greater ability to suppress colitis. We treated DCs from C57BL/6 mice with soluble Delta1-Fc and cotransplanted them with naive CD4$^+$ T cells into Rbpj$^{-/-}$;Rag1$^{-/-}$ mice. The body weight loss was milder in Rbpj$^{-/-}$;Rag1$^{-/-}$ mice transplanted with control DCs (Fig. 6C). CD4$^+$Foxp3$^+$ T cell numbers were increased by transferring DCs stimulated with Delta1-Fc compared with control DCs (Fig. 6D). Those data demonstrated that the loss of Notch signaling in CD11c-positive cells resulted in reduced expression of Aldh1a2, and this was responsible for the enhancement of colitis.

Discussion

RA has multiple functions in the immune systems, and its synthesis is regulated by Aldh1a2 (20). In this study, we demonstrated that Rbpj-deficient DCs have Th17-promoting activity with lower levels of Aldh1a2 expression than control DCs. *Rbpj* deficiency in DCs did not affect secretion of IFN-γ or IL-4 from CD4$^+$ T cells, although Notch in T cells regulates Th1, Th2, and Th17 programs (21). Overexpression of Aldh1a2 in Rbpj-deficient DCs could negate Th17-promoting ability. Transfer of naive CD4$^+$ T cells into Rag1$^{-/-}$ mice that lacked Rbpj in CD11c$^+$ cells exhibited more severe colitis than did control Rag1$^{-/-}$ host. ChIP assays revealed that Notch directly regulated the promoter activity of *Aldh1a2*. Those data demonstrate that Notch in DCs is a crucial modulator of iTreg/Th17 differentiation through its control of RA synthesis.

The high level of expression of Aldh1a2 was initially reported in DCs in mesenteric lymph nodes and Peyer's patches (22), which revealed the contribution of RA to mucosal immunity. Subsequent studies showed that, outside of the gut, the majority of peripheral DCs express Aldh1a2 when they are upregulated by GM-CSF (23) and the TLR2 agonist zymosan, suggesting the important function of RA in DCs in peripheral tissues. Our ChIP assays demonstrated that Notch signaling directly regulated *Aldh1a2* gene transcription. Although one article reported that overexpression of a constitutively active form of Notch1 in the regenerating fin led to increased expression of *aldh1a2* in Zebrafish (24), the direct regulation of *Aldh1a2* by Notch has not been reported. *Aldh1a2*-deficient mice die by embryonic day 10.5 with impaired hindbrain development, failure to turn, lack of limb buds, heart abnormalities, reduced otocysts, and a truncated frontonasal region (25). *Rbpj*-deficient mice also showed embryonic lethality before embryonic 10.5 d, with severe growth retardation and developmental abnormalities, including incomplete turning of the body axis, microencephaly, anterior neuropore opening, and defective somitogenesis (26). Therefore, it is difficult to assess the genetic interaction between Notch and *Aldh1a2* with those two null strains. However, we detected decreased expression of *Aldh1a2* in DCs

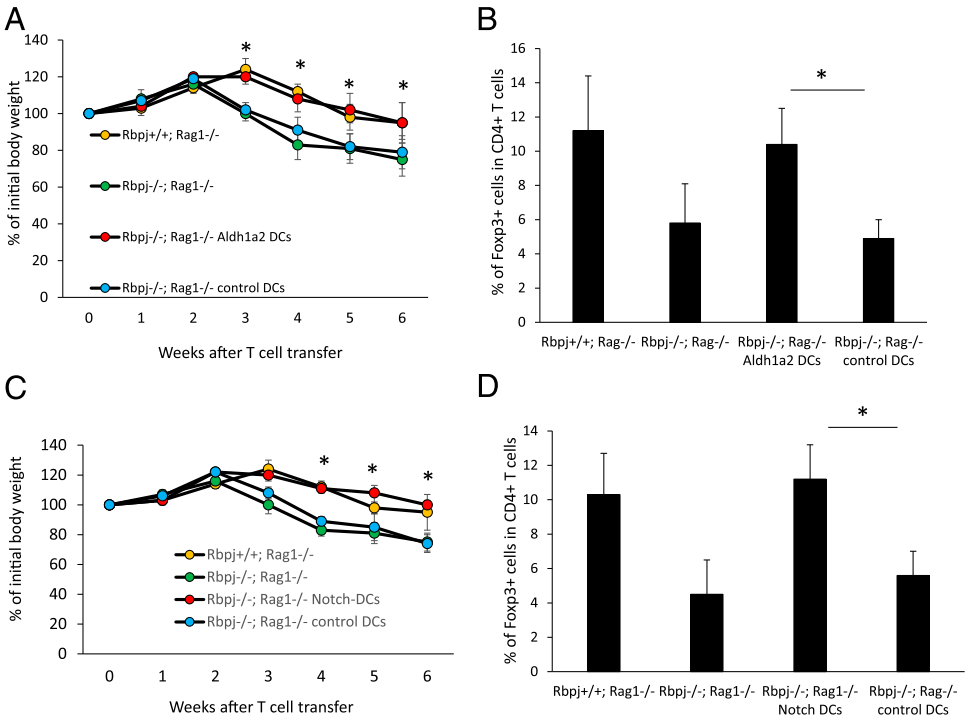

FIGURE 6. Aldh1a2 in DCs regulates colitis. (**A**) Splenic naive CD4$^+$ T cells (4×10^5 cells) were i.v. injected into Rbpj$^{-/-}$;Rag1$^{-/-}$ (green) or Rbpj$^{+/+}$;Rag1$^{-/-}$ (yellow) mice ($n = 6$ in each group). DCs from Rbpj$^{-/-}$ mice infected with control retrovirus (blue) or retrovirus-carrying *Aldh1a2* (red) were transferred into Rbpj$^{-/-}$;Rag1$^{-/-}$ mice at 5-d interval that began at T cell transfer. Data are mean ± SD. *$p < 0.05$ (between blue and red). (**B**) Percentage of Foxp3$^+$ cells in CD4$^+$ T cells in (A). (**C**) Rbpj$^{-/-}$;Rag1$^{-/-}$ (green) or Rbpj$^{+/+}$;Rag1$^{-/-}$ (yellow) mice ($n = 6$ in each group) were i.v. injected with splenic naive CD4$^+$ T cells (4×10^5 cells). DCs from Rbpj$^{+/+}$ mice stimulated with control IgG (blue) or Delta1-Fc (red) were transferred into Rbpj$^{-/-}$;Rag1$^{-/-}$ mice at 5-d interval that began at T cell transfer. Data are mean ± SD. *$p < 0.05$ (between blue and red). (**D**) Percentage of Foxp3$^+$ cells in CD4$^+$ T cells in (C). The data are representative of three independent experiments.

derived from bone marrow from Rbpj$^{-/-}$ mice as well as CD8α^{-} DCs from Rbpj$^{-/-}$ mice, supporting the direct regulation of *Aldh1a2* by Notch signaling in vivo.

Notch regulates the survival of CD8α^{-} DCs in the spleen, as demonstrated by their reduced numbers in the spleens from Rbpj$^{-/-}$ mice (9). The deletion of *Notch2* causes the loss of CD11b^{+} CD103^{+} DCs in the intestinal lamina propria with a decrease of IL-17–producing CD4^{+} T cells in the intestine (10). We detected decreased expression of Aldh1a2 in bone marrow–derived CD8α^{-} DCs and in CD8α^{-} splenic DCs. Thus, Notch is required for not only survival, but also Aldh1a2 expression in CD8α^{-} DCs in the spleen. In contrast, we did not detect reduced expression of Aldh1a2 in CD8α^{+} splenic DCs, suggesting cell type–specific regulation of Aldh1a2 by Notch signaling.

Reports have shown that *Rbpj*-deficient DCs express less CD80 and CD86 than wild type (27), and have a reduced ability for T cell proliferation (28) than control DCs. The discrepancy between those data and our present findings might be explained by the *Cre* transgenic mice used to delete *Rbpj*. Previous reports used Mx-Cre transgenic mice in which poly(I:C) treatment is used for deleting *Rbpj*. The treatment of mice with poly(I:C) might affect the phenotypes of DCs or bone marrow cells in vivo, which might change the activation status or functions of DCs. Another possibility is the difference of deletion efficiency or timing of deletion of *Rbpj* between Mx-Cre and CD11c-Cre transgene. For instance, CD11c-Cre mice would delete *Rbpj* later than Mx-Cre mice. In any case, it will be important to analyze the impact of Notch on the activation or differentiation of DCs during each differentiation step of DCs.

Rbpj$^{+/+}$ mice that were transplanted with naive CD4^{+} T cells in the presence or absence of Rbpj in DCs developed colitis to a lesser degree than Rbpj$^{-/-}$ host mice. In contrast, we did not observe any spontaneous colitis or other inflammatory responses in Rbpj$^{-/-}$ mice, even at ≥7 mo of age. These data do not necessarily negate the importance of Notch signaling in CD11c^{+} cells in the suppression of Th17 differentiation because the production of Aldh1a2 is not completely abolished in Rbpj$^{-/-}$ mice. Therefore, the residual Aldh1a2 activity is still able to help differentiation of iTreg in vivo, which might be sufficient to suppress spontaneous enterocolitis.

We used *Rbpj*-deficient DCs for analyzing the effects of Notch on DC functions. Which Notch receptors are required for Aldh1a2 expression in DCs? DCs express not only Notch1 and Notch2, but also Jagged1, Jagged2, and Delta-like 1 (29). Although we have not tested which Notch or Notch ligand is important for Aldh1a2 expression in vivo, we have shown that stimulation of DCs with Delta-like 1 could strengthen the inhibitory activity on colitis. Identifying which Notch or Notch ligand is required for Aldh1a2 expression would be crucial when considering the therapeutic strategy for targeting the Notch pathways in DCs. In addition, Rbpj might regulate cellular responses not related to Notch. Thus, it will be necessary in the future to test which Notch contributes to Aldh1a2 expression in DCs.

Notch has multiple roles in the immune system as well as other regulatory systems. Our data suggest that stimulation of Notch on DCs or transduction of *Aldh1a2* into DCs might be beneficial for suppressing Th17-mediated disorders. The treatment normalized iTreg numbers, suggesting Notch-mediated Aldh1a2 in DCs balances iTreg and Th17 differentiation. Therefore, Notch stimulation of DCs may represent a novel treatment strategy for Th1- and Th17-mediated autoimmune diseases. In summary, our findings could have important clinical implications in treating autoimmune diseases and inducing immune tolerance in transplantation settings.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

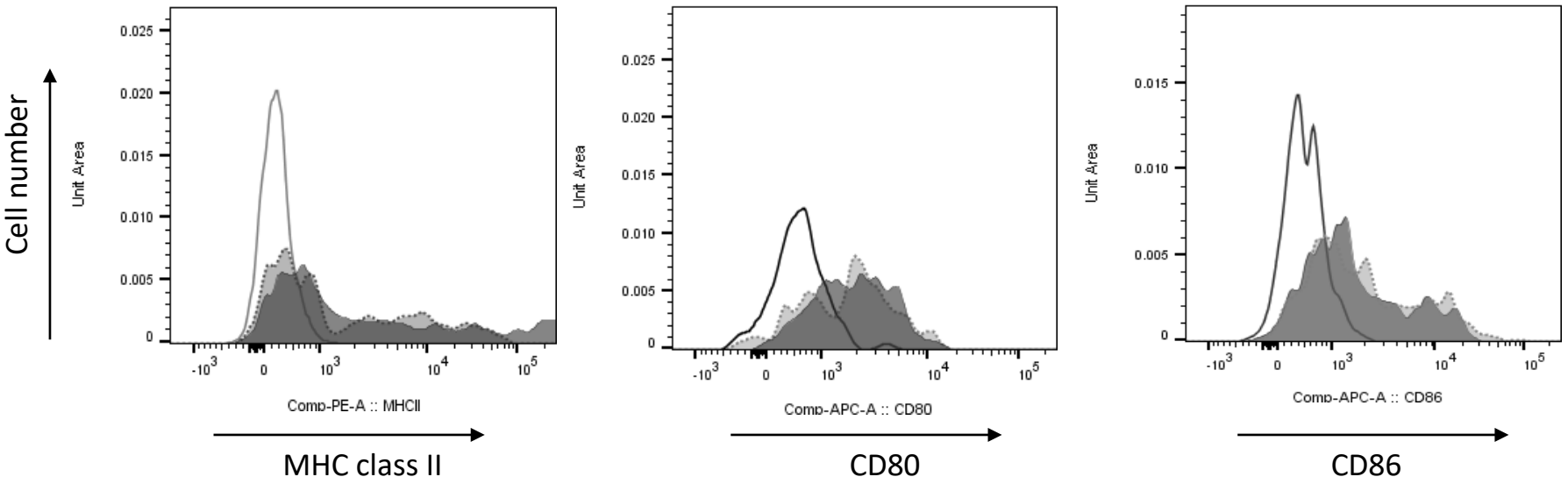
References

1. Hammer, G. E., and A. Ma. 2013. Molecular control of steady-state dendritic cell maturation and immune homeostasis. *Annu. Rev. Immunol.* 31: 743–791.
2. Pulendran, B. 2015. The varieties of immunological experience: of pathogens, stress, and dendritic cells. *Annu. Rev. Immunol.* 33: 563–606.
3. Malissen, B., S. Tamoutounour, and S. Henri. 2014. The origins and functions of dendritic cells and macrophages in the skin. *Nat. Rev. Immunol.* 14: 417–428.
4. Radtke, F., H. R. MacDonald, and F. Tacchini-Cottier. 2013. Regulation of innate and adaptive immunity by Notch. *Nat. Rev. Immunol.* 13: 427–437.
5. Amsen, D., C. Helbig, and R. A. Backer. 2015. Notch in T cell differentiation: all things considered. *Trends Immunol.* 36: 802–814.
6. Maekawa, Y., S. Tsukumo, S. Chiba, H. Hirai, Y. Hayashi, H. Okada, K. Kishihara, and K. Yasutomo. 2003. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 19: 549–559.
7. Maekawa, Y., Y. Minato, C. Ishifune, T. Kurihara, A. Kitamura, H. Kojima, H. Yagita, M. Sakata-Yanagimoto, T. Saito, I. Taniuchi, et al. 2008. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nat. Immunol.* 9: 1140–1147.
8. Maekawa, Y., C. Ishifune, S. Tsukumo, K. Hozumi, H. Yagita, and K. Yasutomo. 2015. Notch controls the survival of memory CD4+ T cells by regulating glucose uptake. *Nat. Med.* 21: 55–61.
9. Caton, M. L., M. R. Smith-Raska, and B. Reizis. 2007. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J. Exp. Med.* 204: 1653–1664.
10. Lewis, K. L., M. L. Caton, M. Bogunovic, M. Greter, L. T. Grajkowska, D. Ng, A. Klinakis, I. F. Charo, S. Jung, J. L. Gommerman, et al. 2011. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* 35: 780–791.
11. Satpathy, A. T., C. G. Briseño, J. S. Lee, D. Ng, N. A. Manieri, W. Kc, X. Wu, S. R. Thomas, W. L. Lee, M. Turkoz, et al. 2013. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nat. Immunol.* 14: 937–948.
12. Benson, M. J., K. Pino-Lagos, M. Rosemblatt, and R. J. Noelle. 2007. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J. Exp. Med.* 204: 1765–1774.
13. Mucida, D., Y. Park, G. Kim, O. Turovskaya, I. Scott, M. Kronenberg, and H. Cheroutre. 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317: 256–260.
14. Xiao, S., H. Jin, T. Korn, S. M. Liu, M. Oukka, B. Lim, and V. K. Kuchroo. 2008. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J. Immunol.* 181: 2277–2284.
15. Morita, S., T. Kojima, and T. Kitamura. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* 7: 1063–1066.
16. Koch, U., and F. Radtke. 2011. Mechanisms of T cell development and transformation. *Annu. Rev. Cell Dev. Biol.* 27: 539–562.
17. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
18. Coombes, J. L., K. R. Siddiqui, C. V. Arancibia-Cárcamo, J. Hall, C. M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J. Exp. Med.* 204: 1757–1764.
19. Brown, C. C., and R. J. Noelle. 2015. Seeing through the dark: new insights into the immune regulatory functions of vitamin A. *Eur. J. Immunol.* 45: 1287–1295.
20. Hall, J. A., J. R. Grainger, S. P. Spencer, and Y. Belkaid. 2011. The role of retinoic acid in tolerance and immunity. *Immunity* 35: 13–22.
21. Bailis, W., Y. Yashiro-Ohtani, T. C. Fang, R. D. Hatton, C. T. Weaver, D. Artis, and W. S. Pear. 2013. Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals. *Immunity* 39: 148–159.
22. Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S. Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21: 527–538.
23. Yokota, A., H. Takeuchi, N. Maeda, Y. Ohoka, C. Kato, S. Y. Song, and M. Iwata. 2009. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int. Immunol.* 21: 361–377.
24. Münch, J., A. González-Rajal, and J. L. de la Pompa. 2013. Notch regulates blastema proliferation and prevents differentiation during adult zebrafish fin regeneration. *Development* 140: 1402–1411.
25. Niederreither, K., V. Subbarayan, P. Dollé, and P. Chambon. 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* 21: 444–448.
26. Oka, C., T. Nakano, A. Wakeham, J. L. de la Pompa, C. Mori, T. Sakai, S. Okazaki, M. Kawaichi, K. Shiota, T. W. Mak, and T. Honjo. 1995. Disruption

of the mouse RBP-J kappa gene results in early embryonic death. *Development* 121: 3291–3301.
27. Feng, F., Y. C. Wang, X. B. Hu, X. W. Liu, G. Ji, Y. R. Chen, L. Wang, F. He, G. R. Dou, L. Liang, et al. 2010. The transcription factor RBP-J-mediated signaling is essential for dendritic cells to evoke efficient anti-tumor immune responses in mice. *Mol. Cancer* 9: 90.
28. Chen, Y. R., F. Feng, L. Wang, S. Y. Qu, Z. Q. Zhang, L. Liu, H. Y. Qin, Y. M. Liang, and H. Han. 2013. Deletion of RBP-J in dendritic cells compromises TLR-mediated DC activation accompanied by abnormal cytoskeleton reorganization. *Mol. Biol. Rep.* 40: 1531–1539.
29. Cheng, P., and D. Gabrilovich. 2008. Notch signaling in differentiation and function of dendritic cells. *Immunol. Res.* 41: 1–14.

Notch and Aldh1a2 regulation in dendritic cells

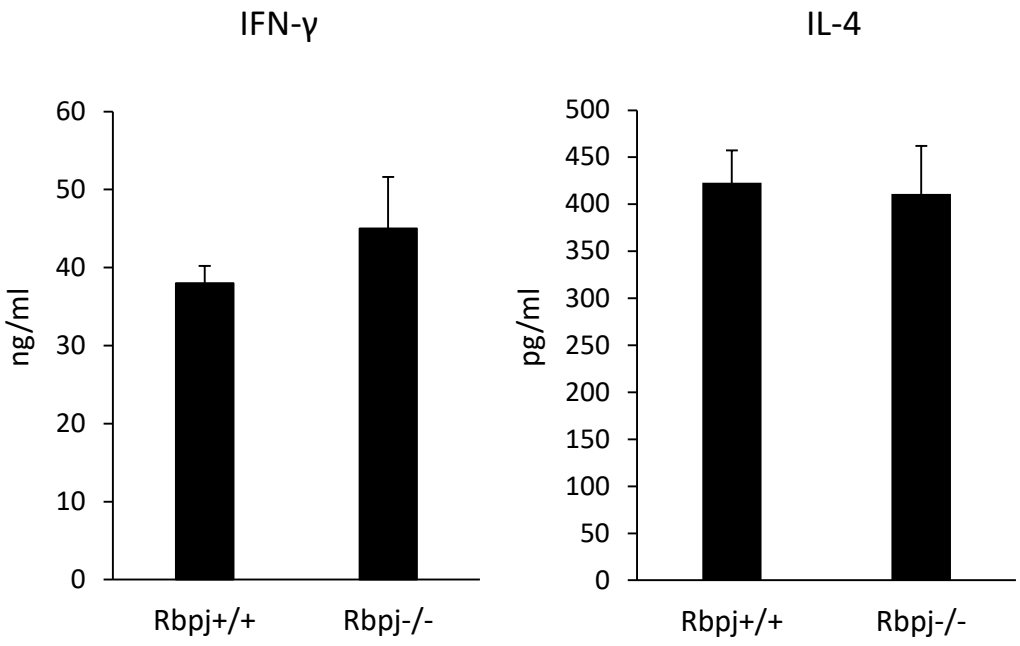
Supplementary Figure 1



Expression of coreceptor molecules on DCs

Bone marrow-derived $CD8\alpha^{-}CD11c^{+}$ cells from $Rbpj^{-/-}$ (dot, light grey) or $Rbpj^{+/+}$ (dark grey) mice were stained with MHC class II, CD80 or CD86. As the negative control (solid line), cells were stained with isotype matched IgG.

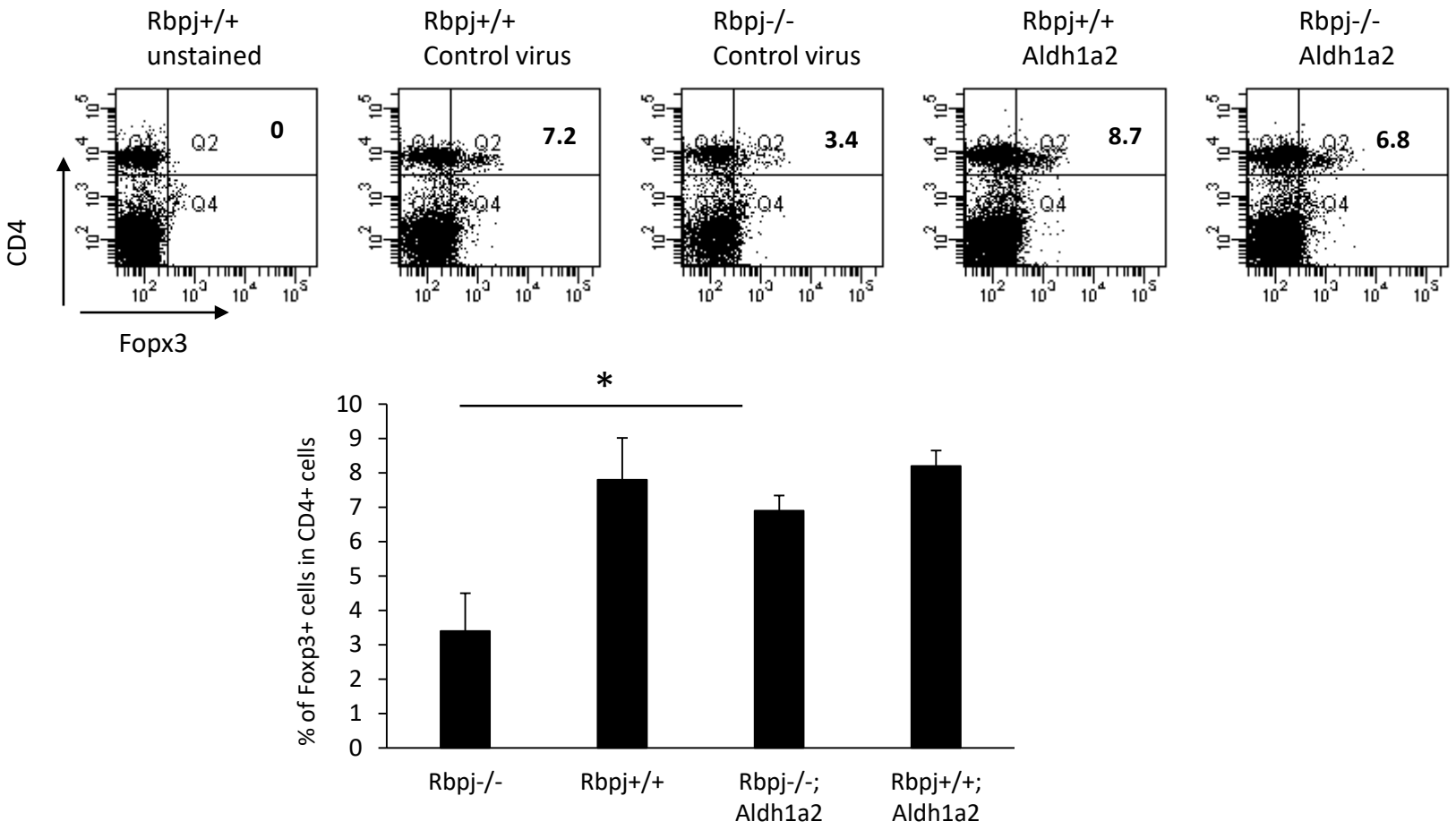
Supplementary Figure 2



Th1 and Th2 differentiations are not affected by *Rbpj* deficiency in DCs

Bone marrow-derived $CD8\alpha^{-}CD11c^{+}$ cells from $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice were cultured with $CD4^{+}$ T cells from OT-II TCR transgenic mice in the presence of OVA peptide for 3 days. The concentration of IFN-γ in the supernatant was evaluated by ELISA. For IL-4 assay, three days after stimulation in the presence of IL-4 (10 ng/ml), cells were washed and restimulated with plate-coated anti-CD3 mAb (1 μg/ml) for 1 day and the concentration of IL-4 in the supernatant was evaluated by ELISA. Data are means ± S.D.

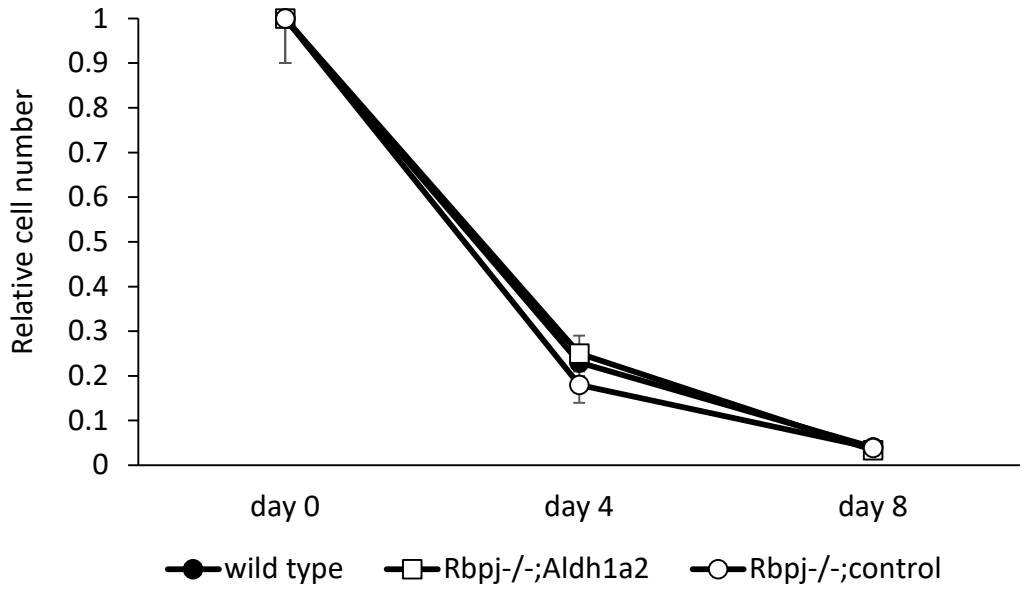
Supplementary Figure 3



iTreg differentiation is restored by overexpressing Aldh1a2 in DCs from Rbpj-/- mice

iTregs were obtained by stimulation of naïve $CD4^+$ T cells by anti-CD3 mAb (1 μg/mL) and bone marrow-derived $CD8\alpha^-CD11c^+$ cells infected with control retrovirus or Adh1a2-carrying retrovirus from $Rbpj^{-/-}$ or $Rbpj^{+/+}$ mice in the presence of TGF-β (2 ng/mL) for 3 days. Then, Foxp3 positive cells in $CD4^+$ cells were evaluated. The number shown in the figure is Foxp3+ cells in CD4+ cells. Cells stained with anti-CD4 antibody alone was used as the negative control. Data from three independent experiments are shown as means ± S.D. *, $P < 0.05$.

Supplementary Figure 4



DCs survival is not affected by overexpressing Aldh1a2 in DCs

DCs (1 x 10^6) from $Rbpj^{-/-}$ mice (CD45.2) infected with control retrovirus (open circle) or retrovirus carrying *Aldh1a2* (open square), or wild type DCs (closed circle) (CD45.1) were transferred into $Rag1^{-/-}$ mice. Then cell numbers of donor cells in the spleen were counted. The relative cell number (cell counts after transfer/1 x 10^6) is shown. Data are means ± S.D (n=5).