Supporting Information

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SI Materials and Methods

Reagents. Fluorescein isothiocyanate-conjugated anti-mouse CD4 (GK1.5; 11–0041), phycoerythrin-cyanine7–conjugated anti-mouse CD4 (GK1.5; 25–0041), Alexa Fluor 488-conjugated anti-mouse CD11c (N418; 53–0114), phycoerythrin-conjugated anti-mouse IL-4 (11B11; 12–7041), allophycocyanin-conjugated anti-mouse CD11b (M1/70; 17–0112), anti-mouse IFN-γ (XMG1.2; 17–7311), anti-mouse Foxp3 (FJK-16s; 17–5773), and Fixable Viability Dye eFluor 660 (65-0864) were from eBioscience. Mouse anti-JAK1 (610231), fluorescein isothiocyanate-conjugated anti-mouse CD19 (1D3; 557398), phycoerythrin-conjugated anti-mouse CD8 (53-6.7; 553032), anti-mouse CD45R/B220 (RA3-6B2; 553090), anti-mouse interleukin 17 (IL-17) A (TC11-18H10; 559502), anti-human CCR6 (559562), and propidium iodide (51-66211E) were from BD Biosciences. Phycoerythrin-conjugated anti-mouse CCR6 (FAB590P) was from R&D Systems. Rabbit anti–phospho-STAT3 (signal transducer and activator of transcription 3) (Tyr^{705}) (9145), rabbit anti–phospho-STAT1 (Tyr^{701}) (9171), rabbit anti-STAT1 (9172), rabbit anti–phospho-STAT5 (Tyr^{694}) (9351), and rabbit anti-STAT5 (9363) were from Cell Signaling. Rabbit anti-STAT3 (sc-482) was from Santa Cruz. Mouse anti–β-arrestin1 was from Abmart. Digoxin was from Prestwick Chemical Library. Recombinant human IL-6 (200-06) was from Peprotech. The custom PCR array plate was from SABiosciences.

Plasmids and Cell Transfection. The plasmid encoding HA-β-arrestin1, HA–β-arrestin1 1–180, and HA–β-arrestin1 181–418 were generated as described (1). β-Arrestin1-RV, β-arrestin1 1–180-RV, and β-arrestin1 181–418-RV were constructed by cloning each fragment into a retroviral vector pGFP-RV (2). FLAG-RORγt was a kind gift from Dan R. Littman (New York University, New York) (3). Transient transfection of HEK293, HEK293T, and Plat-E cells were done with the calcium phosphate method as described (4).

Cell Purification. Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) were isolated from peripheral blood and synovial fluid, respectively, by density gradient centrifugation over HISTOPAQUE-1077 (10771; Sigma). Human $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes were purified by positive selection using CD4 Microbeads (130-045-101), CD8 Microbeads (130-045-201), CD19 Microbeads (130-050-301), and CD14 Microbeads (130-050-201; all from Miltenyi Biotec), respectively, yielding a purity of more than 95% by flow cytometry. $CD4^+CCR6^-$ and $CD4^+CCR6^+$ T cells, as well as mouse $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ myeloid cells, were sorted by using a FACSAria (BD Biosciences) with fluorescencelabeled antibodies.

T-Cell Differentiation. Naïve $CD4^+$ ($CD4^+CD62L^+CD25^-$) T cells from spleen of 8- to 12-wk-old mice were purified by selection using $CD4^+CD62L^+$ T Cell Isolation Kit II (130-093-227; Miltenyi Biotec). Cells were activated with anti-CD3 (2 μg/mL; 145–2C11; 553057) and anti-CD28 (2 μg/mL; 37.51, 553294; both from BD Biosciences) plus cytokines and neutralizing antibodies for the desired polarization as follows: IL-12 (10 ng/mL; 210–12; Peprotech) and anti–IL-4 (10 μg/mL; 11B11; 554432; BD Biosciences) for T_H1 cells; IL-4 (20 ng/mL; 214–14; Peprotech) and anti–IFN-γ (10 μg/mL; R4-6A2; 551216; BD Biosciences) for T_H2 cells; IL-6 (30 ng/mL; 14–8061; eBioscience), TGF-β1 (3 ng/mL; 100–21; Peprotech), anti–IL-4 (10 μg/mL), and anti–IFN-γ (10 μg/mL) for T_H17 cells; TGF-β1 (10 ng/mL), anti–IL-4 (10 μg/mL), and anti–IFN-γ (10 μg/mL) for iT_{reg} cells.

Induction of Collagen-Induced Arthritis. Chicken type II collagen (C9301; Sigma) was dissolved at a concentration of 2 mg/mL in 10 mM acetic acid (A8976; Sigma), and emulsified in an equal volume of Freund's complete adjuvant (CFA). CFA was prepared by mixing 100 mg of heat-killed *Mycobacterium tuberculosis* H37Ra (231141; BD Biosciences) and 20 mL of Freund's incomplete adjuvant (263910; BD Biosciences). Eight- to 12-wk-old mice were immunized intradermally at several sites into the base of the tail with 100-μL emulsion. At day 21 after initial immunization, mice were boosted with the same emulsion of collagen II (CII) and CFA intradermally nearby the primary injection site. Arthritis symptoms were evaluated as described (5).

Micro-CT. Radiography was performed by using a small animal in vivo micro-CT scanner (eXplore CT 120 MicroCT; GE Healthcare) at 50-μm resolution, with an X-ray tube current of 50 mA and a voltage of 100 kV. The 3D bone microarchitecture was generated by Microview software (GE Healthcare).

Histopathology. Hind paws were removed, fixed in 10% neutral buffered formalin (HT501128; Sigma) for 2 d, decalcified in 10% formic acid for 3 d, and embedded in paraffin. Ankle joint sections (5 μm) were mounted onto slides, followed by staining with H&E and safranin O.

ELISA. For titration of CII-specific antibodies in collagen-induced arthritis (CIA), diluted serum was added to CII (5 μg/mL)-coated Nunc microtitre plates (Immuno Module; 469914; Thermo), followed by the addition of HRP-conjugated goat anti-mouse IgG1 (1070-05), IgG2b (1090-05), and IgG3 (1100-05; all from SouthernBiotech). For detection of cytokines in cell culture supernatants, ELISA kit for mouse IL-17 (DY421) and mouse IFN-γ (DY485; both from R&D Systems) were used according to the manufacturer's instructions.

Retroviral Transduction. Retrovirus packaging was performed by transfection of Plat-E cells. After 48 h, retroviral supernatants were collected. Naïve $CD4^+$ T cells were cultured under T_H0-polarizing conditions for 24 h and transduced with supernatants containing retrovirus in the presence of polybrene (107689; Sigma) by centrifugation at $1,230 \times g$ for 90 min at 30 °C. The same procedure for transduction was repeated 24 h later, and cells were cultured under T_H17-polarizing conditions for another 3 d.

Lentivirus Production. The procedures of lentiviral vector construction, lentivirus production, and local administration have been described (6–8). The targeting sequences used were the following: Lenti-sh*Arrb1* (5′-GGG AAG CTC AAG CAT GAG GAC A-3′) and Lenti-control (5′-GCA CGA TAT ACA AGG ATC T-3′). Lentivirus packaging was performed by transfection of HEK293T cells. After 48 h, lentiviral supernatants were collected and concentrated by ultracentrifugation. The concentrated virus stocks were tittered on HEK293T cells based on GFP expression.

Carboxyfluorescein Succinimidyl Ester. Naïve $CD4^+$ T cells were incubated in PBS containing carboxyfluorescein succinimidyl ester (0.5 μM; C34554; Molecular Probes) for 15 min at 37 °C, repelleted, and incubated in fresh medium for another 30 min.

Then, cells were washed and cultured under T_H17-polarizing conditions.

Chemotaxis Assay. Cell migration was performed in 24-well transwell with 5.0-μm pore polycarbonate membrane (3421; Costar). Cells (10^6) were placed on the top chamber, and recombinant mouse CCL20 (250-27; Peprotech) was added to the bottom chamber. After 4 h of incubation at 37 °C, the number of migrated cells was determined by flow cytometry.

Flow Cytometry. For intracellular cytokine staining, cells were stimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/mL; P8139; Sigma) and ionomycin (1 μM; I3909; Sigma) in the presence of Brefeldin A (3 μg/mL; 00–4506; eBioscience). Then, cells were stained for surface molecules, fixed and made permeable with Fixation/Permeabilization Solution Kit (554714; BD Biosciences), and stained with fluorescencelabeled antibodies. For analysis of intracellular Foxp3, cells were stained for surface markers, fixed and made permeable with Foxp3 Staining Buffer Set (00-5523; eBioscience), and stained with fluorescencelabeled anti-Foxp3 before analysis on a FACSCalibur (BD Biosciences). Events were collected and analyzed with FlowJo software (Tree Star).

Immunoprecipitation and Immunoblot. After stimulation for the indicated times, cells were lysed in lysis buffer (C2978; Sigma). For endogenous coimmunoprecipitation, proteins were immunoprecipitated from lysates with the appropriate antibodies, followed by treatment with protein A affinity gel (P6486; Sigma). The immunoprecipitated complexes were separated by SDS/PAGE and blotted with the appropriate antibodies.

ChIP. Cells were fixed with 1% formaldehyde for 10 min at 37 °C and lysed by sonication using Bioruptor (Diagenode). Lysates were immunoprecipitated overnight at 4 °C with anti-STAT3. After washing and elution, cross-links were reversed overnight at 65 °C. Eluted DNA was purified and samples were analyzed by quantitative real-time PCR. Primer pairs are listed in Table S1.

Reverse Transcription and Quantitative Real-Time PCR. Total RNA was extracted with TRI Reagent (T9424; Sigma) according to the manufacturer's instructions. Random hexamer primer and M-MLV Reverse Transcriptase (M5301; Promega) were used for reverse transcription. All gene transcripts were quantified by quantitative real-time PCR performed with JumpStart Taq ReadyMix (D7440; Sigma) supplemented with EvaGreen Dye (31000; Biotium) on a Stratagene Mx3000P (Agilent Technologies). Primer pairs are listed in Table S2.

1. Wang Y, et al. (2006) Association of beta-arrestin and TRAF6 negatively regulates Toll-like receptor-interleukin 1 receptor signaling. *Nat Immunol* 7(2):139–147.
2. Du C, et al. (2009) MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nat Immunol* 10(12):1252–1259.
3. Zhou L, et al. (2008) TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453(7192):236–240.
4. Wang P, et al. (2003) Beta-arrestin 2 functions as a G-protein-coupled receptor-activated regulator of oncoprotein Mdm2. *J Biol Chem* 278(8):6363–6370.
5. Tang W, et al. (2011) The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice. *Science* 332(6028):478–484.
6. Qin XF, An DS, Chen IS, Baltimore D (2003) Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci USA* 100(1):183–188.
7. Lai Kwan Lam Q, King Hung Ko O, Zheng BJ, Lu L (2008) Local BAFF gene silencing suppresses Th17-cell generation and ameliorates autoimmune arthritis. *Proc Natl Acad Sci USA* 105(39):14993–14998.
8. Chalmin F, et al. (2012) Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. *Immunity* 36(3):362–373.

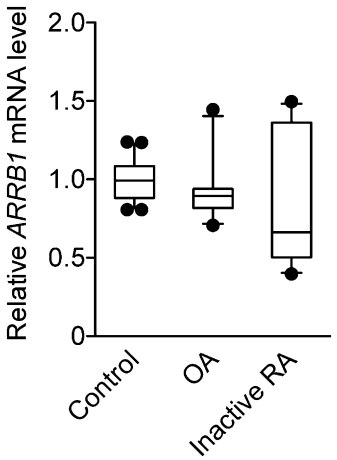

Fig. S1. Comparable expression of β-arrestin1 in $CD4^+$ T cells from normal controls, patients with osteoarthritis (OA), and inactive rheumatoid arthritis (RA). Quantitative RT-PCR of *ARRB1* mRNA in $CD4^+$ T cells from normal controls ($n = 26$), patients with OA ($n = 10$), or inactive RA ($n = 12$). Boxes = 25–75 percentiles. Lines in center of box, median. Whiskers = 10–90 percentiles. Dot, outliers.

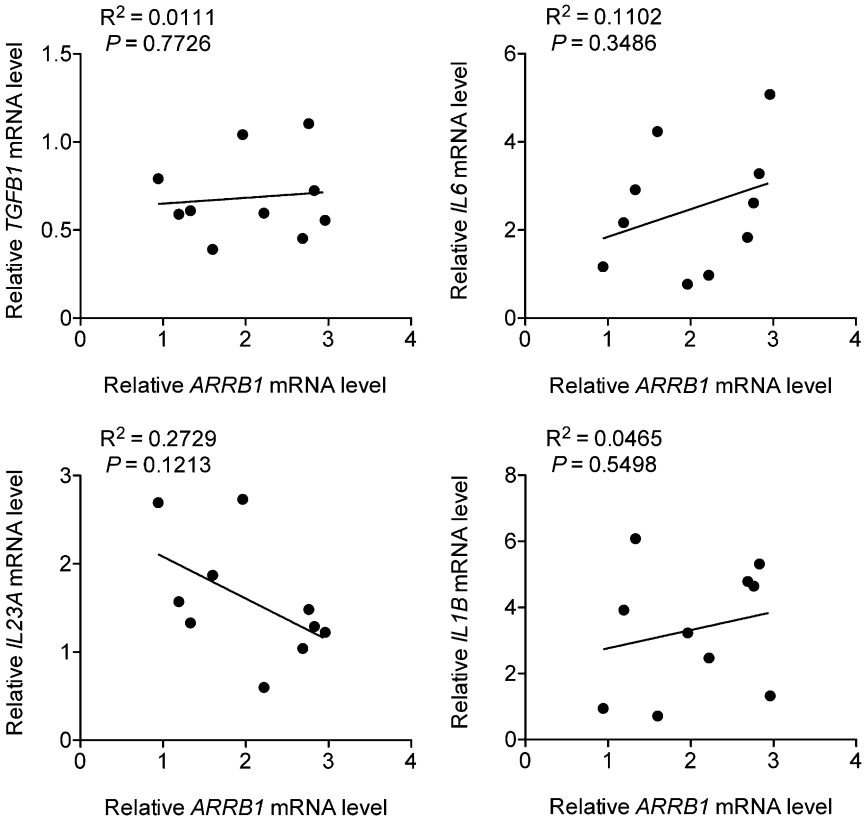

Fig. S2. No significant correlation between the expression of β-arrestin1 and TGF-β1 (*Upper Left*), IL-6 (*Upper Right*), IL-23 (*Lower Left*), or IL-1β (*Lower Right*) in $CD4^+$ T cells from patients with active RA. Linear correlation analysis between transcripts of β-arrestin1 and the cytokines in $CD4^+$ T cells from patients with active RA ($n = 10$).

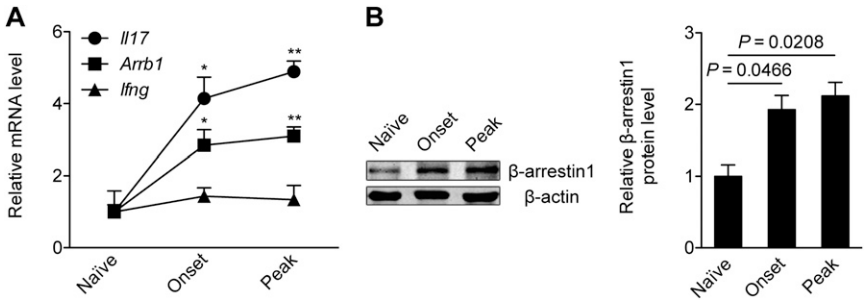

Fig. S3. Expression of β-arrestin1 is up-regulated in $CD4^+$ T cells from mice with CIA. Expression of *Il17*, *Arrb1*, or *Ifng* (*A*) and immunoblot of β-arrestin1 (*B*, *Left*) in $CD4^+$ T cells from naïve mice or mice with CIA (onset or peak) ($n = 4$ per group). Densitometric analysis is shown (*B*, *Right*). $^*P < 0.05$ and $^{**}P < 0.01$, versus naïve mice.

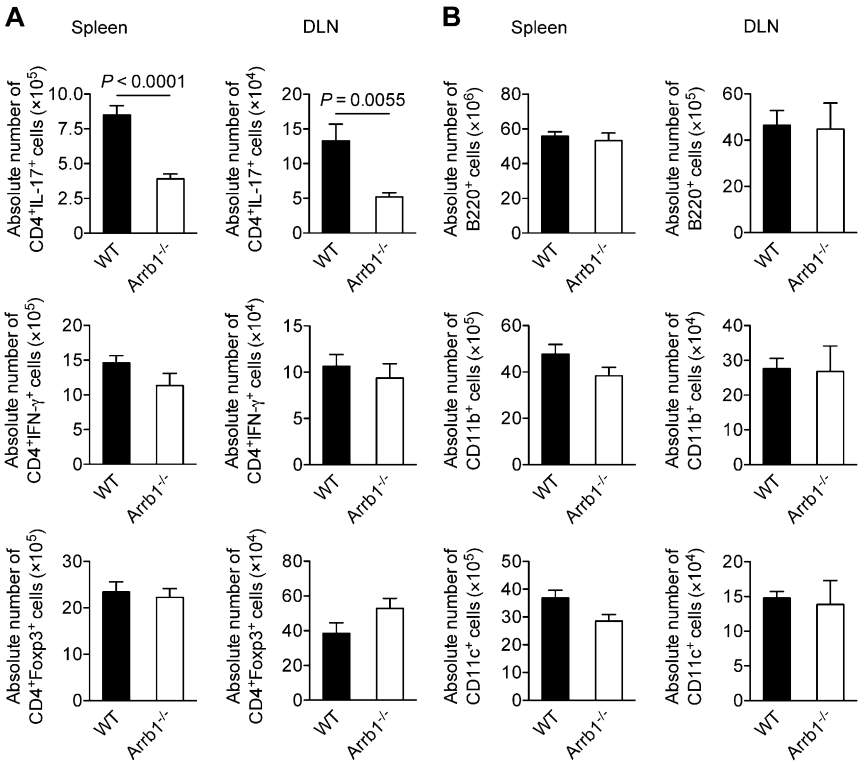

Fig. S4. Knockout of β-arrestin1 reduces the absolute number of T_H17 cells in vivo. Absolute number of $CD4^+IL\text{-}17^+$ (*Top*), $CD4^+IFN\text{-}\gamma^+$ (*Middle*), or $CD4^+Foxp3^+$ cells (*Lower*) (*A*) and $B220^+$ (*Top*), $CD11b^+$ (*Middle*), or $CD11c^+$ cells (*Lower*) (*B*) in the spleen or DLN of CII-immunized *Arrb1*$^{-/-}$ mice and WT littermates ($n = 8$ per group).

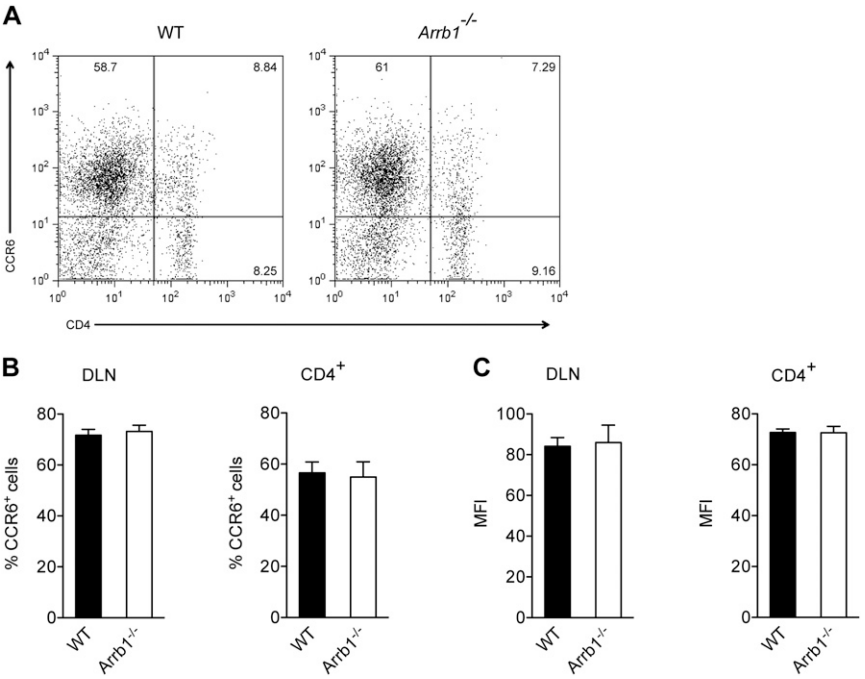

Fig. S5. No significant difference in the frequency of $CCR6^+$ cells and the expression of CCR6 between CII-immunized *Arrb1*$^{-/-}$ mice and WT littermates. (*A*) Flow cytometry of DLN cells from CII-immunized *Arrb1*$^{-/-}$ mice (*Right*) and WT littermates (*Left*) ($n = 3$ per group). (*B*) Percentage of $CCR6^+$ cells in DLN (*Left*) or $CD4^+$ T cells (*Right*) in *A*. (*C*) Mean fluorescence intensity (MFI) of CCR6 in *B*.

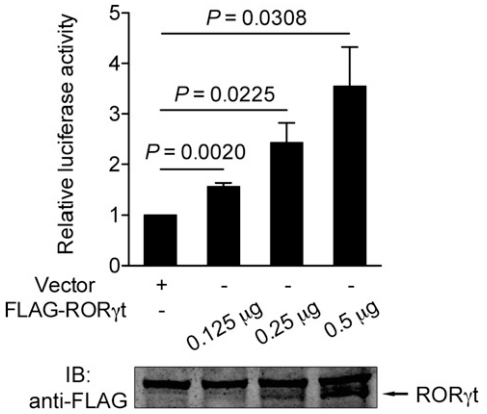

Fig. S6. RORγt enhances mouse *Arrb1* promoter activity. (*Upper*) Luciferase activity of HEK293 cells transfected with luciferase reporter construct containing an intact 2-kb fragment of mouse *Arrb1* promoter plus FLAG-RORγt–expressing vector or empty vector. (*Lower*) Immunoblot of RORγt in tranfected HEK293 cells detected with anti-FLAG.

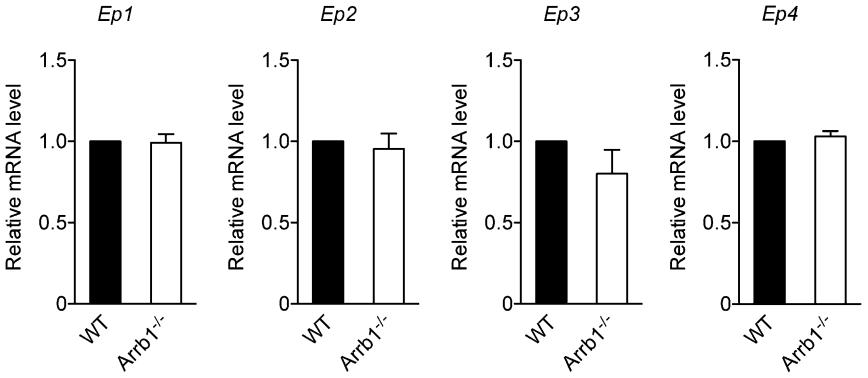

Fig. S7. No significant difference in the expression of prostaglandin E receptors in WT and *Arrb1*$^{-/-}$ T cells. Quantitative RT-PCR of *Ep1* (*Left*), *Ep2* (*Center Left*), *Ep3* (*Center Right*), or *Ep4* (*Right*) mRNA in naïve CD4$^+$ T cells from *Arrb1*$^{-/-}$ mice and WT littermates cultured for 2 d under T_H17-polarizing conditions.

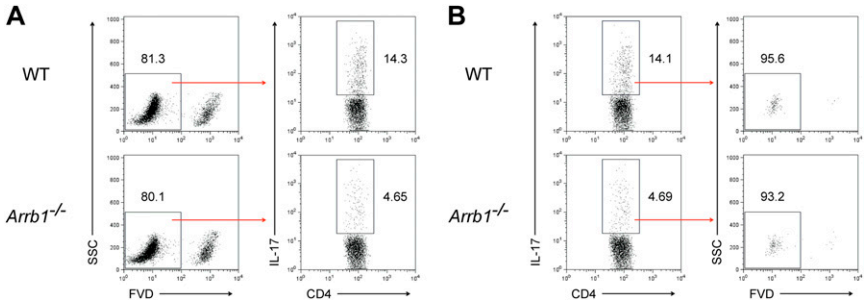

Fig. S8. Depletion of β-arrestin1 impairs T_H17 cell differentiation without affecting cell viability. (*A* and *B*) Flow cytometry of naïve CD4$^+$ T cells from *Arrb1*$^{-/-}$ mice and WT littermates cultured for 4 d under T_H17-polarizing conditions. The cells were first gated on FVD$^-$ live cells (*A*) or CD4$^+$IL-17$^+$ cells (*B*).

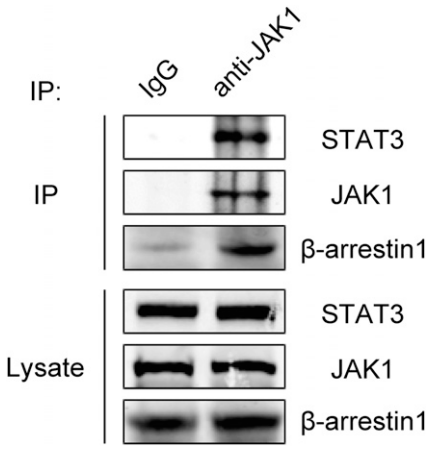

Fig. S9. JAK1, β-arrestin1, and STAT3 associates with each other in T_H17-polarized cells. Endogenous interaction of JAK1/β-arrestin1/STAT3 in naïve $CD4^+$ T cells cultured for 4 d under T_H17-polarizing conditions.

Table S1. Sequences of primers used for ChIP assay

Gene	Full name	Sense	Antisense
Il17	interleukin 17A	GGATTAAGGGCACACGTGTTG	TTTCCCCACTCTGTCTTTCCA
Il17f	interleukin 17F	CCCACAAAGCAACACTCTTGTC	ACTGCATGACCCGAAAGCA
Il21	interleukin 21	TGCCGCTGCTTTACTCATTG	GCACCGTCAGCTTTCAGAGA
Rorc	RAR-related orphan receptor gamma	AGCTTTGCTGTGGAAGATGTTTC	GAAGGGCTGGTAGGGAAGTCA

Table S2. Sequences of primers used for quantitative RT-PCR

Species	Gene	Full name	Sense	Antisense
Mouse	Arrb1	arrestin, beta 1	CCTGGATGTCTTGGGTCTG	TGATGGGTTCTCCGTGGTA
	Batf	basic leucine zipper transcription factor, ATF-like	CCAGAAGAGCCGACAGAGAC	GAGCTGCGTTCTGTTTCTCC
	Bcl2	B cell leukemia/lymphoma 2	GGGAGAACAGGGTATGATAACCG	TAGCCCCTCTGTGACAGCTTA
	Ccl20	chemokine (C-C motif) ligand 20	CGACTGTTGCCTCTCGTACA	CACCCAGTTCTGCTTTGGAT
	Ccr6	chemokine (C-C motif) receptor 6	TCCAGGCAACCAAATCTTTC	GATGAACCACACTGCCACAC
	Ep1	prostaglandin E receptor 1 (subtype EP1)	GGGCTTAACCTGAGCCTAGC	GTGATGTGCCATTATCGCCTG
	Ep2	prostaglandin E receptor 2 (subtype EP2)	GGAGGACTGCAAGAGTCGTC	GCGATGAGATTCCCCAGAACC
	Ep3	prostaglandin E receptor 3 (subtype EP3)	CCGGAGCACTCTGCTGAAG	CCCCACTAAGTCGGTGAGC
	Ep4	prostaglandin E receptor 4 (subtype EP4)	CCATTCCCGCAGTGATGTTCA	TGCGCGACTTGCACAATACTA
	Hprt	hypoxanthine guanine phosphoribosyl transferase	AGTCCCAGCGTCGTGATTAG	TTTCCAAATCCTCGGCATAATGA
	Ifng	interferon gamma	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
	Il1rn	interleukin 1 receptor antagonist	GCTCATTGCTGGGTACTTACAA	CCAGACTTGGCACAAGACAGG
	Il10	interleukin 10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
	Il12a	interleukin 12a	CAATCACGCTACCTCCTCTTTT	CAGCAGTGCAGGAATAATGTTTC
	Il17	interleukin 17A	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
	Il17f	interleukin 17F	TGCTACTGTTGATGTTGGGAC	AATGCCCTGGTTTTGGTTGAA
	Il18	interleukin 18	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTTGTCAACGA
	Il21	interleukin 21	GATCCTGAACTTCTATCAGCTCCAC	GGCATTTAGCTATGTGCTTCTGTT
	Il22	interleukin 22	GTGAGAAGCTAACGTCCATC	GTCTACCTCTGGTCTCATGG
	Il23r	interleukin 23 receptor	ACACTGGGAAGCCTACCTACA	AGCTTGGACCCATACCAAATACT
	Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	TCCAGAATGTCCCAGTCTCC	GAGTCTCAGTTTGGGGTGGA
	Rora	RAR-related orphan receptor alpha	GTGGAGACAAATCGTCAGGAAT	GACATCCGACCAAACTTGACA
	Rorc	RAR-related orphan receptor gamma	AGTGTAATGTGGCCTACTCCT	GCTGCTGTTGCAGTTGTTTCT
	Runx1	runt related transcription factor 1	TACCTGGGATCCATCACCTC	GACGGCAGAGTAGGGAACTG
	Tgfb1	transforming growth factor, beta 1	CACTGATACGCCTGAGTG	GTGAGCGCTGAATCGAAA
Human	ARRB1	arrestin, beta 1	GGGACGCGAGTGTTCAAGAA	ACAAACAGGTCCTTGCGAAAG
	IFNG	interferon, gamma	TCGGTAACTGACTTGAATGTCCA	TCCTTTTTCGCTTCCCTGTTTT
	IL1B	interleukin 1, beta	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
	IL6	interleukin 6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
	IL17	interleukin 17A	CAATCCCACGAAATCCAGGATG	GGTGGAGATTCCAAGGTGAGG
	IL23A	interleukin 23, alpha subunit p19	CTCAGGGACAACAGTCAGTTC	ACAGGGCTATCAGGGAGCA
	RPL13A	ribosomal protein L13a	CGAGGTTGGCTGGAAGTACC	CTTCTCGGCCTGTTTCCGTAG
	TGFB1	transforming growth factor, beta 1	GGCCAGATCCTGTCCAAGC	GTGGGTTTCCACCATTAGCAC