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Supplementary Materials for

Human T_H17 Cells Are Long-Lived Effector Memory Cells

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The PDF file includes:

Materials and Methods

Human subjects and human tissues

We studied healthy humans and patients with GvHD after bone marrow transplantation, chronic ulcerative colitis, colitic-associated colon carcinoma and ovarian cancer. Human tissues were from patients presenting for diagnostic biopsy, prophylactic colectomy, or tumor debulking. Patients received no prior treatment with immunosuppressive or chemotherapeutic agents were recruited for this study. Patients gave written, informed consent. Cells and tissues were obtained from blood and human tissues as we described (*1, 2*). The study was approved by the local Institutional Review Boards. In all the paired studies, different cell subsets utilized were autologous throughout this work, unless specifically described.

Flow cytometry analysis

The cells were first stained extracellularly with specific antibodies against surface antigens, then were fixed and permeabilized with Perm/Fix solution, and finally were stained intracellularly with antibodies to Ki67, caspase 3, orcytokines (BD Biosciences). Samples were acquired on a LSR II (BD) and data were analyzed with DIVA software (BD Biosciences).

Primary T helper (T_H) subsets and T_H polarization

T_H signature cytokines: Primary T_H subsets or polarized T_H subsets were defined on the basis of surface phenotype and specific T_H signature cytokine profile with multiple color flow cytometry analysis (LSR II, BD). All the flow-based phenotype, cytokine profile, proliferation, and survival/apoptosis markers were analyzed with this phenotype definition.

Primary T_H17 cells were initially enriched with magnetic beads and sorted with high speed sorter (FACSAria) with the phenotype of $CD3^+CD4^+CD45RA^-$ $CD45RO^+CCR6^+CD161^+$ (*3-5*) to high purity (99%) and defined with RORγt and IL-17 expression. For *in vivo* functional experiments, primary T_H17 cells were maintained and activated for 72 hours with 2 ng/ml IL-1β, 2 ng/ml IL-23 and 2 µg/ml of anti-IL-4 and anti-IFNγ neutralizing antibodies.

T_H polarization: Naïve $CD4^+$ T cells were stimulated with 2.5 µg/mL anti-CD3 and 1.25 µg/mL anti-CD28 mAb and 3 ng/ml rhIL-2 in the presence of irradiated peripheral blood

mononuclear cells. 10 ng/ml IL-12 and 2 μg/ml of anti-IL-4 or 5 ng/ml IL-4 and 2 μg/ml anti-IFNγ or 5 ng/ml IL-2 plus 10 ng/ml TGFβ or 2 ng/ml IL-1β, 5 ng/ml IL-23 and 2 μg/ml of anti-IL-4 and anti-IFNγ neutralizing antibodies were added for T_H1 or T_H2 or T_{reg} or T_H17 polarization, respectively.

Immunohistochemistry

Immunohistochemistry analysis was performed as described (*1, 2*). For multiple color fluorescence staining, tissues were stained with polyclonal rabbit anti-human-CD3 and polyclonal goat anti-human IL-17 followed by donkey anti-rabbit Alexa Fluor 568 (red) or chicken anti-rabbit Alexa Fluor 488 (green) and/or donkey anti-goat Alexa Fluor 488 (green) conjugated antibodies (all from Invitrogen). For classic immunohistochemistry staining, we used Horseradish Peroxidase or Permanent Red based detection system (EnVision, DAKO).

Quantitative real-time PCR and microarray

General real-time PCR was done as described (*1, 2*). For microarray, real-time PCR was done using 2X SuperArray RT qPCR Master Mix [RT^2 ProfilerTM PCR Array System, SuperArray (PAHS-075A-02)] to quantify expression levels of 84 stem cell transcription factors.

Knockdown HIF-1α expression and function

We previously demonstrated that echinomycin specifically blocked *HIF1A* expression (*6*). T_H17 cells and other T cell subsets were cultured with echinomycin (0.1nM) for 1-3 days as indicated. In other experiments, T_H17 cells were transfected with shHIF-1α expressing lentiviral vector. The lentiviral vector plenti6/V5-TOPO (Invitrogen) was modified by replacing CMV promoter with U6 promoter to drive shRNA-expressing cassette. This is followed by a pGK-driven EGFP cassette. The transfected cells expressed GFP. The core sequences of *HIF1*α−shRNA-1 and *HIF1*α−shRNA-2 were described (*6*).

Notch activation, inhibition and *BCL2* promoter assay

To activate Notch, T cell subsets were transfected with GFP expressing lentiviral vector encoding an active form of Notch (the transmembrane and intracellular domains, comprising residues 1,704–2,531) Notch-IC cDNA (Notch IC) or control vector (*6, 7*). After different time points, T cell gene expression and function were analyzed on the basis of GFP expression. In other experiments, T cell subsets were cultured with γ-

Secretase Inhibitor, Z-Leu-Leu-Norleucine-CHO (0.5 nM, Calbiochem) as indicated. T cell phenotype and function were analyzed.

To detect the effects of HIF-1α and Notch on *BCL2* promoter activity, the human Bcl-2 promoter sequence (-850 to +134, TSS as +1) was initially linked to GFP. HEK293 cells were transfected with viral vector encoding human *BCL2*promoter in conjunction with vector controls or vector containing cDNA encoding *HIF1A*, or *MYC* (genetic control), or *NOTCH-IC* cDNA. The promoter activity was measured by the green fluorescence intensity of transfected cells. Data were shown as the relative intensities. The intensity of control-GFP reporter is defined as 1.0.

In vitro T cell proliferation and division

Primary or polarized T cells were stimulated with 2.5 μg/mL anti-CD3 and 1.25 μg/mL anti-CD28 for 4 days. T cell proliferation was defined by thymidine incorporation on day 3. In some experiments, prior stimulation, T cells were labeled with CFSE (*8*). T cell divisions were determined with flow cytometry analysis.

Apoptosis assay

T cells were activated with anti-CD3 and anti-CD28 for 1-3 days. T cells were stained with Annexin V and 7-AAD. Apoptotic cells were analyzed by flow cytometry (*9*). In other experiments T cells were cultured with cisplatin (20 μm). After 40 hours, cells were harvested Annexin V and 7-AAD staining.

In vivo T cell homeostatic expansion and persistence

Primary T_H17 cells or polarized HLA-$A2^+$ T cell subsets were mixed with HLA-$A2^-$ T cells, and injected into NSG mice. After transfusion, human T cells were recovered from different organs and analyzed by flow cytometry. In other experiments, primary T cells were injected into NSG mice. The mice were administrated with BrdU (1 mg/mice). After 24 to 48 hours, human T cell apoptosis and cycles were analyzed by flow cytometry in mouse spleen.

In vivo tumor formation

To generate tumor associated antigen specific $CD8^+$ T cells, myeloid dendritic cells (DCs) were differentiated as we described (*1, 2, 10*). DCs were loaded with HLA-$A2^+$Her2/neu^+ primary ovarian cancer lysate. HLA-$A2^+CD8^+$ cells (10^5/ml) were stimulated with tumor lysate-loaded DCs (5 x 10^4/ml) in the presence of 5 ng/ml IL-2 and

IL-15 for two weeks. Tumor specific cytotoxicity of the resultant $CD8^+$ T cells was assessed using autologous primary HLA-$A2^+$Her2/neu^+ ovarian cancer cells and HLA-$A2^+$ T2 cells bearing Her-2/neu peptides or matrix control as we previously described (*1, 2, 10, 11*). Autologous T_H17 cells were also stimulated in an identical manner. The origin and cytokine profile of autologous T_H17 cells were described above. HLA-$A2^+$Her2/neu^+ ovarian cancer cells (*1, 11*) were subcutaneously injected into female NSG mice (6-8 weeks old, Jackson Lab, Bar Harbor, Maine) (*1, 2*). The $CD8^+$ T cells (6×10^6) (*1, 2*) or/and autologous T_H17 cells (6×10^6) were injected intravenously into mice on day 10 after human tumor inoculation. Tumor volume was measured and calculated (*1, 2*). Animal protocol was approved by the Unit for Laboratory Animal Medicine of University of Michigan and was conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Statistical analysis

The Wilcoxon signed-rank test was used to determine pairwise differences and the Mann–Whitney U test was used to determine differences between groups. $P < 0.05$ was considered as significant. All statistical analysis was done on Statistica software (StatSoft Inc.).

Supplementary Figure 1

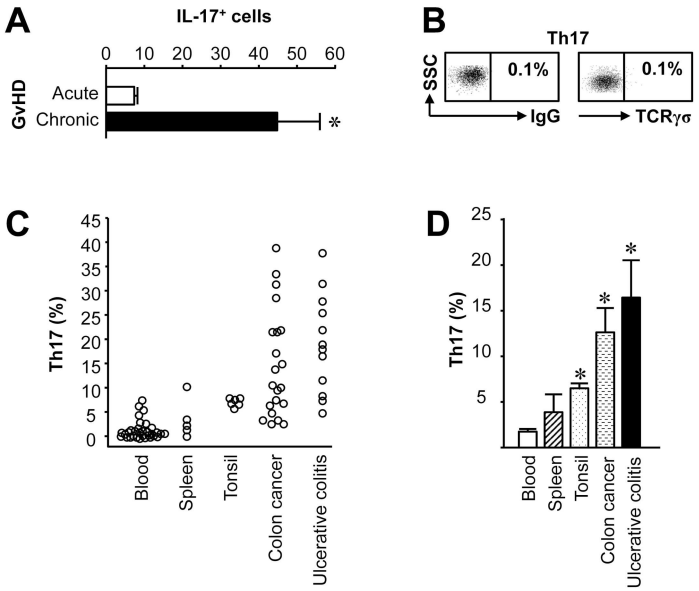

Fig. S1. T_H17 cells in different tissues/organs. Case numbers, GvHD (6), blood (31), spleen (5), tonsil (6), colon cancer (21) and colitis (12). (**A**) IL-17$^+$ cells in oral mucosa tissues in patients with GvHD. Consecutive sections of oral mucosa biopsies were stained for IL-17. IL-17$^+$ cells were quantified in 5 high power fields. Results are expressed as the absolute mean numbers of IL-17$^+$ cells ± SEM per field. $P < 0.001$, acute vs chronic. (**B**) Phenotype of IL-17$^+$ cells. IL-17$^+$ cells were T_H17 cells, and did not express TCRγδ. Gated on CD3$^+$CD4$^+$IL-17$^+$ cells. (**C, D**) T_H17 cells in inflammatory tissues. T_H17 cells were analyzed with flow cytometry in different tissues. Results are expressed as the actual percentage of T_H17 cells in individual donors (**C**) and the mean percentage of T_H17 cells ± SD in CD4$^+$ T cells (**D**). $P < 0.001$, compared to blood.

Supplementary Figure 2

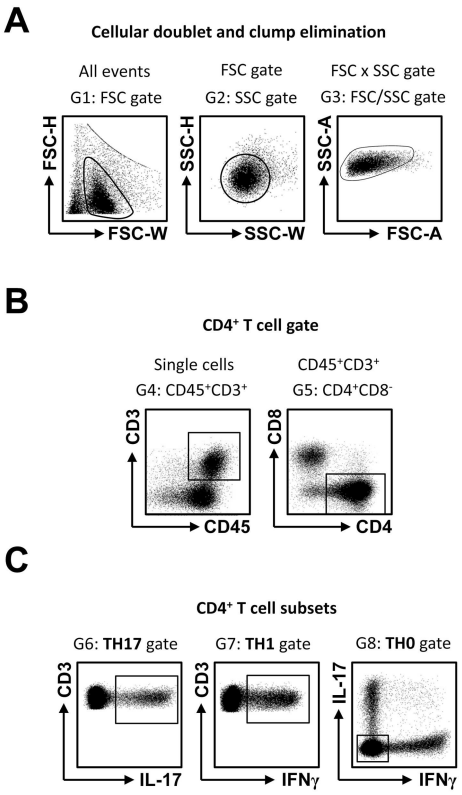

Fig. S2. Flow-based gating for primary T_H cell subsets. Single cell suspension was subjected to staining for CD3, CD45, CD4, CD8, IL-17, and IFNγ. Different T cell subsets were analyzed with a LSR II. (**A**) Single cells. In order to avoid cellular doublets and clumps, the cell suspension was initially analyzed on the basis of FSC and SSC in three dimensions with H, A, and W axes. Three gates (G1, G2, and G3) were accordingly utilized to exclude cellular doublets and clumps, and obtain real single cells. (**B**) $CD4^+$ T cells. Gates 4 and 5 were used to gate T cells, and $CD4^+$ T cells. (**C**) Primary T_H subsets. Gates 6, 7 and 8 were used to gate T_H17, T_H1, and T_H0 cells. All the flow-based phenotype and functional analyses were based on these gates.

Supplementary Figure 3

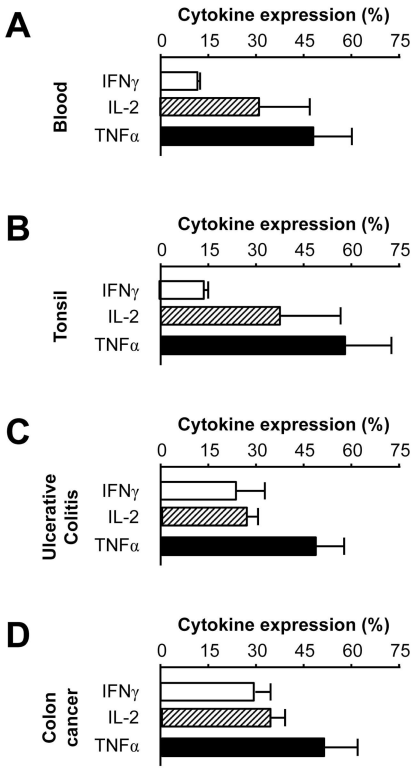

Fig. S3. Cytokine profile of primary T_H17 cells. Effector cytokine profile of primary T_H17 cells was analyzed with LSR II in blood (**A**), tonsil (**B**), ulcerative colitis (**C**) and colon cancer (**D**). Results are expressed as the mean percentage of cytokine expressing cells ± SD in T_H17 cells. Tonsil: 6; Blood: 31; Ulcerative colitis 12; Colon cancer, 21.

Supplementary Figure 4

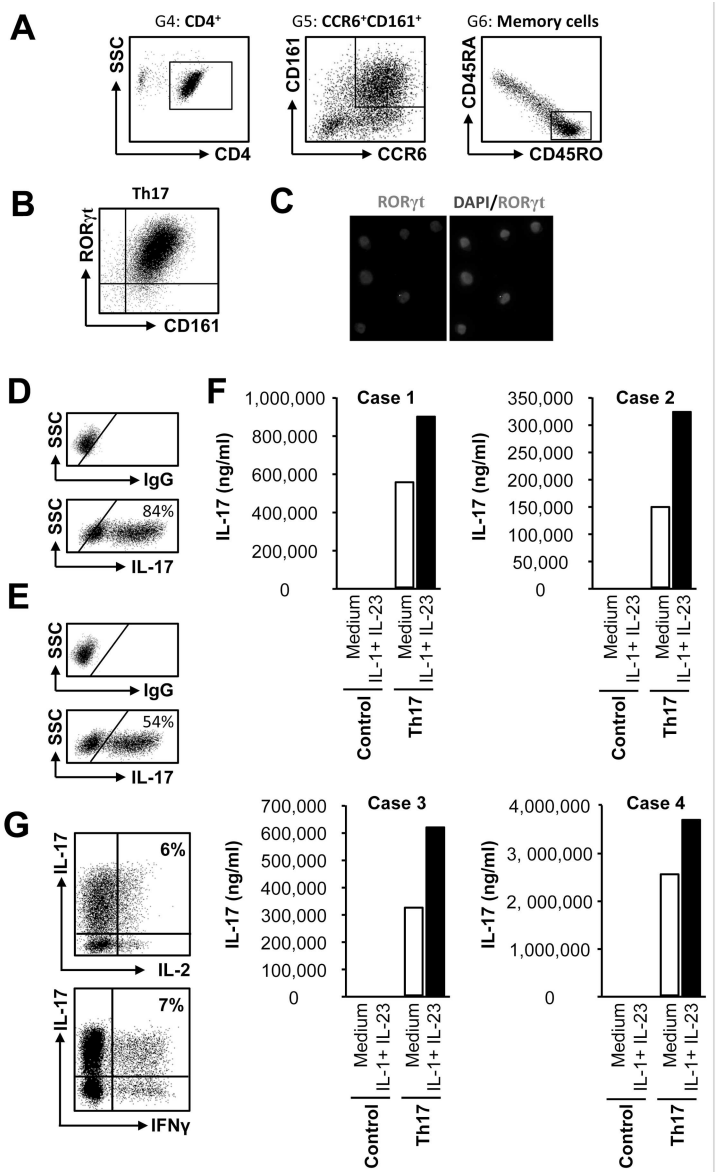

Fig. S4. Flow-based gating and sorting for T_H17 cells. $CD4^+$ T cells were enriched with magnetic beads through negative selection. The cells were stained for CD3, CD4, CD45RA, CD45RO, CCR6, and CD161, and analyzed with a LSRII. Cellular doublets

and clumps were excluded with gates G1-3 (Fig. S2). (**A**) Surface phenotype of T_H17/T_H17 precursor cells. Different gates were utilized to identify $CD4^+$ T cells (G4), $CCR6^+CD161^+CD4^+$ T cells (G5) and $CCR6^+CD161^+CD4^+CD45RA^-CD45RO^+$ T cells (G6). (**B, C**) ROR expression in T_H17/T_H17 precursor cells. We sorted $CCR6^+CD161^+CD4^+CD45RA^-CD45RO^+$ T cells with high-speed sorter (LSR II). The cells were stained for ROR and CD161 expression, and analyzed with LSR II (**B**). Or, the cytospin slides were made with the sorted cells, and stained with anti-ROR, and analyzed with fluorescence microscope. ROR is localized in the nuclear. ROR expression (red), nuclear staining (DAPI, blue). (**D, E**) IL-17 expression by the sorted T_H17/T_H17 precursor cells. The sorted $CCR6^+CD161^+CD4^+CD45RA^-CD45RO^+$ T cells (G6) were maintained and polarized for 72 hours with IL-1 and IL-23. The resultant T_H17 cells were termed as polarized primary T_H17 cells. The cells were sampled for intracellular IL-17 staining. Based on the isotype control, more than 80% of the polarized primary T_H17 cells expressed intracellular IL-17 (**D**), and more than 50% of them expressed high levels of intracellular IL-17 (**E**). (**F**) IL-17 production by the sorted T_H17/T_H17 precursor cells. The sorted $CCR6^+CD161^+CD4^+CD45RA^-CD45RO^+$ T cells (G6) were maintained and polarized for 72 hours with IL-1 and IL-23. The cells were sampled for measuring IL-17 in the supernatants. $CCR6^+CD161^+CD4^+CD45RA^-CD45RO^+$ T cells released large amount of IL-17, and IL-1/IL-23 further enhanced their IL-17 production. Four cases are shown. (**G**) IFNγ and IL-2 expression in the polarized primary T_H17 cells. The polarized primary T_H17 cells were stained for intracellular IL-2 and IFNγ, and were analyzed with LSR II.

Supplementary Figure 5

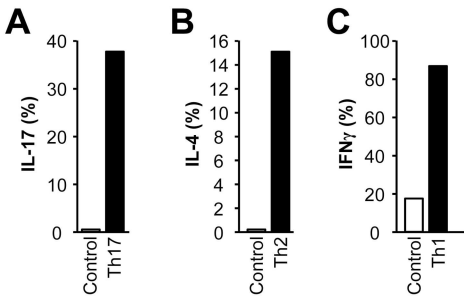

Fig. S5. Polarized T cell subsets. Naïve $CD4^+$ T cells were polarized to T_H1, T_H2, and T_H17 as described. T_H signature cytokines were analyzed with LSR II. Results were expressed as the percentage of signature cytokine expressing cells. One of 8 is shown.

Supplementary Figure 6

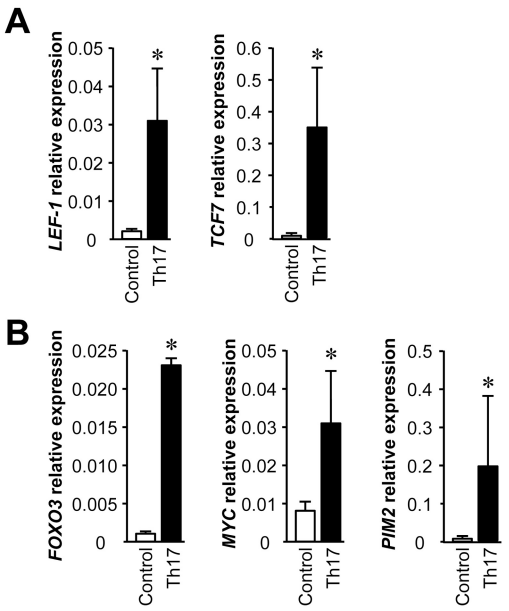

Fig. S6. T_H17 cells express high levels of stem cell genes. Selective stem cell-associated gene expression was quantified by-real time PCR. (**A**) shows several Wnt/β-catenin signaling genes; and (**B**) shows other stem cell-associated genes including *MYC*, *FOXO3* and *PIM2*. Results are expressed as the mean relative values ± SEM. n = 8, $P < 0.05$.

Supplementary Figure 7

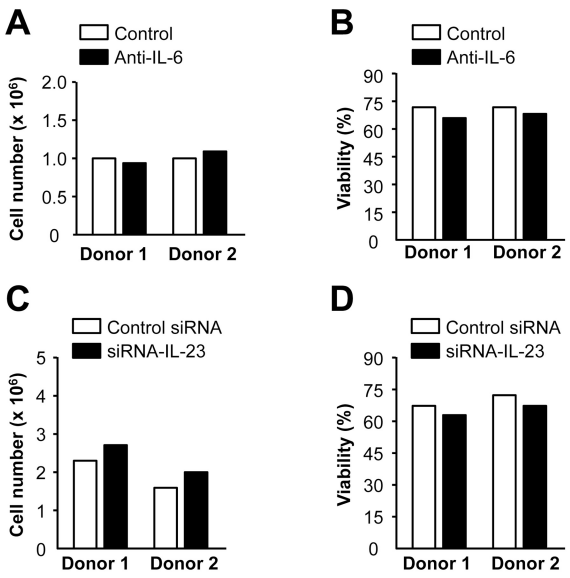

Fig. S7. Effects of IL-6 and IL-23 on T_H17 cell apoptosis. Naïve T cells were polarized into T_H17 cells in the presence of APCs. Anti-IL-6 was added into the culture (**A, B**) or IL-23 was blocked in APCs with siRNA-IL-23 (**C, D**). The total viable cells were recorded and the viability was calculated. Results are expressed as the mean values ± SD. Two donors with duplicates.

Supplementary Figure 8

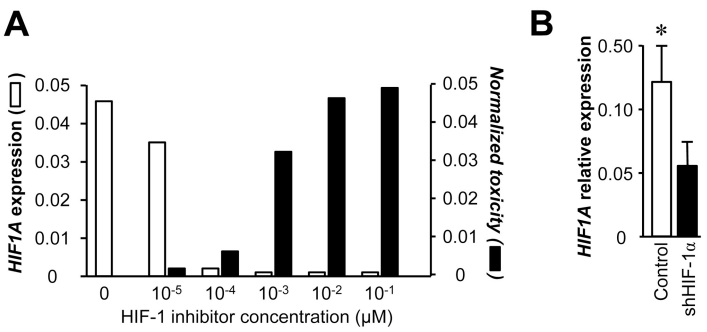

Fig. S8. HIF-1 blockade with echinomycin and shHIF-1α. (**A**) Optimization of echinomycin concentration. T_H17 cells were cultured with titrations of echinomycin. The toxicity was calculated based on the cell loss. 0.1 nM echinomycin resulted in potent reduction of HIF-1 expression (left scale) with negligible cell loss (right scale). This concentration was utilized in the experiments. (**B**) ShHIF-1α efficiently blocked *HIF1A* expression. Two sets of shHIF-1α were selected. After transfection, both efficiently blocked *HIF1A* expression in human primary T cells.

Supplementary Figure 9

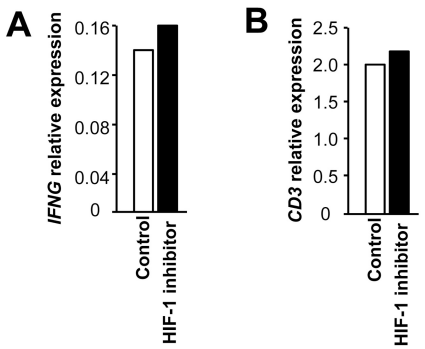

Fig. S9. The HIF-1 inhibitor echinomycin had no effects on the human *IFNG* and *CD3* expression in vivo. Human T cells were collected from mouse spleen as described in "Materials and Methods". Human *IFNG* (**A**) and *CD3* (**B**) gene expression was quantified by real time PCR relative to human housekeeping genes (*GAPDH*). Results are expressed as the mean relative values ± SEM. n = 3, P > 0.05.

Supplementary Figure 10

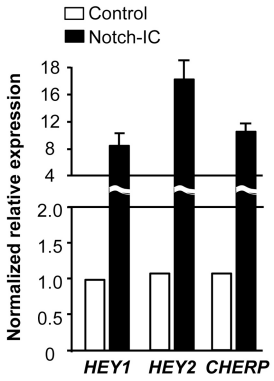

Fig. S10. Notch-IC activated Notch signaling gene expression. T_H17 cells were transfected with Notch-IC. After 40 hours, Notch signaling gene expression was quantified by real-time PCR. Results are expressed as the mean relative values ± SD. n = 4, $P < 0.001$ Notch-IC vs control.

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