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Notch Signaling Regulates Mouse and Human Th17 Differentiation

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Th17 cells are known to play a critical role in adaptive immune responses to several important extracellular pathogens. Additionally, Th17 cells are implicated in the pathogenesis of several autoimmune and inflammatory disorders as well as in cancer. Therefore, it is essential to understand the mechanisms that regulate Th17 differentiation. Notch signaling is known to be important at several stages of T cell development and differentiation. In this study, we report that Notch1 is activated in both mouse and human in vitro-polarized Th17 cells and that blockade of Notch signaling significantly downregulates the production of Th17-associated cytokines, suggesting an intrinsic requirement for Notch during Th17 differentiation in both species. We also present evidence, using promoter reporter assays, knockdown studies, as well as chromatin immunoprecipitation, that IL-17 and retinoic acid-related orphan receptor γt are direct transcriptional targets of Notch signaling in Th17 cells. Finally, in vivo inhibition of Notch signaling reduced IL-17 production and Th17-mediated disease progression in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. Thus, this study highlights the importance of Notch signaling in Th17 differentiation and indicates that selective targeted therapy against Notch may be an important tool to treat autoimmune disorders, including multiple sclerosis. The Journal of Immunology, 2011, 187: 692–701.

The regulation of Th17 differentiation from naive CD4+ T cells is an area of active investigation. Th17 cells and the proinflammatory cytokines (IL-17A, IL-17F, IL-21, and IL-22) produced by these cells have been implicated in several autoimmune and inflammatory disorders (1, 2). The importance of this subset in autoimmune diseases was first recognized when mice lacking expression of the p19 subunit of IL-23, a cytokine involved in differentiation and expansion of Th17 cells, failed to develop certain autoimmune disorders (3, 4). The pathogenic role of IL-17 as well as Th17 cells has now been documented in numerous autoimmune diseases, including multiple sclerosis (5), rheumatoid arthritis (6), psoriasis (7), Crohn’s disease (8), and systemic lupus erythematosus (9).

Several factors are known to affect Th17 differentiation, including antigenic stimuli (10, 11), expression of particular transcription factors (12), and epigenetic changes in the IL-17 gene locus (13). The cytokine milieu leading to Th17 differentiation is the most carefully studied factor. In mice it is known that IL-6, along with proinflammatory cytokines TGF-β and IL-21, promotes differentiation of naive CD4+ T cells into the Th17 lineage (14). Manel et al. (15) have similarly shown that human Th17 differentiation requires exposure to low doses of TGF-β in concert with IL-1β, IL-6, IL-21, and/or IL-23. In addition to the cytokine environment, transcription factors are important determinants of Th17 differentiation (14, 16, 17). The transcription factor retinoic acid-related orphan receptor (ROR)γt, in cooperation with RORα, controls Th17 differentiation (18). Th17 differentiation also is regulated by histone-3 acetylation and H3Lys4 methylation in both the IL-17A and the IL-17F promoters in a lineage-dependent manner (13). Despite great progress in understanding the molecular mechanism of Th17 differentiation, the contribution of cell surface proteins found on CD4+ T cells is not well understood.

Notch proteins are type I transmembrane proteins known to play a crucial role in cell fate determination in many cell lineages, including early T cell development in the thymus (19). Four Notch receptors (Notch1, -2, -3, and -4) are found in mammals. In developing T cells, Notch1 has been reported to regulate αβ versus γδ T cell differentiation (20), T cell versus B cell fate determination (21), and CD4+ versus CD8+ T lineage decision (22). Notch1 is also present on naive (23) and activated CD4+ T cells (24). Additionally, we and others have shown that Notch1 signaling is activated upon crosslinking of the TCR (24, 25).

Canonical Notch signaling is induced when one of the four mammalian Notch receptors (Notch1, -2, -3, or -4) encounter one of the five known ligands (Jagged1, -2, -3 or -4 and Delta-like 1 or 2) on a neighboring cell. This interaction initiates a proteolytic cleavage

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of the transmembrane Notch peptide near the extracellular surface by an ADAM protease, which, in turn, induces a conformational change that allows access and cleavage of the Notch transmembrane domain by the γ-secretase complex. Cleavage of Notch receptors by γ-secretase results in the release of an intracellular Notch fragment, which rapidly translocates to the nucleus where it interacts with the DNA binding protein known as CSL (CBF-1, suppressor of hairless, Lag-1). In the absence of Notch signaling, CSL is bound to DNA in a complex with several repressor proteins. Intracellular Notch translocation to the nucleus and binding to CSL results in disruption of the repressor complex followed by recruitment of several coactivator proteins resulting in the initiation of transcription of genes located downstream of Notch/CSL complexes (reviewed in Refs. 26, 27).

Notch is reported to play a critical role in Th1- (28, 29) and/or Th2-mediated (30, 31) immune responses. Data from several laboratories suggest that APCs expressing Delta-like 4 drive the differentiation of Th1 cells (32, 33) while APCs expressing Jagged1 or Delta-like 4 drive the differentiation of Th17 cells (34). In this study, we examined the role of Notch signaling in Th17 polarization. We used pharmacologic inhibitors as well as knockdown approaches to establish a role for Notch signaling in Th17 polarization. Promoter analysis and chromatin immunoprecipitation (ChIP) assays demonstrated regulation of both the IL-17 and RORγt promoters by Notch1. Lastly, we present in vivo data demonstrating that inhibition of Notch signaling ameliorates the severity of experimental autoimmune encephalomyelitis (EAE), a murine autoimmune disease that displays several characteristics of human multiple sclerosis. These data provide further understanding of the Th17 differentiation pathway and suggest opportunities for exploiting the Notch signaling pathway to treat Th17-mediated autoimmune disorders.

Materials and Methods

Drugs and chemicals

γ-Secretase inhibitors compound E (Alexis Biochemicals, San Diego, CA) and IL-CHO, a small peptide inhibitor of γ-secretase (25), were resuspended in DMSO and used in concentrations as indicated in the figure legends.

Antibodies

For detection of human Notch 1, anti-Notch1 (C20; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-activated Notch 1 (Rockland Immunochemicals, Gilbertsville, PA) Abs were used. Anti-Notch2 (Abcam, Cambridge, MA), anti-Notch3 (M134; Santa Cruz Biotechnology), and anti-Notch4 (H225; Santa Cruz Biotechnology) were also used. For detection of mouse activated Notch1, anti-Notch 1 (mN1A clone; ebioscience, Cambridge, MA), anti-Notch3 (M134; Santa Cruz Biotechnology), and anti-Notch2 (Abcam, Cambridge, MA) Abs were used. β-actin Ab (Sigma-Aldrich, St. Louis, MO) was used as a loading control.

Cell culture and mouse in vitro polarization

For mouse in vitro polarization assays, naive CD4+ T cells were isolated from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) using the IMag magnetic system (BD Pharmingen, San Jose, CA), according to the manufacturer’s protocol. Cells (2.5 × 10^6/ml) were pretreated in vitro at 37°C for 30 min with 0.1% DMSO or with γ-secretase inhibitor (GSI) (25 μM IL-CHO or 4 μM compound E) and then plated onto 12- or 6-well plates precoated with 1 μg/ml each anti-CD3 and anti-CD28. To polarize CD4+ T cells to a Th17 phenotype, 20 ng IL-6 (R&D Systems, Minneapolis, MN), 5 ng TGF-β1 (R&D Systems), and 5 μg anti–IFN-γ (BD Pharmingen) and anti–IL-4 (BD Pharmingen) were used per milliliter of cells (35, 36). Cells were polarized for 24, 48, or 72 h. The activation supernatants were evaluated for IL-17A (BD Biosciences), IL-17F (R&D Systems), and IL-21 (R&D Systems) by ELISA. To study the effect of Notch inhibition on fully differentiated Th17 cells, naive CD4+ T cells were differentiated toward the Th17 lineage for 4 d. These cells were then treated with either DMSO or IL-CHO followed by culturing in anti-CD3-coated plates. After 24 h, supernatants were collected and analyzed for IL-17 cytokine by ELISA (BD Biosciences).

Cell culture and human in vitro polarization

Human in vitro Th17 polarization was performed using a modified protocol from Manel et al. (15). Naive CD4+ T cells were purified from PBMCs by negative selection using MACS separation according to the manufacturer’s instructions (Miltenyi Biotec, Sunnyvale, CA) and were cultured at 37°C in 5% CO2, in serum-free X-VIVO 10 media (BioWhittaker, Walkersville, MD) Naive cells (2 × 10^6/ml) were plated in 24-well plates with beads coated with anti-CD3 and anti-CD28 (Dynabeads; Invitrogen Dynal, Oslo, Norway) at a concentration of one bead per cell. Abs and cytokines were added at the time of plating at the following concentrations: 10 U/ml IL-2, 5 ng/ml TGF-β1, 10 ng/ml IL-6, 10 ng/ml IL-23, 10 ng/ml IL-21, 10 ng/ml IL-1β, 10 μg/ml anti-IL-4, and 10 μg/ml anti–IFN-γ. All Abs and recombinant cytokines were purchased from R&D Systems. IL-17A, IL-17F, IL-21, and IL-22 protein levels in the activation supernatants were quantified by ELISA (eBioscience). To evaluate the effect of Notch inhibition on differentiated Th17 cells, naive CD4+ T cells were cultured in Th17 polarizing conditions for 4 d following by treatment with either DMSO or IL-CHO. Supernatants were collected after 24 h and analyzed for IL-17 and IL-22 cytokines (eBioscience).

Cell lines and constructs

HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM medium (Mediatech, Manassas, VA) supplemented with 10% FBS (Cellgro; Mediatech), 2 mM glutamine, and 1 mM pyruvate. Supplements were from Lonza (Walkersville, MD). The intracellular portion of Notch1 (Notch1ΔC) encoding plasmid construct was generated by cloning Notch1ΔC cDNA into BarnHI and EcoRI sites of pcDNA3.0 (37).

Retroviral expression vector and transduction

The sequence encoding Notch1ΔC was subcloned into the retroviral vector LZRS and viral particles were produced as described previously (38). For transduction of virus, naive human CD4+ T cells were isolated and stimulated with anti-CD3/CD28–coated beads for 24 h and transduced with retroviral supernatant in the presence of 8 μg polybrene as described before (38). Transduced cells were then differentiated to Th0 or Th17 conditions. The cells were transduced again the following day with retroviral supernatants and cultured for an additional 48 h.

Dual-Luciferase assay

HEK 293T cells were plated on 60-mm dishes and cotransfected with Notch1ΔC (1 μg) expression vector constructs cloned into pcDNA3.0 along with a human IL-17 (~1125 bp) promoter luciferase construct (1 μg) provided by Dr. Sarah Gaffen (University of Pittsburgh) (39) and 0.1 μg Renilla luciferase construct as the internal transfection control. Luciferase assays (Dual-Luciferase assay system; Promega, Madison, WI) were performed according to the manufacturer’s instructions.

Intracellular staining and cell surface staining

Mouse CD4+ T cells were polarized toward a Th17 phenotype as described above. After 72 h, the cells were stimulated by adding 80 nM PMA and 2.5 μM ionomycin for 1 h in addition to brefeldin A for 5 h. Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Fluorescent Abs (anti-mouse CD4-FITC, anti-mouse IL-17A-allophycocyanin, and anti-mouse IFN-γ-PE) were obtained from BD Biosciences. Anti-mouse Notch1-PE was obtained from eBioscience. Cells were analyzed on a FACS LSR II (BD Biosciences).

Real-time PCR

Human naive CD4+ T cells were polarized to Th17 as described above. RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). The RNA was then DNAase I treated (Qiagen) and cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was then performed using 18S rRNA to normalize following the 2ΔΔCT method (40). The primer sequences used were: Notch1, forward, 5’-GTC AAC GCC GTA GAT GAC C-3’, reverse, 5’-TTG TTA GCC CCG TCC TTC AG-3’; RORγt (15), forward, 5’-TTT TCC GAG GAT GAG ATT GC-3’, reverse, 5’-CCT TCC ACA TCG TGG CTA CA-3’; 18S rRNA, forward, 5’-GCC GCC CCC TCG ATG CTC TTA G-3’, reverse, 5’-GCT CGG GCC TGC FT TTA GAA CAC TCT-3’.
by quantitative real-time polymerase (Stratagene Ms3000P system). Prim- 
er sequences were: IL-17, forward, 5'-CTC CAG AAG GCC CTC AGA 
CTA C-3', reverse, 5'-AGC TTT CCC TCC GCA TTG AGC CAG-3' (41); 
RORyt, forward, 5'-TTT GGA ACT GCC TTG CCA TC-3', reverse, 
5'-AAG ATC TGC AGC TTT ACA-3'. The expression of each gene 
was normalized to the expression of β-actin by the 2^(-ΔΔCt) method (40).

RNA interference
To knock down the expression of Notch1, CD4+ T cells were purified and nucle- 
larly segmented with interfered RNA (siRNA) specific for Notch1 or 
scrambled siRNA (Santa Cruz Biotechnology) using an Amxax nucle- 
ator program. Briefly, 5–10×10^6 CD4+ T cells were resuspended in 100 
μl Nucleofector solution and transfected with 100 nM siRNA using the 
U-014 Amxax Nucleofector program (Lonza, Basel, Switzerland). After 
transfection, the cells were incubated for 6 h at 37˚C and stimulated with 
anti-CD3/CD28–coated magnetic beads under Th17 polarizing conditions 
for 48 h.

MTS assay
Cytotoxicity assay was performed using CellTiter 96 AQueous One solution 
reagent (Promega) as per the manufacturer’s instructions.

ChIP assay
ChIP assays (Upstate Cell Signaling Solutions) were performed using 1× 
10^5 naive CD4+ T cells stimulated with anti-CD3/CD28–coated magnetic 
beads (one bead per cell) under Th0 (no cytokines) or Th17 conditions 
and pretreated with DMSO or GSI (IL-CHO) for 24 h. The following primers 
were used for quantitative as well as standard PCR. IL-17 primer sets 
were: 17CSL1, forward, 5'-TTG ACC CAT AGC ATA GCA GC-3', 
reverse, 5'-TTC AGG GGT GAC ACC ACT TT-3'; 17CSL2, forward, 5'- 
GAA AAT CTC GTG TCT CTT GAA CC-3', reverse, 5'-TTC CTC 
ACA GTT TCC TTG GC-3'; 17CSL3, forward, 5'-TTC CAC TTT CCA 
CTT ACC-3', reverse, 5'-TTC CTC CCT GTC TCT CTA TC-3'; 17CSL4, 
forward, 5'-CAA TTT GGA AAA GCA AT-3', reverse, 5'-CCC TAC 
GTC TCC CCC TCT AC-3'. RORyt primer sets were: RCBF1, forward, 
5'-ATC TCC AGC TGC AGT TTG GA-3', reverse, 5'-GAT GCC CCT 
GTT TTC TGT AG-3'; RCBF2, forward, 5'-AGA GAG ACT CCT 
TGC CTC TC-3', reverse, 5'-TCA AGG CTG AGG CTG GAG AT-3'. Abs 
used were rabbit anti-Notch1 or normal rabbit IgG (Santa Cruz Biotechnology). 
Conditions for real-time PCR were 50˚C for 2 min, 95˚C for 10 min, 95˚C 
for 15 s, 60˚C for 1 min (40 cycles); conditions for semiquantitative PCR 
were 95˚C for 5 min, 95˚C for 30 s, 55˚C for 1 min, 95˚C for 30 s (35 
cycles), 72˚C for 2 min.

In vivo GSI treatment
For the EAE experiments, 8- to 12-wk-old female SJL/J mice were pur- 
chased from Charles River Laboratories (Wilmington, MA). All mice were 
housed in the Animal Care Facility at the University of Massachusetts. 
Animals were fed in accordance with the Institutional Animal Care and Use 
Committee guidelines. The GSI administered in vivo was LY-411575 (LY) 
by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from

Statistical analysis
Statistical analyses were performed using GraphPad Prism version 4.0. 
Unpaired t tests (α = 0.05) were used when comparing two conditions.

Results

GSi during murine Th17 polarization results in reduced 
Th17-associated cytokine production
The effect of GSI on murine production of IL-17A, IL-17F, and IL- 
21 was tested by treating Th17-polarized cells with IL-CHO and 
compound E, two chemically distinct GSIs that block γ-secretase 
by different mechanisms. IL-CHO is a competitive peptide alde- 
hyde inhibitor of γ-secretase activity that is thought to modify the 
active sites, whereas compound E is a nonpeptide, nontransition 
state, noncompetitive inhibitor of γ-secretase. Naive CD4+ T cells 
were isolated from spleens of 8- to 12-wk-old C57BL/6 mice, 
pretreated with GSI or DMSO control for 30 min at 37˚C and 
cultured in Th17 polarizing conditions for 24, 48, or 72 h and IL- 
17A, IL-17F, and IL-21 cytokine levels were assessed. The level of 
IL-17A produced by Th17 cells treated with GSI was signifi- 
cantly reduced in comparison with DMSO-treated Th17-polarized 
cells (Fig. 1A). Similarly, a reduction in IL-17F and IL-21 cyto- 
kine levels were observed after GSI treatment as compared with 
DMSO (Fig. 1A). The observed cytokine profiles demonstrate that 
GSIs reduced Th17-associated cytokines from in vitro-differen- 
tiated Th17 cells. Interestingly, we also observed that Notch1 
is upregulated in Th17-polarized cells as compared with Th0 
conditions (Fig. 1B).

Notch is a primary target of GSI in CD4+ T cells, and to ensure 
that GSI was effective at reducing Notch1 activation, intracellular 
levels of Notch1 were assessed by immunoblot (Fig. 1B) and 
intracellular staining (Fig. 1C). These data revealed that Notch1 
protein expression was reduced in Th17-polarized murine CD4+ 
T cells treated with GSI.

To determine the effect of GSI on IL-17A production on a per 
cell basis, intracellular staining of IL-17A was also performed in 
Th17-differentiated cells pretreated with either DMSO or GSI (IL- 
CHO). We observed a reduction in intracellular IL-17 levels in GSI- 
treated Th17 cells as compared with DMSO (Fig. 1D). Addi- 
tionally, the effect of Notch inhibition on already differentiated 
Th17 cells was assessed. Naive CD4+ T cells were cultured in 
Th17 polarizing conditions for 4 d followed by treatment with 
both DMSO or GSI. Interestingly, no changes in IL-17 levels 
were detected (Fig. 1E).

The inhibition of γ-secretase during human Th17 polarization 
results in decreased Th17-associated cytokine levels
To determine whether Notch signaling also plays a role in human 
Th17 differentiation, we treated in vitro human Th17-polarized 
cells with two different GSIs (IL-CHO and compound E). Naive 
CD4+ T cells (CD4+CD45RA-) were purified from PBMCs, pre- 
treated with either GSI or DMSO for 30 min and cultured in Th17 
polarization conditions for 24, 48, and 72 h. IL-17A, IL-17F, and 
IL-22 secreted by human Th17 cells were significantly reduced in 
the presence of GSI compared with DMSO (Fig. 2A). Surprisingly, 
we did not detect significant levels of IL-21 in human in vitro-differ- 
entiated Th17 cells (data not shown). Consistent with the 
murine data, Th17 polarization of human CD4+ T cells resulted in 
increased levels of activated Notch1, compared with those acti- 
vated under neutral conditions (Fig. 2B). An MTS assay was 
performed to confirm that the decrease in IL-17 secretion by GSI 
was not due to an effect on cell proliferation (data not shown).

Next we assessed the effect of Notch inhibition in fully differ- 
entiated Th17 cells. Naive CD4+ T cells were differentiated in
Th17 conditions for 4 d followed by treatment with either DMSO or GSI. As seen in murine cells, no changes in IL-17A and IL-22 cytokines were observed (Fig. 2C). Taken together, data in Figs. 1 and 2 show that GSI treatment blocks the differentiation of naive CD4+ T cells into Th17 cells. Moreover, treatment with GSI affects Th17 differentiation at earlier time points, but not in cells already committed to the Th17 lineage, suggesting a requirement for Notch signaling at early stages of Th17 differentiation.

Delivery of Notch1 siRNA to human naive CD4+ T cells leads to decreased IL-17 secretion

GSI blocks targets of γ-secretase, including all members of the Notch family of proteins. To determine whether Notch1 is a functional target of GSIs during Th17 polarization, expression of Notch1 was reduced by delivery of siRNA to naive CD4+ T cells. Naive CD4+ T cells were nucleoporated with Notch1-specific siRNA and subsequently polarized to the Th17 lineage and harvested 48 h after transfection. Western blot analysis of Notch1 protein and quantitative RT-PCR confirmed that Notch1 siRNA reduced the expression of Notch1 protein (Fig. 3A) as well as mRNA (Supplemental Fig. 2A). Western blot of Notch2, -3, and -4 was also performed to confirm the specificity of Notch1 siRNA (Supplemental Fig. 1). Notch1 knockdown significantly inhibited IL-17A and IL-17F production under Th17 polarizing conditions (Fig. 3B). Surprisingly, we did not observe a significant reduction in IL-22 production upon Notch1 knockdown (Fig. 3B). An MTS assay was performed to check whether the reduction in IL-17 in Notch1 siRNA-treated cells...
was due to differential cell survival revealed no change between scrambled siRNA and Notch1 siRNA (Supplemental Fig. 2B).

The role of Notch1 in Th17 differentiation was confirmed by overexpressing activated Notch1 (intracellular domain of Notch1 cloned in the LZRS retroviral construct) in naive human CD4+ T cells followed by Th17 polarization. An immunoblot for Notch1 confirmed overexpression (Fig. 3C). Naive CD4+ T cells overexpressing Notch1IC LZRS produced higher levels of IL-17 compared with control cells (Fig. 3D). Interestingly, Notch1 overexpression also increased IL-17 secretion in cells activated under Th0 conditions.

**Notch1 binds to the RORγt promoter**

The orphan nuclear receptor RORγt is a key transcription factor that regulates the differentiation of the Th17 effector cell lineage. Thus, we explored whether Notch may regulate its expression.

RNA was isolated from mouse CD4+ T cells polarized under Th17 conditions. cDNA was then synthesized to perform quantitative RT-PCR. RORγt mRNA expression was reduced by 2-fold in Th17-polarized cells treated with GSI compared with DMSO-treated cells (Fig. 4A). To determine whether Notch1 influences human Th17 polarization by regulating RORγt expression, naive human CD4+ T cells were purified and nucleoporated with Notch1-specific siRNA, followed by culture under Th17 polarizing conditions. Quantitative RT-PCR of RORγt demonstrated that Notch1 knockdown resulted in decreased levels of RORγt transcripts (Fig. 4B). Taken together, these data indicate that Notch1 regulates the expression of RORγt.

We then explored the possibility that Notch1 may directly regulate the human RORγt promoter. Analysis of this promoter

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**FIGURE 2.** GSIs significantly reduce Th17 cytokine levels in human in vitro Th17 polarization assays. A, ELISA of IL-17A, IL-17F, and IL-22 in supernatants of Th17-polarized naive human CD4+ T cells treated with GSIs or DMSO as a vehicle control. Purified human CD4+ T cells were pretreated with GSIs (2 μM IL-CHO and 5 μM compound E) or DMSO as a vehicle control and then cultured in Th0 and Th17 polarizing conditions. Supernatants were collected at 24, 48, and 72 h and were analyzed for IL-17A, IL-17F, and IL-22. B, Whole-cell lysates were prepared from naive CD4+ T cells unstimulated (US) or differentiated under Th0 and Th17 conditions and immunoblotted for active Notch1IC. β-actin was used to confirm equal loading. C, Naive CD4+ T cells were activated in vitro under Th17 polarizing conditions for 4 d, followed by treatment with either DMSO or GSI. Supernatants were collected after 24 h and IL-17A and IL-22 ELISAs were performed. Data shown are representative of three independent experiments done in triplicates. *p ≤ 0.05, **p ≤ 0.001.
revealed two potential CSL sites within the proximal 3-kb promoter upstream of the RORγt transcriptional start site (Fig. 4C). ChIP analysis using an anti-Notch1 Ab was then performed to determine whether Notch1 binds directly to the RORγt promoter. The data presented in Fig. 4D indicate that Notch1 binds directly to putative CSL binding sites in the human RORγt promoter (Fig. 4D). In particular, Notch1 bound at the CSL1 site, which could be inhibited by treatment with GSI.

**Notch1 regulates IL-17 promoter activity**

Because Notch has been reported to regulate and bind directly to the IFN-γ and IL-4 promoters, it is possible that Notch may also regulate the IL-17 promoter in addition to the RORγt promoter. Mouse CD4+ T cells were differentiated in vitro toward the Th17 lineage in the presence of either DMSO or GSI. Transcript levels of IL-17 were reduced by 9-fold in GSI-treated Th17 cells as detected by quantitative real-time PCR (Fig. 4A), suggesting that Notch may directly regulate IL-17 promoter. Furthermore, cotransfection of HEK 293T cells with a human IL-17 promoter luciferase construct in combination with an activated Notch1 expression vector construct (Notch1IC) revealed that Notch1 expression significantly increased IL-17 promoter activity (Fig. 5A). This suggests that Notch1 regulates the IL-17 promoter. The human IL-17 promoter (3 kb) upstream of the transcription start site was therefore analyzed for putative CSL binding sites (Fig. 5B). We found four putative CSL sites within this region (Fig. 5B). ChIP analysis of cells polarized under Th17 conditions showed that Notch1 binds to putative CSL binding sites in the human IL-17 promoter, particularly CSL1 and 4 (Fig. 5C), but not CSL2 and 3 (data not shown). The binding was inhibited by pretreatment with GSI (Fig. 5C). Thus, Notch1 directly binds to both RORγt and IL-17 promoters and regulates Th17 differentiation.

**GSI ameliorates the severity of EAE-induced inflammation and Th17 differentiation in vivo**

In vitro experiments demonstrate that reducing Notch activation causes a significant decrease in IL-17 levels. To investigate whether the in vitro observations seen could be replicated in an in vivo setting, an EAE model was used in this study. SJL/J mice were fed control chow or GSI (LY) chow. LY is an orally active GSI that is chemically similar to compound E. We have previously used LY incorporated into chow in mouse models of immune disorders and determined that doses between 2.5 and 5 mg/kg/day are safe and effective in reducing Notch activity systemically. Higher doses cause the well-known secretory diarrhea due to goblet cell metaplasia of the intestine. We have previously reported that GSI treatment ameliorates EAE progression (28). However, the role of Th17 cells in this model was not understood at the time, and in that report we explored exclusively Th1 responses. To induce EAE, mice were treated with PLP peptide emulsified in CFA and injected with pertussis toxin. The initial signs of EAE were observed 8 d after immunization of the control group and 10 d after immunization for the GSI-treated group. At the peak of disease the clinical mean score for the control group was 2, whereas it was 0.8 for the GSI-treated group (Fig. 6A). Therefore, GSI treatment significantly delayed the disease progression as well as reduced the severity of EAE symptoms, as previously shown (28).

To determine whether Th17 responses were affected by GSI in vivo, IL-17 levels in supernatants of peptide-stimulated splenocytes cultured from GSI- or control-treated mice were measured by ELISA. The GSI-treated group showed significantly lower IL-17 levels than did the control group (Fig. 6B). Similarly, supernatants obtained from mononuclear cells isolated from spinal cords showed lower IL-17 levels in the GSI-treated mice than in the control group (Fig. 6C). We also detected lower levels of IFN-γ in peptide-restimulated splenocytes from GSI-fed mice as compared with control mice (Fig. 6D). To determine whether the effect of GSI-mediated inhibition of IL-17 cytokines in vivo is due to overall decrease in T cells number rather than Th17 cell differentiation, we determined the number of CD4+ and CD8+ T cells in spinal cord infiltrates. We found there were no significant differences in the number of cells infiltrating the spinal cord between...
Additionally, no significant differences in CD4+ and CD8+ cells were observed in the spleens of GSI-fed and control mice (data not shown). Indeed, we have maintained animals on GSI chow for as long as 6 mo and not observed differences in CD4+ or CD8+ cell numbers (data not shown). This suggests that the decrease in IL-17 in the group of mice fed with GSI was not due to a difference in infiltrating cell numbers, but is more likely due to the effects of GSI on Th17 differentiation. Additionally, we performed intracellular staining of IL-17 in CD4+ T cells of splenocytes treated in vivo and observed significant decrease in mean fluorescence intensity of IL-17 in GSI-fed mice as compared with control mice (Fig. 6E). Interestingly, we did not observe a decrease in the percentage of CD4+ T cells producing IL-17, suggesting that inhibiting Notch signaling does not affect the number of CD4+ T cells producing IL-17 but rather their inherent ability to produce Th17-associated cytokines (Supplemental Fig. 3).
Discussion

In this study, we addressed the role of Notch signaling in the development of a Th17 response in human and mouse CD4+ T cells. We employed several strategies to investigate the function of Notch in driving a Th17 response. Our data demonstrate that treatment with GSIs, compounds known to block γ-secretase function, also decreases Th17 differentiation and Th17-associated cytokine secretion. Additionally, we have shown that specific inhibition of Notch1 expression through the use of Notch1 siRNA abrogates IL-17A and IL-17F production in polarized human Th17 cells. Surprisingly, we did not observe a significant decrease in IL-22 cytokine levels upon Notch1 knockdown. Comparing our GSI data and specific Notch1 siRNA data, it may be possible that IL-22 is regulated by other downstream targets of γ-secretase. Alternatively, IL-22 may be regulated by other Notch family members, particularly Notch2 as reported before (45).

We also provide further insights into the role of Notch in Th17 induction by demonstrating that blockade of Notch, either through inhibition of γ-secretase or through siRNA-mediated knockdown, results in reduced expression of RORγt, the transcription factor known to be required for effective induction of Th17 cells. These data, coupled with experiments showing Notch1 binding to both the RORγt and the IL-17 promoters, suggest that Notch1 directly regulates the development of the Th17 subset of cells, at least in part through the regulation of these two promoters. The biological consequences of Notch1 effects on Th17 development are highlighted in our in vivo EAE experiments where GSI-mediated blockade of Notch activation results in reduced clinical disease as well as reduced levels of IL-17 produced by restimulated CD4+ T cells isolated from EAE-induced animals treated with GSI. Taken together, these data provide compelling evidence for a key role for Notch signaling in the development of an effective in vitro and in vivo Th17 response. We have also provided evidence that Notch signaling plays a role in early stages of Th17 differentiation, as blocking Notch after 4 d activation has no significant effect on Th17-associated cytokines in both mouse and human cells.

Notch1 has been implicated in the induction of both the Th1 and Th2 subsets of CD4+ T cells (28, 30, 31). Amsen et al. (23) suggested that different Notch ligands expressed on APCs drive differing T cell responses. In particular, this group provided evidence that Delta-like ligands preferentially drive a Th1 cell fate whereas Jagged ligands drive a Th2 fate. Lukacs and colleagues (10) recently revisited and expanded this observation and determined that Delta-like 4 expression is induced on APCs by pathogen-associated signals and that this ligand promotes expression of RORγt and expansion of Th17 CD4+ T cells. The role of Notch signaling in mutually exclusive Th1, Th2, and Th17 differentiation may be mediated by different Notch ligands. Alternatively, it may be due to upregulation of different Notch family members or by differential expression of the same Notch paralog (with Notch1 being the most likely candidate). Notch signaling has been studied extensively but most experimental systems interrogate the conventional Notch signaling pathway, where activation of Notch leads to the production of intracellular Notch, which translocates to the nucleus and drives CSL-dependent transcription. More recent data indicate that activation of Notch also influences NF-κB signaling (46, 47), suggesting cross-talk between these two signaling pathways in T cells. Additionally,
evidence from several groups in a variety of vertebrate and invertebrate systems reveals a role for Notch in the cytosol and point toward a non-nuclear role for Notch in activation of cell survival pathways (48, 49). Therefore, it is possible that different ligands activate different Notch signaling pathways, which, in turn, drive different outcomes that influence T helper differentiation and development. For example, the number of intracellular Notch molecules generated after activation and/or the duration of activation may dictate whether the canonical pathway or combinations of nuclear and cytoplasmic pathways are activated. Further experimentation is required to test this hypothesis.

In summary, in this report we describe a role for Notch signaling in the development of both human and murine Th17 responses. A broad range of diseases require an active Th17 response, from multiple sclerosis to solid tumors. Our data suggest that Notch signaling inhibitors may act in vivo at least by suppressing the Th17 response and may be useful in a variety of clinical situations where Th17 responses are required for disease pathogenesis.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1
Supplementary Figure 2

**A**

Notch1 (Relative transcript levels)

- siC
- siN1

**B**

Absorbance (490nm)

- siC
- siN1

* Indicates a significant difference.
Supplementary Figure.3

Unimmunized Control GSI

IL-17+ CD4+ T cells (%)

0 50 0 50 0 50

Unimmunized Control GSI
Supplementary Data Figure legends:

**Supplementary Fig.1:** Western blot of Notch1IC, Notch2IC, Notch3IC and Notch4IC after nucleoporation of Notch1 siRNA in naïve CD4+ T cells followed by Th17 differentiation. 293T cells transfected with Notch1IC, Notch2IC, Notch3IC and Notch4IC constructs were used as a positive control.

**Supplementary Fig.2:** (A) Quantitative RT-PCR using Notch1 specific primers to assess Notch1 transcript levels. (B) Cells were assayed for viability after nucleoporation using Cell titer 96 Aqueous One solution cell proliferation assay. Data depicted here is absorbance of formazan product measured at 490nm using ELISA plate reader.

**Supplementary Fig.3:** Intracellular staining of IL-17A *in-vivo* in un-immunized, GSI fed or control chow fed mice. Data depicted here is percentage of IL-17+ cells gated in CD4+ T cells.