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Notch Signaling Regulates Mouse and Human Th17 Differentiation

Shilpa Keerthivasan,* Reem Suleiman,†‡ Rebecca Lawlor,‡ Justine Roderick,† Tonya Bates,† Lisa Minter, † Juan Anguita, † Ignacio Juncadella, † Brian J. Nickoloff, ‡§ I. Caroline Le Poole, ‡§ Lucio Miele, ‡ and Barbara A. Osborne†

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.

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Notch proteins are type 1 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 δβ 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 naïve (23) and activated CD4+ T cells in the Th17 lineage (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.

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*Department of Molecular Biology, Loyola University Medical Center, Maywood, IL 60153; †Department of Veterinary and Animal Sciences, Molecular and Cellular Biology Program, University of Massachusetts, Amherst, MA 01003; ‡Department of Pathology, Oncology Institute, Loyola University Chicago, Maywood, IL 60153; §Department of Microbiology and Immunology, Oncology Institute, Loyola University Chicago, Maywood, IL 60153; and ¶Department of Medicine, Cancer Institute, University of Mississippi Medical Center, Jackson, MS 39216.
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 hairy, 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 promote differentiation of Th2 cells (23, 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 Bio-technology, 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, San Diego, CA) was 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, naïve 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-β (R&D Systems), and 10 μg both 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, naïve 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). Naïve 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). Naïve 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 recombiant cytokines used in this polarization 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, naïve CD4+ T cells were cultured in Th17 polarization conditions for 4 d followed 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 (Mediumtech, 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 (Notch1IC) encoding plasmid construct was generated by cloning Notch1IC cDNA into BamHI and EcoRI sites of pcDNA3.0 (37).

Retroviral expression vector and transduction

The sequence encoding Notch1IC was subcloned into the retroviral vector LZRS and viral particles were produced as described previously (38). For transduction of virus, naïve 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 Notch1IC (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 Gaffin (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-PE, 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 naïve 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 DNase 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 2^-ΔΔCt method (40). The primer sequences used were: Notch1, forward, 5′-GTCAACGCCGTAATGACGAC3′, reverse, 5′-TTGTTTACCCCGGCTTCTAC3′; RORγt, (15), forward, 5′-TATCCAGATGAGATTGC3′, reverse, 5′-CTCTTACACTCTGATCTA3′; 18SrRNA, forward, 5′-GGCCGCCCGCTCGATCCTG3′, reverse, 5′-GCTCGCCGCCCGTTTGAACAC3′. Mouse CD4+ T cells cultured as above were harvested and total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX). Total RNA samples were subjected to treatment with DNase using the TURBO DNA-free kit (Ambion), cDNA was synthesized, and transcripts were amplified.

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by quantitative real-time polymerase (Strategene Mx3000P system). Primer sequences were: IL-17, forward, 5′-CGT CCT CCC GGC GCA CCG-3′; reverse, 5′-AGC TTT CCC TTC GGA TTA ACA CAG-3′ (41); RORγt, forward, 5′-TTT GGA ACT GGC TTT CCA TC-3′; reverse, 5′-AAG ATC TGC AGT TTC 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 nucleoporated with small interfering RNA (siRNA) specific for Notch1 or scrambled siRNA (Santa Cruz Biotechnology) using an Amaxa nucleoporator system. Briefly, 5×10^6 naive CD4+ T cells were resuspended in 100 μl Nucleofector solution and transfected with 100 μM siRNA using the U-014 Amaxa 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 1×10^6 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′-CTG ACT CAT AGC ATA GCA GC-3′; reverse, 5′-TTC AGG GGT GAC ACC ATT TT-3′; 17CSL2, forward, 5′-GAA AAT CTC GTG TCT CTT GAA CC-3′; reverse, 5′-TTC CCT ACA GAT GCC TTC TTC CTT CCA CCT AC-3′; reverse, 5′-TTC CCT CCT GTC GTG TCT CC-3′; 17CSL4, forward, 5′-CAGAATTG GAA AAA GAG ACT AT-3′; reverse, 5′-CCCT CAC TGC GCC TCC TTC AC-3′; RORγt primer sets were: RCBF1, forward, 5′-ATC TCC AGT GCC AGC TTC GA-3′; reverse, 5′-GAT GCC CCT GTT TTT AGG AGC-3′; RCBF2, forward, 5′-AGA GGG ACT TCC GCT TTC TC-3′; reverse, 5′-TCA AAG 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 rabbit anti-Notch1 or normal rabbit IgG (Santa Cruz Biotechnology).

**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 aldehyd inhibitor of γ-secretase activity that is thought to modify the active sites, whereas compound E is a nonpeptide, nontransient state, noncompetitive inhibitor of γ-secretase. Naïve 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 significantly reduced in comparison with DMSO-treated Th17-polarized cells (Fig. 1A). Similarly, a reduction in IL-17F and IL-21 cytokine 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-differentiated 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). Additionally, the effect of Notch inhibition on already differentiated Th17 cells was assessed. Naïve CD4+ T cells were cultured in Th17 polarizing conditions for 4 d followed by treatment with either 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). Naïve CD4+ T cells (CD4+CD45RA-+) were purified from PBMCs, pretreated 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-differentiated 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 activated or not activated 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 differentiated Th17 cells. Naïve 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 reduced viability.
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
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, co-transfection 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).

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FIGURE 4. Notch1 regulates RORγt promoter activity. A, In vitro IL-CHO treatment downregulates RORγt and IL-17 mRNA expression. Total RNA was isolated from CD4+ T cells pretreated with 25 μM IL-CHO or DMSO as a vehicle control and cultured in Th17 polarizing conditions and analyzed by quantitative real-time PCR. B, Human naive CD4+ T cells (1 × 10⁶) were nucleoporated with Notch1-specific siRNA or scrambled siRNA followed by in vitro Th17 polarization. Cells were harvested and RORγt expression was determined by quantitative RT-PCR. Transcript abundance was normalized to 18S rRNA expression. C, Schematic representation of putative CSL binding sites in human RORγt promoter. D, Specific primers were used to amplify putative CSL binding sites on human RORγt promoter. A ChIP assay was performed to determine recruitment of Notch1 on human RORγt promoter. Data shown represent fold recruitment of Notch1 on RORγt promoter with respect to isotype control IgG normalized to input DNA. Semiquantitative PCR was also performed (2 μl DNA eluates) using specific primers against different putative CSL binding sites in human IL-17 promoter to confirm transcript size. Data shown represent the mean ± SD of three independent experiments done in triplicates. *p ≤ 0.05, **p ≤ 0.01. US, unstimulated.

FIGURE 5. Notch1 regulates human IL-17 promoter activity. A, HEK 293T cells were cotransfected with intracellular activated Notch expression vector construct (Notch1IC) along with a human IL-17 promoter construct cloned upstream of firefly luciferase gene. A luciferase assay was performed and data were normalized to Renilla luciferase depicted as relative luciferase units (RLU). B, Schematic representation of putative CSL binding sites in human IL-17 promoter. C, A ChIP assay was performed to determine the recruitment of Notch1 on human IL-17 promoter. Data shown represents fold recruitment of Notch1 on human IL-17 promoter with respect to isotype control IgG normalized to input DNA. Semiquantitative PCR was also performed (2 μl DNA eluates) using specific primers against different putative CSL binding sites in human IL-17 promoter to confirm transcript size. Data shown represent the mean ± SD of three independent experiments done in triplicates. *p ≤ 0.05. US, unstimulated.
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 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.

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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).

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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

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References


