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Notch3 Inhibition in Myelin- Reactive T Cells Down-Regulates Protein Kinase Cθ and Attenuates Experimental Autoimmune Encephalomyelitis

Maciej Jurynczyk,* Anna Jurewicz,* Cedric S. Raine,* and Krzysztof Selmaj*‡

Among its varied functions, Notch signaling is involved in peripheral T cell responses. The activation and polarization of CD4+ T cells toward a Th1 lineage are essential steps in the pathogenesis of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis. Inhibition of all four Notch receptors with a γ-secretase inhibitor was shown to block Th1-type polarization and to attenuate the symptoms of experimental autoimmune encephalomyelitis. In this study, we have examined the role of individual Notch receptors in proliferation, cytokine production, and encephalitogenic potential of PLP-reactive T cells. Specific induction of Notch1 and Notch3 transcripts were noted in PLP-reactive T cells upon Ag stimulation. However, using γ-secretase inhibitor and Abs blocking distinct Notch receptors, we have found that selective inhibition of Notch3, but not Notch1, receptor abrogated proliferation, Th1- and Th17-type responses of PLP-reactive T cells. Moreover, Notch3 inhibition in T cells correlated with the down-regulated expression of protein kinase Cθ, a kinase with important regulatory function within mature T cells. Thus, selective inhibition of the Notch3 receptor may have important effects on peripheral T cell responses and may offer a new attractive target in treating autoimmune diseases, including multiple sclerosis. The Journal of Immunology, 2008, 180: 2634–2640.

M ultiple sclerosis (MS) is a chronic demyelinating disease of the human CNS. Histologically, MS is characterized by CNS lesions displaying inflammation, demyelination, and axonal damage (1). Although its etiology is not known, MS is generally considered to be an autoimmune Th1-mediated disease (2). The morphology of the acute lesion and the susceptibility to MS apparently conferred by certain HLA Ags suggest that autoreactive CD4+ T cells have a crucial role in the disease process. Data supporting this concept also come from studies on experimental autoimmune encephalomyelitis (EAE), an animal model with many similarities to MS. Induced by the s.c. injection of myelin Ags, EAE may be transferred to naïve recipients by CD4+ T cells, but not by autoantibodies. Thus, the activation and polarization of CD4+ T cells toward a Th1 lineage are essential steps in the pathogenesis of MS and EAE.

Notch is a family of four heterodimeric transmembrane receptors (Notch1–4) that regulates cell fate decisions during development of many organs (3). Notch signaling is initiated by the interaction between the receptor and a ligand from the Jagged or Delta family. Upon ligand binding, two cleavages mediated successively by ADAM 17 and γ-secretase, release the intracellular form of Notch (ICN). ICN migrates to the nucleus and activates transcription of target genes (3). Inhibitors of γ-secretase (GSI) block signaling from Notch receptors.

Among its varied properties, Notch is involved in peripheral T cell responses. Naïve CD4+ T cells express Notch1 and Notch2 (4), while activated CD4+ T cells show high expression of all four Notch receptors (5). The effect of Notch signaling on T cell activation, proliferation, and polarization remains unclear. Under different experimental conditions, Notch signaling may have an inhibitory or stimulatory effect on IL-2 production and T cell proliferation (5–7). T cell polarization and the profile of cytokine production may depend on the ligand interacting with the Notch receptor (4, 8–11). It has been shown that Jagged1 promotes IL-4 expression and stimulates Th2-type responses (4), while Delta1 and Delta4 induce differentiation along a Th1-type pathway (9, 11). The up-regulation of expression of all four Notch receptors after T cell activation via TCR suggests that all Notch receptors may play a role in peripheral CD4+ T cell responses. Although the pathway seems to be common for all four Notch receptors, activation of individual Notch receptors may lead to different cell fate decisions. For example, ICN1 was shown to activate a Th2 differentiation program (4), while ICN3 promoted Th1 development (9).

In this study, we have examined the role of distinct Notch receptors in the activation and polarization of myelin-reactive CD4+ T cells and have observed that γ-secretase inhibition of Notch signaling decreased proliferation of autoreactive T cells in response to myelin Ag, reduced IFN-γ production, and attenuated a model of adoptively transferred EAE. By blocking individual Notch receptors, we also found that selective inhibition of Notch3 was more effective in abrogating Th1-type responses than was the inhibition of γ-secretase. This effect correlated with a decrease in protein kinase C isoform, PKCθ, activity in anti-Notch3-treated CD4+ T cells.
Materials and Methods

Subcutaneous immunization with a myelin Ag

Six- to 8-week-old female SJL/J mice were used. Animals were housed and maintained in an accredited facility, the Animal Care Department of the Medical University of Lodz. Animals were injected s.c. with PLP190-195 in CFA (Difco). On day 0, each mouse received 0.25 ml of mixture of 0.15 mg of PLP190-195 dissolved in 0.1 ml of PBS and 0.75 mg of Mycobacterium tuberculosis, in 0.15 ml of CFA, injected s.c. in four abdominal sites. On day 10, mice were sampled and lymph nodes isolated. Procedures for all animal experiments were approved by the University Ethics Committee.

Lymph node cell (LNC) culture

LNC derived from PLP-immunized SJL/J mice were cultured in T cell culture medium (RPMI 1640, medium containing 10% FCS, 100 μ/ml penicillin/streptomycin mixture, 2 mM glutamate, and 0.05 mM n-2-ME), in the presence of γ-secretase inhibitor IX (Sigma-Aldrich), at a concentration of 15 μM, or neutralizing anti-Notch1 or anti-Notch3 Ab (R&D Systems) at a concentration of 10 μg/ml, or vehicle. Cells were plated at a density of 4 × 10⁶/ml in 24-well plates at 37°C in 5% CO₂. After 30 min of incubation, cells were stimulated with PLP190-195 at a concentration of 50 μg/ml.

CD4⁺ T cell isolation

Mouse CD4⁺ T cells were isolated from LNC by depletion of non-CD4⁺ T cells using a CD4⁺ T cell isolation kit (Miltenyi Biotec). This system of isolation is based on the magnetic labeling of non-CD4⁺ T cells which are depleted by being retained on a column. LS columns and a MidiMACS Separator (both from Miltenyi Biotec) were used. The experiment was conducted per the manufacturer’s instructions. The purity of each CD4⁺ T cell population was assessed on FACSCalibur (BD Biosciences) with the use of anti-CD4 and anti-TCRβ Fluorescent Abs (BD Pharmingen) and was at 94%.

RNA isolation and real-time PCR

Notch1, Notch2, Notch3, and Notch4 mRNA expression was assessed in CD4⁺ T cells from mice immunized with PLP190-195. CD4⁺ T cells were isolated either directly after lymph node removal from PLP190-195-immunized mice or after 72 h PLP stimulation of LNC in culture. Cells were lysed in RLT buffer (Qiagen). Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from 2 μg of total RNA using a SuperScript II RNase H-Reverse Transcriptase, oligo(dt)12-18 primers, and 10 mM dNTP Mix (Invitrogen Life Technologies). For reverse transcription, a thermal program of 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min was used. The cDNA served as a template for real-time PCR. Primer sequences were as follows: Notch1 forward primer, 5'-TCC AGA GTG CCA CCT AGT T-3'; Notch1 reverse primer, 5'-TCC ACC GGC TCA TCC TTC AC-3'; Notch2 forward primer, 5'-ACC CTC CGC GGA GAC TCT-3'; Notch2 reverse primer, 5'-TCC CAG AAC CAA TCA GGT TAG C-3'; Notch3 forward primer, 5'-CAG GCG AAA GCG AGA ACA C-3'; Notch3 reverse primer, 5'-GCG GAC GTT GCT CAT TCC AA-3'; Notch4 forward primer, 5'-TGT CCT CTC CCC CAT AGA GTA TGC A-3'; Notch4 reverse primer, 5'-CTC GAA ATC AAC TTT GTC CTC TTG-3'; β-actin forward primer, 5'-GAA GTC CCT CAC CCT CCC AAA-3'; and β-actin reverse primer, 5'-GGC ATG GAC GGC ACC A-3' and have been used previously (the sequences for Notch1, 2, and 4 came from Ref. 12, Notch3 from Ref. 5, and β-actin from Ref. 4). Each reaction contained cDNA, 100 nM of each primer, and SYBR Green PCR Master Mix (Applied Biosystems). Samples were run on an Applied Biosystems 7500 sequence detection system.

Induction of passive EAE

After 3 days of culture, transfer populations were suspended in sterile PBS and injected into the tail vein of naive recipients at 2 × 10⁶ cells/mouse. Mice were observed for 35 days after cell transfer and scored daily in a blinded fashion on a 5-point clinical scale: 1 = limp tail, 2 = hind limb paresis, 3 = forelimb paresis and hind limb plegia, 4 = tetraplegia, and 5 = moribund/death.

Histopathology

Ten days after LNC transfer, representative animals from all examined groups were perfused with 20 ml of cold-buffered 2.5% glutaraldehyde. Brain and spinal cords were dissected from each animal and 1-mm slices were made from 10 levels of the neuraxis (optic nerve, cerebral hemispheres, cerebellum/brainstem, spinal cord at C7, Th3, L2, L5, L6, and S1, and spinal nerve roots), postfixed in cold 1% osmium tetroxide for 1 h, dehydrated through a graded series of ethanol, cleared in propylene oxide, and embedded in epoxy resin (Epon 812; Electron Microscopy Sciences). One-micrometer sections of epoxy-embedded tissue were stained with toluidine blue and examined by light microscopy. A score for inflammation and demyelination ranging from 0 to 4 was determined according to established criteria (13), and remyelination and Wallerian degeneration of axons were similarly assessed.

1HJThymidine incorporation assay and CFSE proliferation assay

LNC from mice preimmunized with PLP190-195 were cultured in triplicate with 100 μg/ml PLP190-195 and GSI, anti-Notch1 Ab, anti-Notch3 Ab, or vehicle for 96 h in 200-μl flat-bottom microtiter well plates. For the final 24 h, 1 μCi/well (1HJthymidine (Amersham Biosciences) was added, and uptake was measured using a liquid scintillation beta counter (Pharmacia). Results were expressed as cpm. For CFSE analysis, LNC were labeled with 0.5 μM/cell CFSE and cultured under similar conditions in 24-well plates in the presence of anti-CD3 and anti-CD28 Abs (BD Pharmingen). Fluorescence was measured with a FACS-Calibur (BD Biosciences) and was analyzed with CellQuest software (BD Biosciences). In this method, fluorescence intensity reflects cell proliferation as it halves with each cell division.

Cytokine production assessment

Supernatant fluids from LNC cultures were collected after 72 h of incubation. The concentration of IFN-γ, IL-17, IL-4, and IL-5 was assessed by ELISA (R&D Systems) according to the manufacturer’s instructions. Results were reported as the mean obtained from three experiments ± SD.

Flow cytometry analysis of regulatory T cells

After 72 h of culture, LNC were prepared and stained per the manufacturer’s protocol (Mouse Regulatory T Cell Staining Kit no.3; eBioscience) and analyzed on a FACS Calibur (BD Biosciences) using CellQuest software (BD Biosciences). Cells were stained with FITC-conjugated mAbs against mouse CD4 and PE-conjugated mAbs against CD25 (eBioscience). For intracellular staining of FoxP3, cells were treated according to the manufacturer’s instructions (eBioscience) and stained with PE-Cy5-conjugated anti-mouse FoxP3. PE-Cy5-conjugated rat IgG2a was used as an isotype control.

Western blotting

For Notch1 and Notch3 immunoblotting, spleen cells from naive SJL/J mice were incubated with GSI, blocking Notch1 or Notch3 Abs, or vehicle, and stimulated with anti-CD3 and anti-CD28 Abs (BD Pharmingen) for 16 h. For PKCθ blotting, LNC from immunized SJL/J mice were cultured in the same conditions as transfer populations. Spleen cells or LNC were collected, counted, and placed in the lysis buffer (TBS, 1% Triton X-100, 1 mg/ml BSA, 0.2 U/ml aprotinin, and 1 mM PMSF) at a concentration of 2 × 10⁶ LNC/75 μl. Lysates were sonicated, centrifuged (14,000 rpm, 30 s, 4°C), and dissolved in SDS sample buffer (0.1 Tris-HCl, 4% SDS, 20% glycerol, 0.05% bromophenol blue, and 5% 2-ME). Samples were electroblotted on 8% SDS-PAGE (25 mA, 45 min. at room temperature) and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline (0.01 M Tris, pH 7.4, 0.15 M NaCl, 0.1% Tween 20) overnight, probed with primary Ab, washed, and probed with HRP-conjugated secondary Ab. Blots were developed using ECL Plus (Amersham Pharmacia) per the manufacturer’s protocol. Abs included: anti-Notch1 (M-20), anti-Notch3 (M-20), PKCθ (C-18), and anti-goat HRP Ab (all from Santa Cruz Biotechnology).

Statistical analysis

Data were expressed as means and SD. Statistical analysis was performed using the Student t test and the Mann-Whitney U test.

Results

Notch1 and Notch3 mRNA expression increases after specific Ag stimulation of myelin-reactive CD4⁺ T cells

We first asked which Notch receptor played the most important role in peripheral T cell responses of myelin-reactive CD4⁺ T cells. To investigate this, we compared the expression level of mRNA for all four Notch receptors in CD4⁺ T cells before and after stimulation in vitro with a myelin Ag. SJL/J mice were
immunized s.c. with PLP_{139–151} (proteolipid protein, peptide 139–151), and on day 10, draining lymph nodes were removed, and LNC were isolated. Using a magnetic labeling system, CD4^+ T cells were sorted from LNC either directly after isolation or after a 72-h culture in the presence of myelin Ag. The mRNA level of all four Notch receptors was measured by real-time PCR (Fig. 1A). Autoreactive CD4^+ T cells stimulated ex vivo with a specific Ag strongly up-regulated mRNA for Notch1 (7.6-fold) and Notch3 (9.2-fold). The level of Notch2 did not change and the Notch4 mRNA level was not increased. These results suggested that all Notch receptors, Notch1 and Notch3 played the most significant role in myelin-reactive T cell activation. The specificity of Notch1 and Notch3 induction in Ag-specific T cells was examined in vitro using a GSI, as well as neutralizing Abs to Notch1 and Notch3. After 16 h of culture, the GSI at a concentration of 15 μM down-regulated both the Notch1 and Notch3 intracellular forms (~120 kDa) in CD4^+ T cells stimulated with PLP_{139–151}. Notch1-blocking Abs selectively inhibited Notch1 receptor and Notch3-blocking Abs (both at a concentration of 10 μg/ml) selectively inhibited Notch3, respectively.

**Inhibition of signal transmitted through Notch receptors with GSI decreases severity of EAE**

The ability of Notch receptors to influence the encephalitogenic potential of autoreactive T cells was examined in an adoptive transfer model of EAE. Using a GSI, we blocked signaling from Notch receptors in LNC from SJL/J mice immunized with PLP_{139–151}. Isolated LNC were restimulated in vitro with PLP_{139–151} in the presence of 15 μM GSI, or vehicle, and then adoptively transferred into SJL/J naive recipients. Clinical signs of EAE appeared 8–12 days after transfer of LNC treated with vehicle (Fig. 2). Severity of disease was higher in vehicle-treated mice than in mice receiving GSI-treated LNC. Differences between both groups reached statistical significance 10 days postinduction of EAE (vehicle score 2.8 ± 0.6 vs GSI score 1.3 ± 0.8; p = 0.0089) and remained significant until the end of the observation period. Thus, Notch inhibition in LNC preparations substantially reduced the severity of adoptively transferred EAE.

**Inhibition of Notch3, but not Notch1, ameliorates the clinical course of passive EAE**

Whether inhibition of distinct Notch receptors may affect the clinical course of passively transferred EAE was tested using PLP_{139–151}-stimulated LNC from mice preimmunized with the myelin Ag which were incubated with anti-Notch1- or anti-Notch3-neutralizing Abs at a concentration of 10 μg/ml each for 72 h. EAE induced by LNC treated with anti-Notch3 Abs had a significantly milder clinical course than that of control EAE (statistically significant from day 10 after cell transfer, Notch3 score 0.6 ± 0.5 vs vehicle score 2.8 ± 0.6; p = 0.0018). EAE induced by LNC with inhibited Notch3 was also less severe than EAE induced with GSI-treated cells (day 11, Notch3 score 0.8 ± 0.2 vs GSI score 1.9 ± 0.2; p = 0.0038; Fig. 2). Differences were statistically significant until the end of the period of observation. EAE induced by cells treated with anti-Notch1 Abs, although more severe at several time points during the disease, was not significantly different from that of control EAE mice (Fig. 2). Thus, selective inhibition of Notch3 was more effective in the abrogation of the encephalitogenic potential of myelin-reactive LNC than was inhibition of all other Notch receptors.

**Mice with EAE induced by cells treated with GSI or anti-Notch3 Abs display milder pathology of the CNS**

Histopathology of CNS tissue from all groups of animals was conducted under code by light microscopy. Animals were sampled 15 days posttransfer. The results showed that the animals fell into two groups, one with more intense inflammation and destructive pathology and one with less inflammation and no demyelination (Table I). After breaking the code, we found that the first group represented EAE induced by either control LNC or LNC treated with Notch1-neutralizing Abs. EAE in the second group was found to be transferred by LNC treated with GSI or anti-Notch3 Abs. Control and anti-Notch1-treated cells displayed inflammation, appreciable nerve fiber damage (Wallerian degeneration), evidence of
demelination, and no remyelination. EAE induced by LNC with Notch inhibited by γ-secretase or anti-Notch3 Ab was characterized by milder pathology with less inflammation, no traits of demelination, and only isolated fibers undergoing Wallerian degeneration. In the Notch3 group, we also observed small areas of remyelinated axons (Table I). Differences in pathology were in accord with the clinical course of adoptively transferred EAE induced by myelin-reactive T cells pretreated with distinct anti-Notch Abs.

**γ- and Notch3-blocking Abs decrease the proliferation of myelin-reactive LNC**

To explain the effect of Notch inhibition on the encephalitogenic potential of autoreactive T cells, we measured the proliferation of myelin-reactive LNC, incubated with GSI or blocking Abs, in response to PLP<sub>139–151</sub>. GSI-treated LNC showed reduced specific proliferation to PLP<sub>139–151</sub>. Similar results were obtained when LNC were incubated with anti-Notch3 Abs. In contrast, Notch1 inhibition did not affect proliferation of myelin-specific T cells (Fig. 3, A and B). These results in vitro correlated well with the effect in vivo of Notch3 inhibition on EAE protection.

**Notch3-blocking Abs attenuate Th1- and Th17-type responses**

To examine the effect of Notch inhibition on the polarization of autoreactive T cells, we assessed in PLP<sub>139–151</sub>-specific T cells the production of a Th1-type cytokine, IFN-γ, Th2-type cytokines IL-4 and IL-5, and a Th17-type cytokine, IL-17. We found that both GSI and anti-Notch3 Abs reduced the release of IFN-γ and IL-17, while Notch1-neutralizing Abs strongly promoted IL-17 production (Fig. 3C). Notch inhibition did not affect IL-5 production (Fig. 3C) and the level of IL-4 in supernatants from PLP<sub>139–151</sub>-stimulated LNC was below the level detection (<10 pg/ml). These results suggest that anti-Notch3 treatment reduces the encephalitogenicity of T cells by reducing Th1- and Th17-type responses. They also confirm that Notch1 and Notch3 exert distinct functions in peripheral T cells.

### Table I. Histopathology of adoptive EAE groups

<table>
<thead>
<tr>
<th>Adoptively Transferred EAE</th>
<th>Inflammation</th>
<th>Demyelination</th>
<th>Wallerian Degeneration</th>
<th>Remyelination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control LNC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Anti-Notch1 Ab</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>GSI</td>
<td>±</td>
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<td>–</td>
</tr>
<tr>
<td>Anti-Notch3 Ab</td>
<td>±</td>
<td>–</td>
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*Scored on a 0–3+ scale for inflammation, demyelination, Wallerian degeneration, and remyelination from 6 to 10 blocks from four levels of spinal cord (L2,5,6, and S1) from each animal. Note the more severe pathology in the EAE induced by control and anti-Notch1-treated cells vs EAE transferred by GSI and anti-Notch3-treated cells.

**FIGURE 3.** γ-Secretase inhibition and selective Notch3 inhibition reduced proliferation of myelin-reactive T cells and abrogated Th1 and Th17 responses. A, Using [³H]thymidine, we assessed proliferation of LNC isolated from mice 10 days after PLP<sub>139–151</sub> immunization and cultured for 96 h with anti-Notch agents. Proliferation of cells stimulated with PLP<sub>139–151</sub> was 20% lower in GSI-treated cells and 16% lower in cells treated with Notch3-blocking Abs than in control LNC. Anti-Notch1 treatment did not affect T cell proliferation. Bars represent mean cpm ± SD from six experiments. B, To confirm these results, cells treated in similar conditions were stained with CFSE. In comparison to control cells (background), anti-Notch1 treatment did not alter T cell proliferation, while anti-Notch3 treatment reduced it. C, IFN-γ, IL-17, and IL-5 production in LNC treated with anti-Notch agents measured by ELISA. GSI and anti-Notch3 Abs diminished IFN-γ and IL-17 production. Anti-Notch1 Abs did not affect IFN-γ, but strongly stimulated IL-17 secretion. IL-5 production did not depend on Notch signaling inhibition. Bars represent mean ± SD from five experiments.

same at 72 h (Fig. 4). Thus, Notch inhibition of LNC did not affect the population of regulatory T cells.

**Notch3 inhibition down-regulates PKCθ in PLP-activated T cells**

Notch may affect lymphocyte function by interactions with several target genes. Intracellular factors which have been suggested to act downstream of Notch signaling in mature T cells include the AKT/GSK-3β pathway molecules, T-bet, NF-κB, and others (6, 7, 15, 16). One of the molecules proposed to act downstream of Notch3...
signaling in immature lymphocytes is PKC\(\alpha\), a PKC isoform expressed selectively in T cells (17). Being recruited to the immunological synapse after TCR engagement, PKC\(\alpha\) plays an important role in peripheral T cell activation (18). We have investigated the effect of Notch inhibition on the expression of PKC\(\alpha\) in autoreactive CD4\(^+\) T cells. First, we isolated LNC from naive SJL/J mice, sorted out the CD4\(^+\) T cells, and stimulated them via the TCR with anti-CD3 and anti-CD28 Abs. PKC\(\alpha\) was not present in naive CD4\(^+\) T cells but, as expected, it appeared after 72 h of TCR stimulation in vitro (Fig. 5A). We also found high expression of PKC\(\alpha\) in CD4\(^+\) T cells isolated from mice immunized with PLP\(_{139-151}\) and subsequently stimulated in culture with the same Ag for 16 h (Fig. 5B). However, 16 h after peirucination of T cells with anti-Notch3 Ab, PKC\(\alpha\) was no longer detectable in T cells. At the same time, peirucination of T cells with GSI reduced the level of PKC\(\alpha\), while Notch1 inhibition had no effect (Fig. 5B). Thus, Notch3 inhibition resulted in the down-regulation of PKC\(\alpha\) in CD4\(^+\) T cells.

Notch3 inhibition does not affect JNK signaling in myelin-reactive T cells

We next assessed the effect of Notch inhibition on the function of JNK, a stress-activated MAPK. JNK is induced in T cells in response to Ag stimulation (19) and has been shown to be an activation target of PKC\(\alpha\) (20–22). JNK-deficient mice have been shown to exhibit aberrant T cell activation and differentiation (23–25). Recently, Kim et al. (26) reported that the active form of Notch1 negatively regulates the JNK pathway by antagonizing JNK-interacting protein 1-mediated activation of JNK signaling. It has also been shown earlier (23) and again demonstrated in the present experiments that T cells exposed to Ag showed significant JNK induction (Fig. 6A). However, 72 h of inhibition of Notch signaling in vitro with GSI or Notch Abs did not affect the level of JNK activity (Fig. 6B) in PLP-reactive T cells.

Discussion

Notch receptors regulate many cell fate decisions, including T cell activation and differentiation. During Ag presentation, Notch receptors on CD4\(^+\) T cells interact with their ligands, Delta and Jagged, expressed on dendritic cells. Notch signal is thus initiated and through a series of events it determines the peripheral T cell responses. The effect of Notch signaling manipulation on the course of autoimmune inflammatory diseases has been examined. In a recent study, treatment with Jagged1- and Delta1-Fc fusion proteins differentially modulated the course of EAE (27). With \(\gamma\)-secretase inhibitors, it has been shown that blocking the Notch pathway prevents Th1 polarization and IFN-\(\gamma\) secretion (15). Administration of GSI in vivo delayed the onset of EAE and substantially reduced clinical signs (15). We had previously reported that inhibition of \(\gamma\)-secretase within the CNS of EAE mice leads to faster clinical recovery from the disease (28). However, this effect was mainly attributed to enhanced remyelination observed within the CNS of GSI-treated animals. In the present study, we used GSI to block Notch signaling directly in lymph node cells derived from mice immunized with the myelin peptide PLP\(_{139-151}\). We observed that GSI reduced T cell proliferation and diminished IFN-\(\gamma\) production. Moreover, GSI-treated LNC transferred via tail vein injection into naive animals induced less severe EAE than vehicle-treated LNC.

\(\gamma\)-Secretase inhibitors block Notch signaling from all four Notch receptors. However, distinct Notch receptors may have different effects on peripheral T cell responses. In two recent studies, Notch1 and Notch2 were shown to directly regulate GATA3 expression and to promote Th2-type polarization (29, 30). In another study, CD4\(^+\) T cells isolated from transgenic mice expressing a Notch1 antisense construct produced reduced amounts of IFN-\(\gamma\) (15). These cells, expressing 30–40\% less Notch1 protein than normal CD4\(^+\) T cells, were able to adopt a Th2 phenotype. Moreover, naive CD4\(^+\) T cells derived from mice which conditionally
inactivate Notch1 in mature T cells had comparable Th1- and Th2-type immune responses to those of CD4+ cells from control animals (31). Notch3 overexpression by T cells has been shown to increase IFN-γ production and decrease IL-4 secretion (9). In the presence of a Delta1-Fc fusion construct, Notch3 antisense DNA inhibited Th1 development (9). In our study, we began by asking which Notch receptors played the most significant role in the activation and polarization of myelin-specific CD4+ T cells. The level of transcripts encoding all four Notch receptors was compared in CD4+ T cells from PLP-immunized mice before and after a 72-h PLP stimulation in vitro. We found that such stimulation significantly increased Notch1 and Notch3 transcription in CD4+ T cells, which suggested that these two Notch receptors played the most important role in Th1-type responses of myelin-reactive CD4+ T cells. To further characterize their role, we selectively inhibited Notch1 or Notch3 receptor in myelin-reactive LNC in vitro using specific neutralizing Abs. Notch1 inhibition did not affect the proliferation of autoreactive T cells, did not alter IFN-γ production, and did not affect the clinical course of adoptively transferred EAE. However, it stimulated IL-17 production.

In contrast, Notch3 neutralization had effects different from those of Notch1 on the immunological properties of PLP-specific T cells. Anti-Notch3 treatment decreased T cell proliferation and attenuated Th1-type and Th17-type responses. It also significantly diminished the encephalitogenic potential of autoreactive T cells that transferred a milder EAE than did vehicle-treated LNC. Moreover, LNC with blocked Notch3 receptor induced an EAE with a less severe course than GSI-treated LNC and a pathology that was correspondingly less destructive with evidence of myelin repair. These findings suggest that the selective inhibition of Notch3 inhibition may be more effective in abrogating Th1-type responses than the inhibition of all four Notch receptors.

Notch3 has been also implicated in the development of regulatory T cells. For example, mice with transgenic expression of activated Notch3 generate increased numbers of regulatory CD4+CD25+ T cells and fail to develop experimental autoimmune diabetes (14). Therefore, we have asked whether the effect of Notch3 inhibition on EAE, which ameliorated and reduced autoimmune T cell transfer, may have been due to the expansion of regulatory T cells. We assessed expression of CD25 and regulatory T cells before and after Notch3 neutralization and found no differences in the numbers of CD4+CD25+FoxP3+ regulatory T cells. Thus, it seemed that the decrease in encephalitogenic potential of PLP-specific T cells after anti-Notch3 treatment was more related to the inhibition of IFN-γ and IL-17 production than to the generation of regulatory T cells.

Notch signaling has been reported to be implicated in several intracellular events in T cells, including the activation of the NF-κB (7) and PI3K/AKT pathways (6), and the transcription of genes for IL-4 (4) and T-bet (9, 15). When we measured the expression of PKCθ, a downstream target of Notch signaling in immature thymocytes (17) and an important regulator of the mature T cell activation and polarization (32), we observed a decrease in PKCθ protein in CD4+ T cells incubated with GSI or anti-Notch3-blocking Abs. In contrast, there was no change in the PKCθ level in CD4+ T cells inhibited with anti-Notch1. Thus, Notch3 inhibition in T cells and decreased encephalitogenic potential of T cells correlated with diminished activation of PKCθ. Since JNK was shown to be an activation target of PKCθ (20–22), we tested the effect of Notch inhibition on the function of JNK and found no differences. However, the relevance of PKCθ-induced JNK activation remains unclear, as mature T cells from PKCθ knockouts displayed intact JNK function (33). Moreover, PKCθ-deficient mice have been shown to be resistant to EAE and are unable to mount Th1-type responses (34, 35). Thus, we conclude that the reduced encephalitogenic potential and attenuated Th1-type responses of PLP-specific T cells after Notch3 inhibition may result from PKCθ activation. Taken in concert, selective inhibition of Notch3 inhibition may offer a new strategy for the treatment of autoimmune diseases, including multiple sclerosis.

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**Disclosures**

The authors have no financial conflict of interest.

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