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Jagged1 and Delta1 Differentially Regulate the Outcome of Experimental Autoimmune Encephalomyelitis[1]

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Notch signaling plays an important role during T cell development in the thymus and in T cell activation but the role of Notch in autoimmunity is not clear. We investigated the role of Jagged1 and Delta1 in experimental autoimmune encephalomyelitis. During experimental autoimmune encephalomyelitis, Delta1 expression is up-regulated on dendritic cells and B cells after priming while Jagged1 is up-regulated only on dendritic cells. Administration of anti-Jagged1 Ab exacerbated clinical disease while that of anti-Delta1 Ab reduced the severity of the clinical disease. In contrast, administration of Jagged1-Fc protected from disease, that of Delta1-Fc exacerbated disease. Treatment with Jagged1-Fc was associated with increased IL-10-producing Ag-specific cells in the CNS, while anti-Jagged1 decreased the frequency of IL-10-producing cells. Treatment with Delta1-Fc increased Th1 cells in the CNS, while anti-Delta-1 decreased the frequency of Th1 cells. Manipulation of Delta1 or Jagged1 had no effect on the frequency of Th17 cells or FoxP3$^+$ cells. Moreover, Jagged1 may play a role in CNS homeostasis because murine astrocytes specifically express Jagged1 that is up-regulated by TGF-β, whereas IFN-γ, TNF-α, and IL-17 decrease Jagged1 expression. Our study provides novel data about differential roles of Notch ligands in regulating inflammation in the periphery as well as in the CNS. ***The Journal of Immunology,*** **2007, 179: 5990–5998.**

The immune system has developed powerful mechanisms to prevent unnecessary activation of T cells and thus autoimmunity (1), by promoting negative T cell costimulatory pathways (2). Overall, the balance between the positive and negative signaling costimulatory pathways decides the fate of T cells after antigenic encounter (3).

Notch signaling is an evolutionarily conserved pathway controlling diverse aspects of development and tissue homeostasis. Mammals express multiple receptors (Notch1–4) and ligands (Delta-like 1, 3, 4 and Jagged-1, 2) (4). Notch signaling requires cell-cell interactions leading to Notch cleavage via a γ-secretase. The intracellular (IC)[3] fragment of Notch translocates to the nucleus and binds a Cp-binding factor/recombination signal sequence-binding protein (RBP)-Jκ/suppressor of hairless family transcription factor (termed Cp-binding protein in humans, RBP-Jk in mice), converting it from repressor to transcriptional activator through the recruitment of proteins such as Mastermind and CREB-binding protein/p300 (5, 6).

Unlike the extensive studies of Notch pathway in the developmental stage of T cells (7–13), the precise role of Notch pathway in mature T cell physiology remains unclear. In peripheral lymphoid organs, naive CD4$^+$ T cells express Notch1 and Notch2 receptor mRNA (14–16), while they do not express Notch3 and Notch4 mRNA (14). However, mRNA expression of all Notch receptors is increased after Ag stimulation of T cells (15). T cells also express the Notch ligands Jagged1 (16), Jagged2 (17), and Delta1 (18). Dendritic cells (DCs) express the Notch ligands Jagged1 and Jagged2 as well as Delta4 that are induced by LPS treatment (14, 16, 17, 19). Loss or gain of function strategies have been used to study the role of Notch pathway in T cell maturation. Although triggering the Notch pathway in vivo seems to be associated with inhibition of T cell priming and induction of tolerance (20–22), manipulation of Notch signaling in vitro has produced more conflicting results. The group of Yasutomo (23) first demonstrated that stimulation of naive CD4$^+$ T cells with Delta1 promotes the differentiation toward Th1 and that overexpression of Notch3-IC but not Notch1-IC in activated CD4$^+$ T cells also promoted Th1. Subsequently, Amsen et al. (14) reported that Delta1-expressing fibroblasts enhanced IFN-γ production of Ag-stimulated T cells. They also showed that stimulation by Jagged1 increased IL-4 production suggesting that engagement of T cell Notch by Jagged1 directed Th2 cell differentiation (14). In contrast, other investigators suggested that stimulation of human CD4$^+$ T cells by Jagged1 induced Ag-specific regulatory T cells (Tregs) instead of Th2 (16, 24, 25). These conflicting data suggest that the role of Jagged1 in CD4$^+$ T cell differentiation may be different between mice and humans. In vivo, use of a γ-secretase inhibitor, a pharmacological inhibitor of Notch signaling, inhibited only Th1 cell differentiation and suppressed experimental autoimmune encephalomyelitis (EAE), a T cell-mediated disease that is

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[3] Abbreviations used in this paper: IC, intracellular; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; Treg, regulatory T cell; MOG, myelin oligodendrocyte glycoprotein; GFAP, glial fibrillary acidic protein; RT, reverse transcription; MS, multiple sclerosis; LCM, laser capture microdissection; RBP, recombination signal sequence-binding protein; CSL, Cp-binding protein/RBP-Jκ/suppressor of hairless/LAG-1.

used as a model for the study of multiple sclerosis (MS) (26). In another study, mice that lack the downstream of Notch signaling (inactivation of CSL/RBP-J) in $CD4^+$ T cells fail to develop a protective Th2 cell response against the gastrointestinal helminth *Trichuris muris* (27). Thus, it has not yet been resolved whether Notch regulates Th1 cell differentiation, Th2 cell differentiation, or both.

The Notch pathway is involved in myelination in the developing CNS. Re-expression of Notch in the adult CNS has been proposed to hamper remyelination in MS (28). Previous studies also revealed that pharmacological inhibition of Notch signaling ameliorated EAE (29). However, in a toxin-induced demyelination model, constituents of the Notch-signaling pathway were expressed in remyelinating lesions, indicating that remyelination may occur in the presence of Notch signaling (30, 31).

Factors triggering autoimmune diseases are still poorly understood, but an imbalance between Th1 effector T cells and Tregs may contribute to the pathogenesis. In EAE, Ag-specific $CD4^+$ Th1 cells mediate inflammatory damage in the CNS, with consequent demyelination, manifested clinically by progressive paralysis (32). Recently, Th17 (IL-17-producing cells) were shown to contribute to the immunopathogenesis of the disease (33, 34). In this report, we examined the role of the Notch-signaling pathway in regulating EAE, using blocking mAbs against and agonistic fusion proteins of Jagged1 and Delta1. We found that while blockade of both Delta1 and Jagged1 increased peripheral activation of T cells, Jagged1 signaling protected from EAE while Delta1 was deleterious. Jagged1 increased the frequency of IL-10-producing Ag-specific cells while Delta1 increased IFN-γ-producing cells. Jagged1 also plays a role within the CNS, where it is expressed by astrocytes. Th1 and Th17 cytokines down-regulated the astrocyte expression of Jagged1, while TGF-β up-regulated this expression. Our findings suggest that the Notch pathway plays a critical role in the regulation of T cell effector functions in autoimmunity.

Materials and Methods

Mice and EAE induction with myelin oligodendrocyte glycoprotein (MOG)

Female wild-type C57BL/6 mice were purchased from The Jackson Laboratory. MOG-specific TCR-transgenic mice (2D2) were provided by V. Kuchroo (Center for Neurologic Diseases, Harvard Medical School, Boston, MA) (35). Foxp3GFP knockin (*foxp3gfp*.KI) mice were generated as described (34). MOG35–55 peptide (M E V G W Y R S P F S R O V H L Y R N G K) corresponding to the mouse sequence was synthesized by Quality Controlled Biochemicals (division of BioSource International) and purified to >99% by HPLC. Mice were immunized s.c. in the flanks with 100–150 μg of MOG peptide in 0.1 ml of PBS and 0.1 ml of CFA containing 0.4 mg of *Myobacterium tuberculosis* (H37Ra; Difco) and injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories) on the day of immunization and 2 days later. Animals were kept for at least 30 days and EAE was scored as follows: grade 1, limp tail or isolated weakness of gait without limp tail; grade 2, partial hind and front leg paralysis; grade 3, total hind leg; grade 4, total hind leg and partial front leg paralysis; grade 5, moribund or dead animal. Mice were housed in the New Research Building Animal Facility at Harvard Medical School. All animal experiments were done in compliance with the approval of the Harvard Medical Area Standing Committee on Animals.

Abs and reagents

The mouse Jagged1-Fc and Delta1-Fc-fusion proteins were generated as previously described (36). The anti-Jagged1 (HMJI-29, hamster IgG) and anti-Delta1 (HMDI-5 hamster IgG) mAbs were generated against Jagged1-Fc and Delta1-Fc, respectively (details will be described elsewhere). These mAbs and fusion proteins were produced by Bioexpress cell culture and administered i.p. at 200 μg starting from the day of the immunization and every other day until day 10 postimmunization. Delayed therapy consisted of the same dose regimen but was administered starting on day 10 postimmunization. Control hamster IgG were administered to the same protocol. Recombinant mouse IFN-γ, TNF-α, and TGF-β1 were purchased from Roche. Recombinant mouse IL-17 was obtained from R&D Systems. Purified rat anti-mouse IFN-γ- and TNF-α-neutralizing Abs were purchased from BD Pharmingen. *N*-(*N*-(3,5-difluorophenacetyl)-L-alanyl)-*S*-phenylglycine *t*-butyl ester was purchased from EMD Biosciences.

Preparation of CNS mononuclear cells

Mice were perfused through the left cardiac ventricle with cold PBS. The spinal cords were flushed out with PBS by hydrostatic pressure. Spinal cords were dissociated by passing the tissues through a cell strainer (70 μm), incubated in HBSS with HEPES (10 mM) and EDTA (2 mM) at 4°C for 1 h. Cells were washed and resuspended in isotonic 37% Percoll. After centrifugation, the supernatant containing floating myelin was removed and mononuclear cells were collected, washed, and resuspended in culture medium for further analysis.

Proliferation assay

Cells were cultured in RPMI 1640/10% FCS supplemented with 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, and 100 U of penicillin/100 μg of streptomycin/ml. Cells were seeded in triplicate at 2×10^6 cells/ml and 200 μl/well was plated with different concentrations of MOG peptide. After 48 h of culture, 1 μCi [^{3}H]thymidine (NEN Life Science Products) was added in 20 μl of medium to each well for another 16–18 h. Cells were harvested on filters mats, dried, and cpm per well was determined by scintillation counting (PerkinElmer). Data presented as mean cpm in triplicate wells.

IC cytokine staining

Cells were isolated from spinal cords of MOG-immunized mice as described and were stimulated in culture medium with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml), and monensin (GolgiStop 1 μl/ml; BD Biosciences) for 4 h at 37°C, in a humidified 10% CO_2 atmosphere. Cells were washed and stained for surface markers of MOG TCR-specific cells (anti-CD4, Vα3.2, and Vβ11) and in some conditions (CD25, chemokine receptors) by incubating at room temperature for 20 min, cells were fixed and permeabilized using Cytofix/Cytoperm and perm/wash buffer from BD Biosciences according to the manufacturer's instructions. Following permeabilization, the PE-conjugated cytokine Abs were added for 20 min at room temperature, and the cells were washed twice and analyzed by using a FACSCalibur Flow Cytometer (BD Biosciences). All Abs to cytokines (IL-4, IL-10, IL-17, and IFN-γ) including the corresponding isotype controls were obtained from BD Biosciences.

Adoptive transfer of MOG TCR-transgenic T cells

To investigate the effect of Delta1 and Jagged1 blockade on Ag-specific T cell proliferation in vivo, adoptive transfer of MOG-specific 2D2 TCR-transgenic T cells was performed as previously described (37). In brief, splenocytes from 2D2 mice containing 3×10^6 $CD4^+$ T cells were injected i.v. into nonirradiated C57BL/6 mice and immunized with 100 μg of MOG/CFA. mAbs were administered every second day and on day 5 after immunization, mice were killed and the draining lymph nodes were collected. The extent of expansion and activation marker expression on $CD4^+V\alpha3.2^+V\beta11^+$ cells was compared in animals that received anti-Delta1, anti-Jagged1, or control rat IgG. For the cytokine profile studies, isolated lymphocytes from spleens of naive 2D2 mice were incubated in vitro with MOG35–55 peptide (20 μg/ml) for 48 h in the presence of IL-2 (100 U/ml) and IL-7 (5 ng/ml). Cells were activated for two rounds of 48 h followed by a final activation with anti-CD3/CD28 (1 μg/ml) for 24 h in the presence of IL-12 (20 ng/ml), IL-18 (25 ng/ml), and IL-23 (10 ng/ml). A total of $1–2 \times 10^6$ cells were transferred in recipient mice i.p. followed by injection of pertussis toxin (67 ng) the day after.

Confocal analysis

Animals were sacrificed, perfused with 4% paraformaldehyde in PBS, and spinal cord tissues were harvested and snap-frozen in OCT, and stored at −80°C until use. The following primary Abs were purchased from BD Pharmingen: rat anti-CD4 (1:100), biotinylated rat anti-CD4 (1:100), mouse anti-glial fibrillary-associated protein (GFAP, 1:100). The rabbit anti-Jagged1 (5 ng/ml) Ab was purchased from Abcam. Spinal cords were sectioned at 20 μm and incubated with the astrocyte marker mouse anti-GFAP, biotinylated rat anti-CD4, and rabbit anti-Jagged1 overnight at 4°C. Sections were washed and incubated with appropriate fluorochrome-conjugated secondary Abs: Alexa 488-conjugated goat anti-rabbit (1:500), Alexa 594-conjugated streptavidin goat anti-rat (1:500), and Alexa 688-conjugated goat anti-mouse (1:500) were obtained from Molecular Probes and they are highly cross-adsorbed to avoid cross-reactivity. Photos were captured using confocal microscopy (LSM 510 laser scanning microscope).

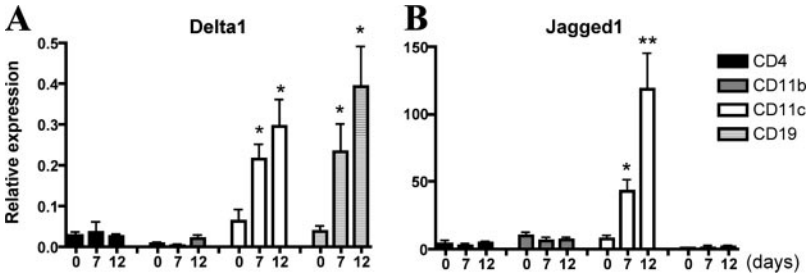

FIGURE 1. Expression profile of Delta1 and Jagged1 on CD4$^+$ T cells and APCs. CD4$^+$ T cells, CD11b$^+$, CD11c$^+$, and CD19$^+$ cells were isolated using FACS sorting from splenocytes and lymph nodes of C57BL/6 naive or mice immunized with MOG35–55 peptide in CFA for 7 and 12 days. Real-time PCR assays for Delta1 (*A*) and Jagged1 (*B*) are shown. cDNA contents were normalized on basis of predetermined levels of GAPDH. *, $p < 0.05$; **, $p < 0.01$ by unpaired *t* test.

Laser capture microdissection (LCM)

Spinal cords from naive and EAE mice were freshly isolated and snap-frozen in OCT and saved at −80°C. Six-micrometer sections of spinal cord tissues were stained with mouse anti-GFAP Ab (1:50; BD Pharmingen) to detect astrocytes and revealed with anti-mouse-HRP method and a diaminobenzidine hydrogen peroxide product as colorimetric substrate. Individual cells were captured with a pulse duration of 2 ms with a pulse power set to 75 mV. At least 50 cells of each group were isolated per spinal cord in duplicate. RNA was extracted using the RNA Absolutely Nanoprep kit according to the manufacturer's protocol (Stratagene).

Primary astrocyte culture

The neonatal mice were anesthetized and the cortex were dissected, cut into small fragments, and digested with 0.25% trypsin-EDTA solution (Invitrogen Life Technologies) in 37°C for 10 min then equal volume of 0.25 mg/ml soybean trypsin inhibitor solution (Invitrogen Life Technologies) were added to stop the reaction. The digested tissues were passed through a fire-polished fine-tip Pasteur pipette for ~20 times to obtain single-cell suspension. The cells were washed with culture medium, and plated into poly-D-lysine-coated T-75 flasks with DMEM supplement with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin (Sigma-Aldrich), and incubated at 37°C with 5% CO_2. At the end of the 10-day culture period, supernatants were removed and replaced with fresh medium containing 20 μM cytosine arabinoside (Sigma-Aldrich) for 72 h followed by fresh medium. The fast growing cells as microglia were eliminated by the treatment. Oligodendrocyte precursor cells and neurons were also gradually eliminated by this culture condition. Purity of the astrocytes was verified by GFAP staining.

Expression analysis by real-time PCR

RNA was purified using the Stratagene RNA kit and transferred directly into the reverse transcription (RT) reagent using the Applied Biosystems TaqMan RT reagents. Samples were subjected to real-time PCR analysis on an Applied Biosystems PRISM 7000 Sequencer Detection System (Applied Biosystems) under standard conditions. Jagged1 and Delta1 were detected using commercially available assays (Applied Biosystems; Mm00496902_m1 and Mm00494477_m1, respectively). Relative mRNA abundance was normalized against GAPDH (Applied Biosystems).

Statistical analysis

The Mann-Whitney *t* test was used for clinical disease analysis. Statistical evaluations of cell proliferation and frequency measurements were performed using the unpaired Student *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

Regulation of Notch ligands expression on CD4$^+$ T cells and APCs during EAE

We examined the expression of Delta1 and Jagged1 on CD4$^+$ T cells and APCs during EAE using quantitative RT-PCR because

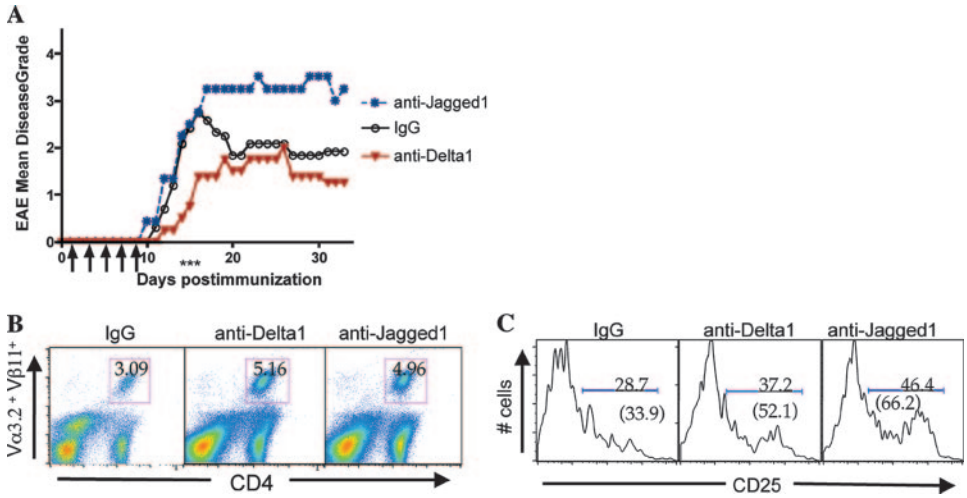

FIGURE 2. EAE outcome in mice treated with anti-Jagged1 or anti-Delta1 blocking Abs. *A*, Disease scores of C57BL/6 mice ($n = 20$ mice/group) immunized with MOG/CFA and that received 200 μg of anti-Jagged1, anti-Delta1, or control IgG every second day between days 0 and 10 postimmunization (arrows). Delta1 blockade significantly decreased the mean maximal score between 10 and 20 days when compared with control IgG-treated mice (mean maximal grade 1.4 ± 0.3 compared with the IgG control group 2.3 ± 0.3; *, $p < 0.05$ by two-tailed Mann-Whitney *U* test) observed on the course of EAE. In contrast, blockade of Jagged1 worsened the disease (mean maximal score 3.3 ± 1 vs 2.3 ± 0.3 in control animals, $p = 0.004$ by Mann-Whitney *U* test). The experiment was repeated three times with similar results. *B*, MOG TCR-specific splenocytes (3×10^6 cells/mouse) were transferred i.p. into C57BL/6 naive recipients ($n = 3$/group) that were immunized with MOG/CFA and treated with 200 μg of anti-Delta1, anti-Jagged1, or control IgG on days 0, 2, and 4 after immunization. Draining lymph nodes were collected 5 days after immunization and the CD4$^+$ transgenic T cells were identified by using anti-Vβ11 and anti-Vα3.2 Abs and quantitated. The data are representative of two similar experiments. *C*, FACS analysis of CD25 expression on transgenic cells on day 5 postimmunization in recipients of anti-Delta1, anti-Jagged1, or control IgG. The numbers in brackets indicate mean fluorescence intensity (MFI).

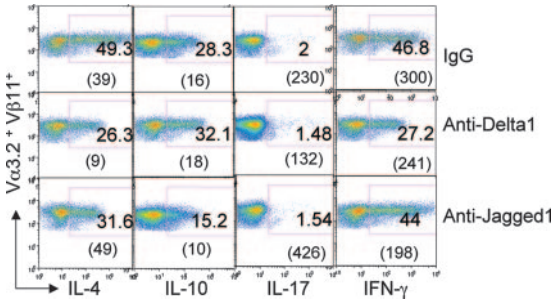

FIGURE 3. In vivo blockade of Jagged1 and Delta1 influences the cytokine profile. Adoptive transfer of MOG TCR-specific cells into C57BL/6 naive mice followed by immunization with MOG/CFA and pertussis toxin injection on days 0 and 2. Mice (n = 3/group) received 200 μg of anti-Delta1, anti-Jagged1, or control IgG every second day until day 10 after transfer. Around the peak of the clinical score (14–16 days posttransfer), spinal cords were flushed out and infiltrated mononuclear cells were isolated by Percoll gradient, activated in vitro with PMA/ionomycin for 4 h at 37°C. Cells were stained for IC cytokines and analyzed by FACS of $CD4^+$ transgenic T cells as identified by using anti-Vβ11 and anti-Vα3.2 Abs. The data are representative of three similar experiments. The numbers in brackets indicate mean fluorescence intensity (MFI).

commercially available Abs could not distinguish the ligands reliably by flow cytometry or Western blots. We used FACS-sorted $CD4^+$ T cells, B cells ($CD19^+$), macrophages ($CD11b^+$), and DCs ($CD11c^+$) from the spleens and lymph nodes of naive, 7 days (preclinical stage), and 12 days (peak of the disease) after immunization. The expression of Delta1 was low on $CD4^+$ T cells and macrophages before and after disease priming (Fig. 1*A*). However, Delta1 expression was enhanced specifically on DCs ($CD11c^+$ cells) and on B cells ($CD19^+$ cells) by 7 and 12 days after immunization (Fig. 1*A*). Interestingly, Jagged1 expression was weak on $CD4^+$ T cells, macrophages, and B cells but was strongly induced on DCs following disease induction (Fig. 1*B*).

Blockade of Delta1 or Jagged1 increases $CD4^+$ T cell activation but differentially regulates EAE

The exact role of the Notch ligands during an autoimmune response has not been described. Using blocking mAbs against Delta1 and Jagged1, we investigated the role of these two ligands in EAE induced by immunization of C57BL/6 mice with MOG35–55. We found a significant decrease in the mean clinical score between days 10 and 20 in mice treated with anti-Delta1 mAb (mean maximal grade 1.4 $\pm$ 0.3 compared with IgG control group 2.3 $\pm$ 0.3, p = 0.038 by two-tailed Mann-Whitney U test). In contrast, blockade of Jagged1 worsened the disease (Fig. 2*A*). Compared with control IgG-treated mice, there was a statistically significant increase in the disease score in the anti-Jagged1-treated mice (mean maximal score 3.3 $\pm$ 1 vs 2.3 $\pm$ 0.3 in control animals, p = 0.004 by Mann-Whitney U test). Overall disease incidence was similar in all groups (100%). Delayed therapy with anti-Jagged1 or anti-Delta1 from days 10 to 20 after immunization had no significant effects on the disease (data not shown).

To study the effect of Jagged1 or Delta1 blockade on the frequency of Ag-specific T cells, we adoptively transferred MOG-specific TCR-transgenic $CD4^+$ T cells (2D2) (35) into C57BL/6 mice that were immunized with MOG/CFA and treated with anti-Delta1, anti-Jagged1, or control IgG on days 0, 2, and 4 after immunization. Draining lymph nodes were collected 5 days after immunization and the 2D2 cells were identified by using anti-Vβ11 and anti-Vα3.2 Abs (Fig. 2*B*). Our data show significantly enhanced expansion of $CD4^+V\alpha3.2^+$ $V\beta11^+$ T cells in mice treated with anti-Delta1 ($28.5 \times 10^6 \pm 6.6$ vs $3.7 \times 10^6 \pm 1.8 \times 10^6$, $p < 0.01$) and anti-Jagged1 ($24.7 \times 10^6 \pm 9.9$ vs $3.7 \times 10^6 \pm 1.8 \times 10^6$, $p < 0.05$) Abs compared with control IgG-treated mice. Moreover, the $CD4^+V\alpha3.2^+V\beta11^+$ T cells showed increased expression of the activation marker CD25 in anti-Delta1 and anti-Jagged1-treated mice compared with control IgG mice, indicating that blockade of either Jagged1 or Delta1 causes an increase in T cell activation (Fig. 2*C*).

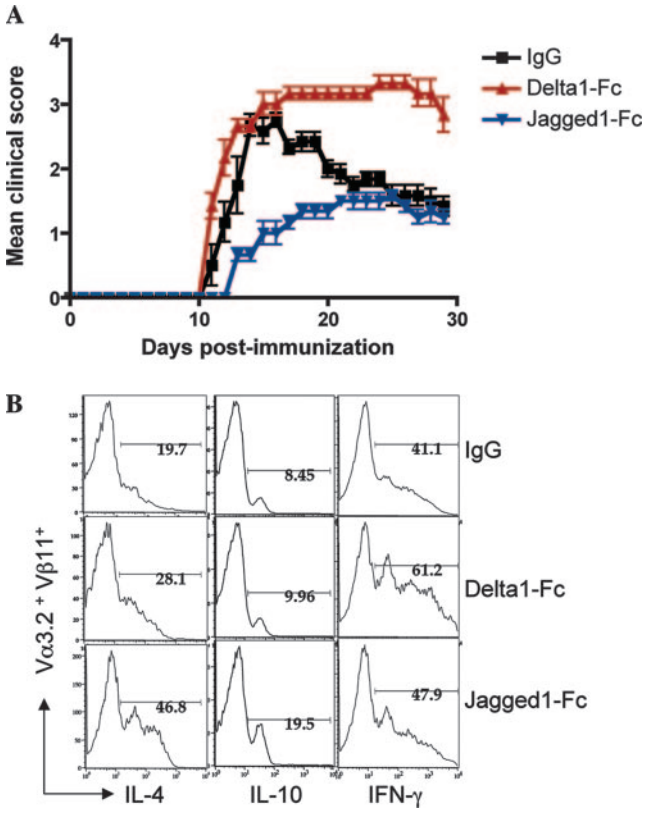

FIGURE 4. Regulation of EAE by Delta1 and Jagged1 ligation. *A*, C57BL/6 mice were immunized with MOG/CFA and given 200 μg of Delta1-Fc, Jagged1-Fc, or rat IgG on days 0, 2, 4, 6, and 8 postimmunization. Animals were followed for 30 days. The data are representative of two similar experiments. *B*, IC staining of adoptively transferred MOG TCR-specific cells in C57BL/6 that were treated with Delta1-Fc or Jagged1-Fc regimen. CNS-infiltrating cells were isolated 12 days after transfer followed by stimulation in vitro with PMA/ionomycin. Cells were stained with the surface molecules, anti-CD4, anti-Vα3.2, and anti-Vβ11 followed by IC staining of IL-4, IL-10, and IFN-γ. Data are representative of three to four mice in each group.

Next, we assessed the effect of blocking Notch signaling in $CD4^+$ T cells in a ligand-independent manner in vitro. $CD4^+$ T cells from C57BL/6 mice were stimulated with anti-CD3 (1 μg/ml) in the presence of syngeneic APCs and exposed to different doses of a γ-secretase inhibitor DAPT that prevents activation of all Notch receptors by inhibiting the final enzymatic cleavage. We show that inhibition of Notch activation with DAPT significantly increased the proliferation of $CD4^+$ T cells (data not shown), thus supporting our findings of Notch ligands blockade in vivo (Fig. 2*B*).

Blockade of Delta1 and Jagged1 has differential effects on T cell maturation during EAE

The role of Jagged1 or Delta1 in T cell differentiation and migration was analyzed in vivo by using 2D2-transgenic T cells as indicator cells. We adoptively transferred 2D2 cells into naive

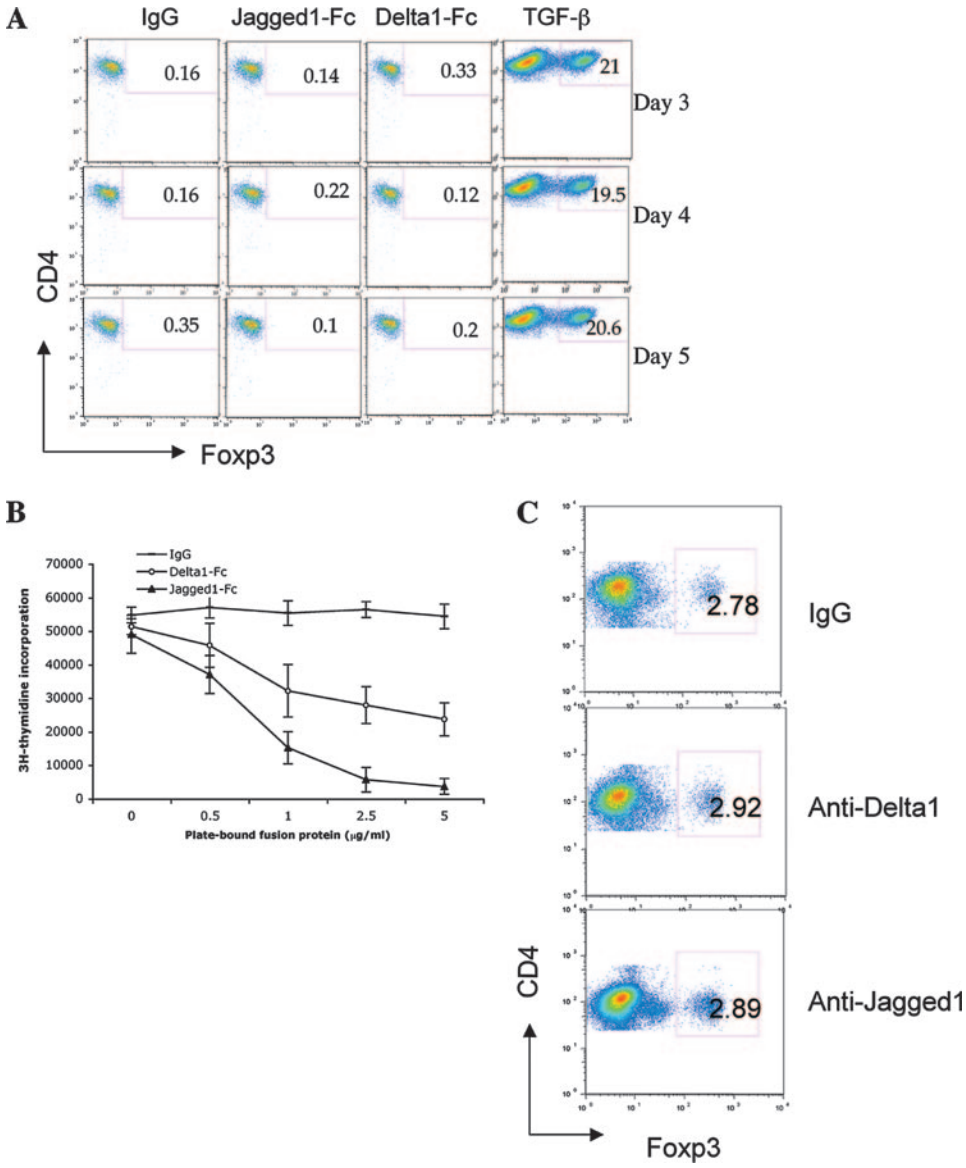

FIGURE 5. Delta1 and Jagged1 do not influence the generation of Foxp3$^+$ T cells. *A*, CD4$^+$/GFP^{neg} cells were FACS sorted from splenocytes of *foxp3gfp*.KI naive mice and stimulated with soluble anti-CD3 (1 μg/ml) and irradiated APCs in the presence of 10 μg/ml plate-bound Delta1-Fc, Jagged1-Fc, or control IgG. The frequency of CD4$^+$Foxp3/GFP$^+$ cells was measured by FACS analysis 3, 4, and 5 days after stimulation. Conversion of CD4$^+$Foxp3^{neg} into CD4$^+$Foxp3$^+$ in the presence of TGF-β was used as a positive control. *B*, The proliferation of CD4$^+$Foxp3$^-$ cells exposed to different doses of Delta1-Fc, Jagged1-Fc fusion proteins or control IgG was measured by [^{3}H]thymidine incorporation. *C*, *Foxp3gfp*.KI mice immunized with MOG/CFA and treated with five injections of anti-Delta1, anti-Jagged1, or control Ig. Cells were isolated from draining lymph nodes on day 12, and the frequency of CD4$^+$Foxp3/GFP$^+$ cells was analyzed by FACS. Data are representative of two similar experiments.

C57BL/6 mice that were immunized and treated with anti-Delta1, anti-Jagged1 or control IgG as described above. At the peak of disease (days 14–16 after immunization), spinal cords were collected ($n = 3$/group) and the infiltrating mononuclear cells were isolated by Percoll gradient. Intracytoplasmic cytokine production by the CD4$^+$ transgenic T cells (Vβ11$^+$Vα3.2$^+$) was measured by flow cytometry. Mice treated with anti-Delta1 but not anti-Jagged1 showed a significant decrease in IFN-γ-producing 2D2 cells compared with IgG control mice (31.6 $\pm$ 5.2 vs 52.9 $\pm$ 6.3 in control animals, $p = 0.003$ by unpaired t test) while IL-4-producing 2D2 cells were decreased in both anti-Jagged1 (25.1 $\pm$ 7.3 compared with 51.6 $\pm$ 5.8 in control mice, $p = 0.007$ by unpaired t test) and anti-Delta1 (29.5 $\pm$ 5.8 compared with 51.6 $\pm$ 5.8 in control mice, $p = 0.006$ by unpaired t test) treated mice. No significant change in frequency of Th17 2D2 cells was observed, suggesting that Delta1 and Jagged1 do not regulate Th17 cells differentiation. Interestingly, anti-Jagged1 treatment significantly decreased IL-10-producing 2D2 cells, suggesting that Jagged1 plays a role in the induction of regulatory T cell 1 cells (12.6 $\pm$ 3.8 compared with 32.3 $\pm$ 4.9 in control mice, $p = 0.01$ by unpaired t test). Data shown in Fig. 3 are representative of three independent experiments.

Notch signaling with Delta1-Fc worsens EAE while Jagged1-Fc protects from EAE

We next used Delta1-Fc and Jagged1-Fc fusion proteins that bind to Notch receptors and activate the Notch pathway (36) to address the effect of Notch engagement by these ligands in EAE. As predicted by the blocking Ab data, Delta1-Fc enhanced clinical disease (mean maximal grade 3.2 $\pm$ 0.2 compared with IgG control group 2.5 $\pm$ 0.3, $p = 0.002$ by two-tailed Mann-Whitney U test) and prolonged the duration of peak disease (12.4 $\pm$ 0.8 compared with IgG control 4.6 $\pm$ 0.9, $p = 0.008$ by two-tailed Mann-Whitney U test), while Jagged1-Fc delayed EAE onset (13.9 $\pm$ 1.9 vs 9.9 $\pm$ 1.1, $p = 0.04$) and ameliorated the clinical score (mean maximal grade 1.3 $\pm$ 0.6 compared with IgG control group 2.5 $\pm$ 0.3, $p = 0.005$ by two-tailed Mann-Whitney U test) (Fig. 4*A*).

Using the 2D2 adoptive transfer system in Delta1-Fc- and Jagged1-Fc-treated C57BL/6, we found that Delta1-Fc increased the frequency of IFN-γ-producing cells isolated from the CNS 12 days after immunization. In contrast, Jagged1-Fc increased Th2/Tr1 cells as shown by the increase in IL-4- and IL-10-producing cells, respectively (Fig. 4*B*).

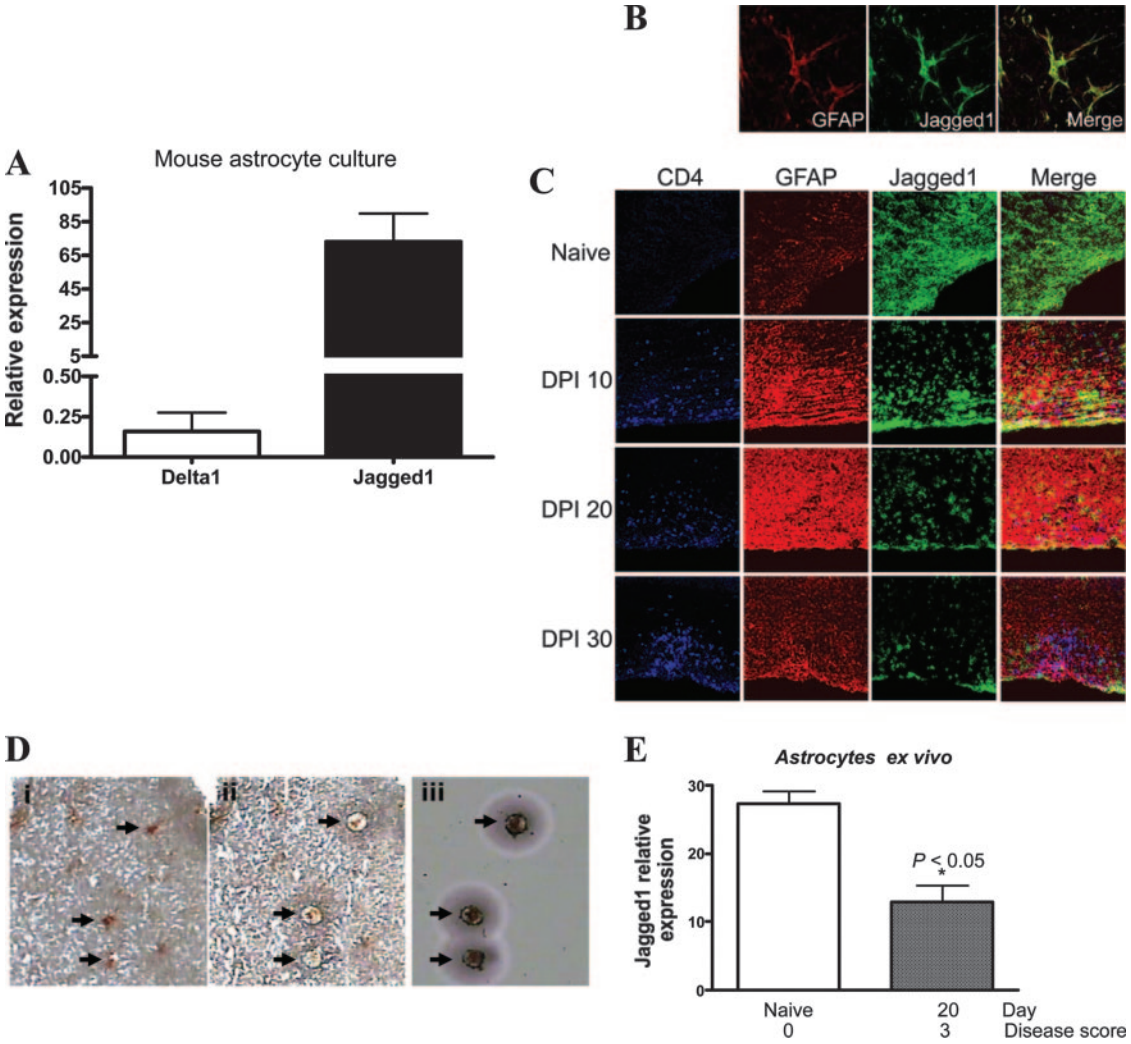

FIGURE 6. Jagged1 expression on astrocytes is down-regulated in EAE. *A*, Total RNA was isolated from primary mouse astrocyte cultures and transcribed to cDNA by RT and cDNA was used for TaqMan PCR. Jagged1 and Delta1 were measured by quantitative PCR and normalized to GAPDH expression, showing that astrocytes express Jagged1 but not Delta1. *B*, Immunostaining of spinal cord tissues from C57BL/6 mice showing the expression of Jagged1 (green) on GFAP-positive cells (red) by confocal microscopy. *C*, A representative experiment of spinal cord tissues from C57BL/6 naive or immunized mice for 10, 20, and 30 days (disease score were 2, 3.5, and 3, respectively) that were dissected and triple-immunolabeled with mouse anti-GFAP (red), rat anti-CD4 (blue), and rabbit anti-Jagged1 (green). *D*, A representative immunostaining of GFAP-positive cells (*i*) that were captured (*ii*) and microdissected (*iii*) (arrowheads) from the white matter of spinal cord sections. *E*, Quantitative TaqMan PCR of Jagged1 expression in astrocytes isolated by LCM from spinal cord samples of naive and mice with EAE (20 days after immunization). Jagged1 expression decreased significantly (∗, $p < 0.05$ by unpaired t test) in astrocytes from EAE mice (disease score = 3). Data represent the mean ± SEM for three to four mice of each group.

$CD4^+Foxp3^+$ Tregs are not regulated by Notch signaling

Previous reports suggest that Notch signaling may play a role in the induction of Tregs (16, 24). To characterize the role of Notch pathway in the generation of Tregs, we used *Foxp3gfp* knockin (*foxp3gfp*.KI) mice with a reporter, GFP, introduced into the endogenous *Foxp3* locus (34). $CD4^+$ T cells were isolated from spleens of naive *foxp3gfp*.KI and the $Foxp3^{neg}$ (GFP^{neg}) $CD4^+$ cells were FACS sorted and exposed to an optimal dose (10 μg/ml) of plate-bound Delta1-Fc, Jagged1-Fc, or control IgG in the presence of soluble anti-CD3 (1 μg/ml) and irradiated APCs. Treatment was conducted for 3, 4, and 5 days and the percentage of $CD4^+Foxp3/GFP^+$ cells was analyzed by FACS staining. Treatment with Jagged1-Fc or Delta1-Fc did not induce conversion of $CD4^+$ T cells into $CD4^+Foxp3^+$ T cells in our in vitro system, although cells exposed to TGF-β (5 ng/ml) exhibited a significant conversion of $CD4^+Foxp3^+$ Tregs (Fig. 5*A*). Time- and dose-dependent treatment with Delta1-Fc or Jagged1-Fc was conducted for 1–6 days at various doses (1–10 μg/ml) (data not shown). However, Jagged1-Fc, and to a lesser extent Delta1-Fc, inhibited proliferation of $CD4^+Foxp3^{neg}$ cells stimulated with anti-CD3 (1 μg/ml) in the presence of irradiated APCs (Fig. 5*B*) in agreement with our findings that blocking anti-Delta1 and anti-Jagged1 Abs increased Ag-specific T cell expansion in vivo (Fig. 2*B*) and increased activation of $CD4^+$ T cell as shown by increase in the frequency of CD25-positive cells (Fig. 2*C*).

To confirm that Delta1 and Jagged1 do not regulate $Foxp3^+$ Treg generation in vivo, we immunized *Foxp3gfp*.KI mice with 150 μg of MOG/CFA and treated them with anti-Jagged1, anti-Delta1, or control IgG every second day up to day 10 (5× 200 μg/mouse). Draining lymph nodes were isolated at the peak of the clinical score and examined for the frequency of $CD4^+Foxp3^+$. As suggested by the in vitro data, anti-Jagged1 or anti-Delta1 Abs did not alter the frequency of $Foxp3^+$ Tregs in the peripheral compartment, indicating that the differential effects of Jagged1 and

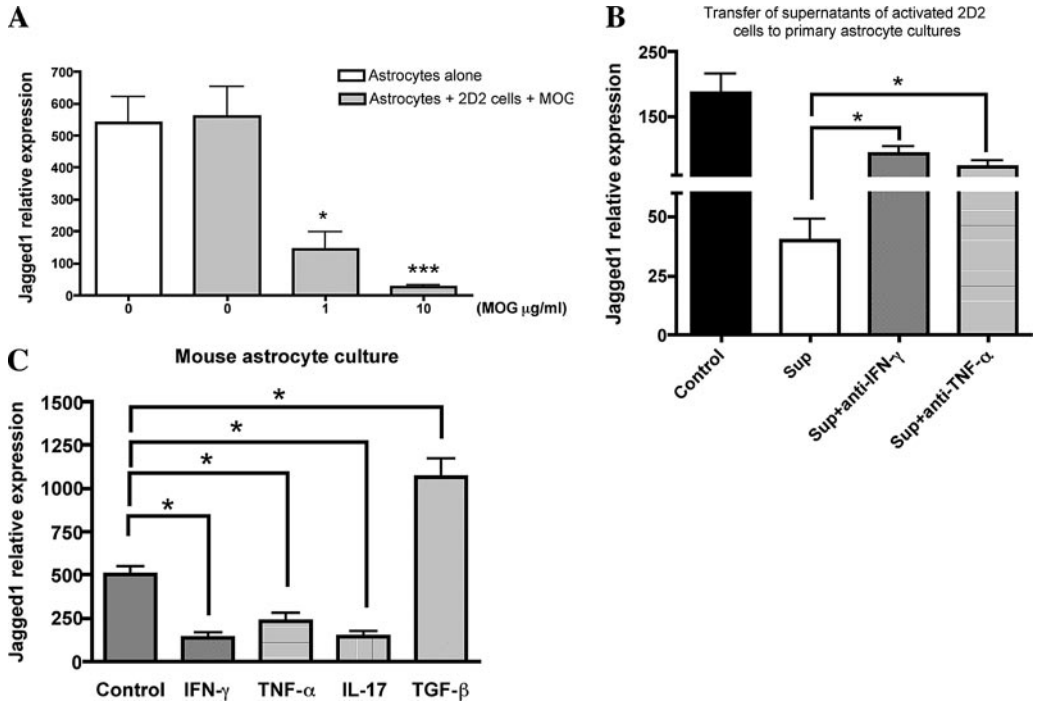

FIGURE 7. Pro- and anti-inflammatory cytokines have opposite effects on Jagged1 expression in primary astrocyte cultures. *A*, Coculture of primary mouse astrocytes with MOG-specific T cells (ratio 1:2) stimulated with MOG35–55 peptide and syngenic APCs shows reduction of Jagged1 expression on astrocytes in a time- and dose-dependent fashion. Twenty-four and 48 hours following activation in vitro, cells in suspension were removed and the attached monolayer astrocytes were washed twice with PBS and harvested using PBS/EDTA. Total RNA was isolated and TaqMan quantitative PCR was conducted. Jagged1 expression decreased significantly in astrocytes 24 and 48 h after activation with MOG35–55 peptide (1 and 10 μg/ml). *B*, MOG-specific T cells were activated with MOG35–55 peptide (10 μg/ml) in the presence of syngenic APCs for 24 h in 6-well plates (10^6 cells/ml) and supernatants were incubated in the presence or absence of neutralizing Abs against IFN-γ or TNF-α for 4 h. Supernatants were transferred to astrocyte cultures and incubated for another 24 h. After treatment, cells were washed and total RNA was isolated from astrocyte cultures and Jagged1 expression was measured using TaqMan PCR (*, $p < 0.05$ and ***, $p < 0.001$, respectively, by unpaired *t* test). *C*, Real-time quantitative PCR for Jagged1 expression in mouse primary astrocytes stimulated with rIFN-γ (10 U/ml), TNF-α (5 ng/ml) for 6 h. rTGF-β (5 ng/ml) was added for 6 h and IL-17 (3.125 ng/ml) treatment was conducted for 24 h. Mean ± SEM of three to four independent experiments.

Delta1 on EAE are independent of Treg expansion (Fig. 5*C*). Mononuclear cells isolated from the spinal cords of anti-Jagged1- or anti-Delta1-treated mice at the peak of clinical disease showed similar frequencies of Foxp3$^+$ Tregs among the groups (data not shown).

Down-regulation of Jagged1 expression on astrocytes during EAE

CD4$^+$ encephalitogenic T cells are reactivated in the CNS, thus we investigated the expression of Notch ligands on CNS-resident cells. Previous studies have shown that Jagged1 is expressed on astrocytes in both murine and human tissues (28, 30) although the regulation of Jagged1 levels is not clear. Because Jagged1 signaling is protective in EAE, we investigated whether astrocyte Jagged1 expression is regulated in EAE. First, we investigated the expression of Jagged1 and Delta1 in mouse primary astrocyte cultures. Using quantitative RT-PCR, we found that Jagged1 is highly expressed on astrocytes whereas Delta1 expression is very weak (Fig. 6*A*). Confocal analysis of spinal cord tissues from naive B6 mice confirmed the expression of Jagged1 on resident astrocytes (Fig. 6*B*).

Next, we analyzed Jagged1 expression in the CNS during EAE. Animals were sacrificed at different time points (10, 20, and 30 days after immunization) and spinal cord sections were triple-immunostained for CD4$^+$ T cells, Jagged1 expression, and astrocytes (GFAP). Animals sacrificed on day 10 showed extensive CD4$^+$ T cell infiltration, astrocytic activation, and decreased expression of Jagged1 when compared with naive samples (Fig. 6*C*). Jagged1 expression reached its lowest level at day 30 correlating with a massive CD4$^+$ T infiltration (Fig. 6*C*). To confirm this observation, we used a LCM technique to isolate astrocytes (GFAP-positive cells) from spinal cords of naive and EAE mice (day 20) (Fig. 6*D*). The level of Jagged1 mRNA was significantly lower in astrocytes isolated from EAE mice when compared with those from naive samples (Fig. 6*E*).

Activated Ag-specific CD4$^+$ T cells down-regulate Jagged1 expression on astrocytes in vitro

We hypothesized that the inflammatory microenvironment and specifically Th1 cytokines from encephalitogenic cells drive the down-regulation of Jagged1 on astrocytes. Thus, we cocultured 2D2 CD4$^+$ T cells with mouse primary astrocyte monolayer in the presence of irradiated syngenic APCs. Cultures were stimulated with MOG35–55 peptide for 24 h, then the astrocytes were washed twice with PBS, lysed, and total RNA was isolated and used for the Jagged1 expression assay. Jagged1 mRNA level was significantly down-regulated in astrocytes cocultured with CD4$^+$ T cells that were activated with MOG (1 and 10 μg/ml) compared with naive astrocytes (Fig. 7*A*). Supernatants of activated 2D2 CD4$^+$ T cells induced a similar down-regulation of Jagged1, but preincubating the supernatants with anti-IFN-γ- or anti-TNF-α-neutralizing Abs reversed this down-regulation compared with control Ig (92.8 ± 11.8 vs 39.7 ± 9.1, $p < 0.05$ and 73.9 ± 8.2 vs 39.7 ± 9.1, $p < 0.05$, by unpaired *t* test, respectively) (Fig. 7*B*).

To confirm the effects of cytokines on Jagged1 expression, we exposed primary mouse astrocyte cultures to rIFN-γ (10 U/ml),

TNF-α (5 ng/ml), TGF-β1 (5 ng/ml), and IL-17 (3.1 ng/ml). Jagged1 transcripts decreased significantly in astrocytes exposed to IFN-γ ($p < 0.05$), TNF-α ($p < 0.05$) as early as 6 h after treatment, and IL-17 also down-regulated Jagged1 $\times$ 24 h ($p < 0.05$) (Fig. 7*C*). In contrast, a significant increase in Jagged1 mRNA levels was detected in cells exposed to 5 ng/ml TGF-β1 for 6 h ($p < 0.05$). The effect of cytokines on Jagged1 expression was time and dose dependent (data not shown). The viability of treated astrocyte cultures was controlled by FACS analysis using 7-aminoactinomycin D/annexin V double staining that excluded toxicity of the recombinant cytokine treatment (data not shown).

Discussion

In this study, we used two opposite strategies of Notch-signaling manipulation: blocking mAbs against Notch ligands, Delta1 and Jagged1 or recombinant Delta1-Fc and Jagged1-Fc fusion proteins to analyze the role of these Notch ligands in the development of autoimmune encephalomyelitis. We have demonstrated that the expression of Delta1 and Jagged1 is regulated during EAE. Jagged1 and Delta1 are weakly expressed on peripheral $CD4^+$ T cells and macrophages. However, Delta1 was dominantly induced after disease priming on DCs and B cells, whereas Jagged1 mRNA levels increased only on DCs during EAE. Furthermore, we provide evidence that Delta1 preferentially increases the frequency of Th1 $CD4^+$ T cells in the CNS thus exacerbating the severity of EAE. In contrast, Jagged1 causes amelioration of the clinical disease by increasing the frequency of Th2 (IL-4) and Tr1 (IL-10) cells in the CNS. Interestingly, Jagged1 is highly expressed on CNS-resident astrocytes and is down-regulated by IL-17, IFN-γ, and TNF-α but up-regulated by TGF-β, suggesting that while expression of Jagged1 by CNS-resident cells may contribute to the "CNS privilege", inflammation leads to down-regulation of this protective molecule.

There is a debate as to whether Notch signaling enhances T cell activation or suppresses T cell function in murine and human systems (38). Notch ligands have been shown to differentially affect T cell differentiation (16, 23–25), but the molecular events underlying the role of Notch in directing naive T cell differentiation toward the Th1, Th2, or Treg lineages are unclear. Our data demonstrate by both blocking and activating approaches that Notch ligands differentially polarize T cell responses. In vivo blockade of Jagged1 during EAE decreased Th2 and Tr1 cells. In contrast, blockade of Delta1 impaired Th1 responses. Our present findings are in agreement with a recent report that showed differential roles of Jagged and Delta in the fate of T cells in vitro (14). Furthermore, it has been shown that human B cells overexpressing Jagged1 cocultured with allogenic $CD4^+$ T cells induced suppressive regulatory cells that produce IL-10 (24, 25). This is consistent with our data where Jagged1 generates, in addition to Th2 cells, Tr1 cells producing IL-10 but fails to generate $Foxp3^+$ regulatory cells in vitro and in EAE mice. Hoyne et al. (16) found that injection of Jagged1-transfected DCs into mice generated Tregs, although this study does not prove the direct contribution of Jagged1 to the differentiation of naive $CD4^+$ T cells to Tregs. Because the differentiation of Th2 induced by Jagged1 was observed in mice (14), the role of Jagged1 in terms of $CD4^+$ T cell differentiation may be different between mice and humans.

Apart from influencing the differentiation into effector cells, several reports suggested a role for the Notch pathway in T cell activation although these data are inconsistent. Eagar et al. (39) demonstrated that a stimulatory anti-Notch Ab, as well as the Jagged1- and Delta1-expressing B cell line, suppressed T cell proliferation in vitro. More recently, Rutz et al. (40) showed that Jagged1 and Delta1 induced a dose-dependent inhibition of early activation markers CD69 and CD25, as well inhibition of proliferation in vitro. In a contradictory study, overexpression of constitutively active Notch1 in $CD4^+$ T cells led to an increase in CD25 expression and cell proliferation (15). In our present report, we show that blocking the Notch signaling by γ-secretase inhibitor has an impact on T cell activation upon anti-CD3 stimulation. Indeed, anti-Jagged1 and to lesser extent anti-Delta1 increased $CD4^+$ T cell proliferation in vivo in conjunction with an increase in CD25 expression.

In sharp contrast, two other studies showed that blocking Notch signaling, using γ-secretase inhibitor, decreased T cell proliferation (41). More recently, it has been shown that γ-secretase inhibitors prevent Th1 polarization and suppress EAE (26). Although the γ-secretase complex can cleave all Notch proteins (Notch 1–4), it can also affect multiple downstream pathways involved in effector T cell functions (42). In the present study, we showed that T cells exposed to DAPT, a well-known γ-secretase inhibitor, exhibited a higher rate of proliferation compared with control-treated cells. These findings raise questions about the specificity of these inhibitors in the complex cell-signaling system. Furthermore, γ-secretase inhibitors do not differentiate between the Notch ligands involved in regulation of T cell functions, and a previous report showed that Notch signaling could be induced independently of γ-secretase in mutant forms of Notch (43) suggesting that the γ-secretase inhibitor may block only a part of Notch's functions. Significant genetic evidence of the existence of CSL-independent Notch signaling has been shown although the molecular components of this pathway and its downstream targets remain largely unknown (44).

In agreement with previous studies (28, 30), we demonstrate that astrocytes express high levels of Jagged1 but not Delta1. Surprisingly, the expression of Jagged1 in the CNS-resident astrocytes was down-regulated during EAE, as shown using confocal microscopy and LCM techniques correlating with $CD4^+$ T cell infiltration at the peak of clinical disease score. Our in vitro coculture data suggest that the proinflammatory cytokines IFN-γ, TNF-α, and IL-17 play a role in the down-regulation of astrocytic Jagged1 while TGF-β up-regulates Jagged1 on astrocytes. It was reported that Jagged1 expression on human astrocytes is up-regulated in MS (28). The authors also showed that TGF-β1 up-regulates Jagged1 in primary cultures of human astrocytes consistent with our present findings. John et al. observed Jagged1 expression by hypertrophic astrocytes within and around active MS plaques lacking remyelination, whereas Notch1 and Hes5 localized to cells with an immature oligodendrocyte phenotype, and interpreted these data as suggesting that Jagged1 induces demyelination in MS brains. The timing of decreased Jagged1 protein expression in the CNS of EAE mice correlates with the known increase in inflammatory cytokines at the peak of disease (45, 46). Interestingly, two recent reports suggest that it is unlikely that Notch signaling is responsible for a failure of remyelination (30, 31).

Collectively, our data indicate a crucial role of Notch pathway in a model of CNS autoimmunity. Therapeutic opportunities that could arise from the manipulation of Notch signaling in immune disorders such as autoimmunity, cancer immunotherapy, and transplantation may prove to be a novel approach to suppress aberrant immune activation.

Disclosures

The authors have no financial conflict of interest.

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