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Impact of Notch1 Deletion in Macrophages on Proinflammatory Cytokine Production and the Outcome of Experimental Autoimmune Encephalomyelitis

Wipawee Wongchana,* Rebecca G. Lawlor,† Barbara A. Osborne,*‡ and Tanapat Palaga*§

Notch signaling is involved in regulating TLR-mediated responses in activated macrophages. In this study, we investigated the impact of deficiency in Notch signaling in activated macrophages in EAE, an adoptive transfer of activated macrophages derived from Notch1−/− (Notch1 knockout [N1KO]) or CSL/Rbp-jcKO2fl/fl (CSL/RBP-Jc KO) mice was performed prior to induction of EAE. Mice receiving activated N1KO macrophages showed decreased severity of EAE compared with mice receiving wild-type or CSL/RBP-Jc KO macrophages. In vitro restimulation of splenocytes by myelin oligodendrocyte glycoprotein 35–55 peptide from these mice revealed that cells from mice receiving N1KO macrophages produced significantly less IL-17 compared with the control mice, whereas IFN-γ production was similar in both groups. We found that activated N1KO, but not CSL/RBP-Jc KO, macrophages produced less IL-6 and had lower CD80 expression compared with wild-type and did not exhibit any defect in IL-12p40/70 production, whereas activated macrophages from CSL/RBP-Jc KO mice phenocopied γ-secretase inhibitor treatment for reduced IL-12p40/70 production. Furthermore, the nuclear translocation of the NF-κB subunit c-Rel was compromised in γ-secretase inhibitor–treated and CSL/RBP-Jc KO but not N1KO macrophages. These results suggest that Notch1 and CSL/RBP-Jc in macrophages may affect the severity of EAE differently, possibly through modulating IL-6 and CD80 expression, which is involved in the Th17 but not Th1 response. The Journal of Immunology, 2015, 195: 5337–5346.

Macrophages are innate immune cells that bridge innate and adaptive immune responses. Signaling through IFN-γ receptors, together with TLRs, activates macrophages, resulting in enhanced phagocytic activity and the production of cytokines. This activation also leads to increased expression of costimulatory molecules that enable macrophages to present peptide Ags to activate Th cells. Furthermore, activated macrophages produce various types of pro- and anti-inflammatory cytokines, such as TNF-α, IL-6, IL-10, and IL-12, that are involved in the activation and differentiation of many cell types, including CD4+ Th cells.

The Notch signaling pathway regulates differentiation, proliferation, survival, and cell fate decisions in both myeloid and lymphoid lineage cells (1). There are four mammalian Notch receptors (Notch1–4) and five ligands (Delta-like 1, 3, and 4 and Jagged 1 and 2). The interaction between Notch ligands and receptors induces the enzymatic cleavage of the Notch receptors, first by an ADAM protease and subsequently by γ-secretase, resulting in the release of the Notch intracellular domain. The intracellular domain of Notch then translocates to the nucleus and forms a complex with the DNA-binding protein CSL/RBP-Jc, which initiates the transcription of the Notch target genes (2). Notch1 is the best studied Notch receptor, and it has been shown to play critical roles in regulating the effector function of immune cells and to be involved in diseases such as cancer and autoimmune disease (3–6).

The dysregulation of cytokine production and hyperactivation of macrophages are linked to many inflammation-related diseases such as sepsis. Additionally, autoimmune diseases such as rheumatoid arthritis (7) are also associated with TLR-activated macrophages. Moreover, it has been shown that the Notch signaling pathway cooperates with TLR signaling in macrophages under pathological conditions that can lead to autoimmune diseases, systemic lupus erythematosus in particular (6).

The progression and severity of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, is well known to be mediated by both Th1 and Th17 CD4+ T cells. Recently, several studies have shown that not only autoreactive T cells but also other factors such as pro- and anti-inflammatory cytokines, chemokines, and costimulatory molecules, which are
produced by other cell types, play crucial roles in sustaining the disease (8–12).

Several reports have elucidated the roles of Notch signaling in the EAE model, especially in T cells. Using a γ-secretase inhibitor (GSI) incorporated into rodent chow (LY chow) to inhibit Notch signaling in vivo, it was demonstrated clearly that Notch signaling regulates Th1 and Th17 responses in the EAE model. A reduction in the severity of EAE-induced inflammation in GSI-treated animals was observed, as were decreases in the signature Th1 (IFN-γ) and Th17 (IL-17) cytokines in restimulated cultures of activated T cells in vitro (13, 14). Additionally, the Notch ligand, Delta-like 4, present on APCs, including macrophages, interacts with Notch receptors on Ag-specific T cells and regulates the trafficking and accumulation of T cells in the CNS (15). These results are similar to a recent study that showed that the induction of EAE in mice overexpressing a dominant-negative form of Notch1 and CSL/RBP-J (Mx1cre/+; [B6.Cg-Tg(Mx1cre)1Cgn/J] mice) KO mice were generated by breeding Notch1fl/fl (Notch1tm2Rikerg) or Rbp-jfl/fl (Rbp-jtm1Hon) mice to Mx1cre/+ mice. To conditionally delete Notch1 or CSL/RBP-J in female mice with the genotype of Notch1fl/fl (N1KO), Notch1tm2Rikerg × Mx1cre/+ (N1KO), Notch1fl/fl × Mx1cre/+ (control), Rbp-jtm1Hon × Mx1cre/+ mice (CSL/RBP-J KO), and Rbp-jtm1Hon × Mx1cre/+ mice (control) were injected with 12–15 μg/kg body weight of poly(I:C) (Ingenex, San Diego, CA) every other day for 5 d. Animals were rested for 3 wk prior to sacrifice and use in experiments. 2D2 TCR transgenic mice [C57BL6/6-Tg(2cd22, Terc2d)1Kuch/J] were maintained by breeding hemizygous mice to WT C57BL6/6 mice. Female mice aged 7–12 wk were used for all experiments. Ten-week-old animals were used for EAE induction. All animals were housed in animal facilities according to the guidelines approved by the Institutional Animal Care and Use Committees at the University of Massachusetts at Amherst and Chulalongkorn University.

Generation of bone marrow–derived macrophages

Bone marrow cells from femur cavities were flushed and incubated in DMEM (Lonza, Walkersville, MD, USA) supplemented with 10% FBS (Life Technologies, Grand Island, NY), HEPES (Lonza), sodium pyruvate (Lonza), streptomycin/penicillin G (Lonza), 5% (v/v) horse serum (Thermo Scientific), and 20% (v/v) L929-conditioned media. Fresh medium was added to the culture at day 4. Cells were harvested on day 7 using cold PBS. Cell surface staining with anti-F4/80–Alexa Fluor 488 and CD11b–Alexa fluoro 647 Abs (Bio-legend, San Diego, CA) was used to confirm a macrophage phenotype (Supplemental Figs. 1A, 2B). The obtained bone marrow–derived macrophages (BMDMs) were cultured in DMEM complete media without horse serum and L929-conditioned media before activation.

Cell culture and activation of BMDMs

BMDMs were primed overnight with reconstituent murine IFN-γ (10 ng/ml) (Biolegend) and washed twice with medium and PBS. Salmonella LPS (100 ng/ml) (Sigma-Aldrich, St. Louis, MO) was added to activate the macrophages for the indicated times. In some experiments, N-[3,5-difluorophenacetyl]-t-allyl-S-phenylglycine t-butyl ester (25 μM) or DMSO (0.01%) (Calbiochem, San Diego, CA) was used to pretreat the macrophages before activation.

Intracellular staining and cell surface staining

The BMDMs were activated as described above. For intracellular staining, brefeldin A (for IL-12p40/70 and IL-6 detection) or monensin (for IL-10 and GM-CSF detection) was added at the beginning of the activation by LPS. The cells were pretreated with Fc Block (BD Biosciences), followed by surface staining and fixation/permeabilization using a BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Anti-mouse F4/80–Alexa Fluor 488 (Biolegend), anti-mouse IL-12p40/70–PE (BD Biosciences and BioLegend), anti-mouse IL-10–allophycocyanin (BioLegend), anti-mouse CD206–PE (BioLegend), and anti-mouse GM-CSF–PE (BioLegend) were used. Anti-mouse Notch1–PE (clone N1A1) was used to stain for Notch1 using the FoxP3 Staining Buffer Set (eBioscience, San Diego, CA) according to the manufacturer’s instructions. In some experiments, anti-mouse CD80, anti-mouse CD86, anti-mouse MHC class II (BD Pharmingen), anti-mouse PD-L1 (BioLegend), and anti-mouse CD4 (BD Biosciences) Abs were used for cell surface staining. The cells were acquired on a FACS LSR II (Becton Dickinson) or Cytomics FC 500 MPL (Beckman Coulter) and analyzed with FlowJo software (FlowJo, Ashland, OR).

ELISA

Culture supernatants from the BMDMs treated as described were harvested at 6 or 24 h after stimulation. Secreted IL-12p70 levels were detected using an IL-12p40/70 ELISA (BD Pharmingen). For some experiments, supernatants were collected and subjected to detection for IL-17 (BD Biosciences), IFN-γ (BD Biosciences), IL-10 (BD Biosciences), IL-2 (BD Biosciences), and GM-CSF (BioLegend) by ELISA according to the manufacturers’ instructions.

Immunofluorescent staining

Cells were cultured in eight-well chamber slides and activated as indicated. After washing with PBS, the cells were fixed with 4% paraformaldehyde and incubated with a rabbit anti–c-Rel polyclonal Ab (Santa Cruz Bio-technology, Santa Cruz, CA), followed by an anti-rabbit IgG [H+L (Fab’)]

Materials and Methods

Animals

Wild-type (WT) C57BL/6 mice were purchased from Harlan Laboratories (South Easton, MA) or the National Laboratory Animal Center, Mahidol University (Salaya, Thailand). All transgenic mice used in this study were purchased from The Jackson Laboratory (Bar Harbor, ME). Notch1 knockout (N1KO) and CSL/RBP-J KO mice were generated by breeding Notch1fl/fl (Notch1tm2Rikerg) or Rbp-jfl/fl (Rbp-jtm1Hon) mice to Mx1cre/+ mice. To conditionally delete Notch1 or CSL/RBP-J, female mice with the genotype of Notch1fl/fl (N1KO), Notch1tm2Rikerg × Mx1cre/+ (N1KO), Notch1fl/fl × Mx1cre/+ (control), Rbp-jtm1Hon × Mx1cre/+ mice (CSL/RBP-J KO), and Rbp-jtm1Hon × Mx1cre/+ mice (control) were injected with 12–15 μg/kg body weight of poly(I:C) (Ingenex, San Diego, CA) every other day for 5 d. Animals were rested for 3 wk prior to sacrifice and use in experiments. 2D2 TCR transgenic mice [C57BL6/6-Tg(2cd22, Terc2d)1Kuch/J] were maintained by breeding hemizygous mice to control WT C57BL6/6 mice. Female mice aged 7–12 wk were used for all experiments.

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fragment)-PE (Cell Signaling Technology, Danvers, MA). The cells were observed under an inverted fluorescent microscope or a confocal microscope.

**Real-time PCR**

BMDMs were activated as above for 4 h. Total RNA was isolated using an RNAqueous kit (Ambion, Austin, TX). cDNA was synthesized, and transcripts were amplified by a quantitative PCR Stratagene Mx3000P. Primer sequences were as follows: Il23p19, forward, 5'-AGC GGG ACA TAT GAA TCT ACT AAG AGA-3', reverse, 5'-GTC CTA GTA GGG AGG TGT GAA GTT G-3'; Il12p40, forward, 5'-AAC CTC ACC TGT GAC ACG CC-3', reverse, 5'-CAA GTC CAT GTT TCT TTG CAC C-3'; arginase 1, forward, 5'-CAG AAG AAT GGA AGA GTC AG-3', reverse, 5'-CAG ATA TGC AGG GAG TCA CC-3'; and β-actin, forward, 5'-ACC AAC TGG GAC GAC ATG GAG AA-3', reverse, 5'-GTG GTG AAG CTG TAG CC-3'. The expression of each gene was normalized to the expression of β-actin by the 2^-ΔΔCT method.

**Adoptive transfer of activated macrophages and EAE disease score evaluation**

BMDMs obtained from control, N1KO, and CSL/RBP-Jk mice were activated as described above for 30 min. The cells were washed three times using warm PBS and the cell number was adjusted to 2 x 10^6 cells in 200 μl PBS. Two hundred microliters activated BMDMs or PBS was injected i.p. into naive C57BL/6 mice. After 4 h, EAE induction emulsion Hooke Kits (Hooke Laboratories, Lawrence, MA) were administered into the flanks of the animals according to the manufacturer’s instructions. Pertussis toxin was injected i.p. at both 2 and 24 h after immunization. The progression and the severity of EAE were monitored and scored from 0 to 5 as follows: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hind- and forelimb paralysis; 5, morbidity and death. The data are reported as the mean daily clinical score (14). The mice that received WT and N1KO macrophages were euthanized during the peak of the disease (days 15–16 postimmunization) and their spleens were collected.

**Coculture of T cells and macrophages**

CD4^+ T cells were negatively isolated from the spleen of 2D2 TCR transgenic mice by a mouse CD4 T lymphocyte enrichment set–DM (BD Biosciences) according to the manufacturer’s instructions. WT and N1KO BMDMs (2 x 10^5 cells) were plated in 48-well plates and activated as described above for 2 h. CD4^+ T cells (5 x 10^5 cells) were added to each well in RPMI 1640 (Hyclone) supplemented with 10% FCS (Life Technologies), HEPES (Lonza), penicillin/streptomycin (Lonza), and 2-ME (Sigma-Aldrich) in the presence of 150 μg/ml MOG35–55 peptide. Five days following the primary stimulation, cells were washed and 5 x 10^5 CD4^+ T cells were stimulated in 48-well plates coated with 1 μg/ml anti-CD3 Ab and incubated for 24 h. Culture supernatants from secondary stimulation conditions were collected and IL-17, IFN-γ, IL-10, and IL-12 were detected by ELISA.

**Tracking macrophages after adoptive transfer**

To observe trafficking of BMDMs after adoptive transfer, 2 x 10^6 BMDMs from WT and N1KO mice were labeled using a CellTrace CFSE cell proliferation kit (Invitrogen) according to the manufacturer’s instructions for adherent cells overnight before activation and following EAE induction. Four hours after the first pertussis toxin injection, cells from fluids of peritoneal cavity, spleens, omentum, and lymph nodes were collected. Omentum and lymph nodes were digested by collagenase type IV (Invitrogen). Cells were stained and gated on the CD11b^+ population.

**FIGURE 1.** Adoptive transfer of N1KO, but not CSL/RBP-Jk KO, macrophages delays the onset and severity of diseases in EAE model. (A) Experimental design for BMDM transfer in EAE mice. (B) Mean clinical scores in EAE mice receiving WT BMDMs, N1KO BMDMs, or PBS for 15 d. **p < 0.05. (C and D) Mean clinical scores in EAE mice receiving WT BMDMs, N1KO BMDMs, CSL/RBP-Jk KO BMDMs, or PBS. The clinical scores were monitored for 27–28 d. **p < 0.05.
Statistical analysis

Statistical analyses were performed using SPSS version 15.0 and GraphPad Prism version 5.0. A one-way ANOVA (α = 0.05) was used when comparing two conditions.

Results

Adoptive transfer of activated macrophages lacking Notch1, but not CSL/RBP-Jk, decreases severity and delays onset of diseases in the EAE model

Macrophages have been identified as one of the effector cells that play a critical role in EAE (17). The adoptive transfer of macrophages activated with LPS with an immune complex that predominantly produces IL-10 in an EAE model resulted in delaying onset of disease and decreasing disease severity (19). Notch signaling is involved in the activation of macrophages and regulates the production of cytokines such as IL-6, IL-12p70, and IL-10 (20–23, 30), which are produced during EAE inflammation. Furthermore, the involvement of Notch signaling in regulating disease severity in the EAE model by using GSI was demonstrated (13, 14). For these reasons, we hypothesized that Notch1 in macrophages may play a crucial role in regulating disease outcome in the EAE model.

BMDMs that were generated from conditional N1KO and control WT mice were used in this study. CSL/RBP-Jk, a gene encoding a DNA-binding protein that plays a central role in the canonical Notch signaling pathway, was also deleted in macrophages and used in the transfer experiment. The expression of two macrophage markers, CD11b and F4/80, were equivalent in the N1KO, CSL/RBP-Jk, and WT control BMDMs, suggesting that the loss of Notch1 or CSL/RBP-Jk did not interfere with macrophage differentiation (Supplemental Figs. 1A, 2B). The loss of expression of Notch1 or CSL/RBP-Jk in the N1KO or CSL/RBP-Jk KO macrophages was confirmed by Western blot (Supplemental Figs. 1B, 2C) and flow cytometry (Supplemental Fig. 1C, 1D). BMDMs from the WT or conditional KO mice were activated by IFN-γ/LPS for 30 min in vitro before adoptive transfer by i.p. injection into the WT recipients 4 h prior to EAE induction (Fig. 1A). We observed a significant delay in the onset of the disease and a decrease in disease severity in the animals that received activated N1KO macrophages, compared with those receiving activated WT macrophages or PBS control (Fig. 1B, 1C). In contrast, no difference in the onset or the severity of diseases was found between animals receiving CSL/RBP-Jk KO macrophages and those of the control (Fig. 1D). These data indicated that Notch1 expression in transferred macrophages influences EAE outcome but the expression of CSL/RBP-Jk in macrophages is dispensable for this effect.

Because Notch signaling in macrophages is required for the optimal production of IL-12p40/70 and IL-6 and Il23p19 mRNA, which are involved in Th cell polarization, and because EAE is a Th1/Th17-driven autoimmune disease, we hypothesized that Notch1 in macrophages could influence the response of Th1 and/or Th17 in EAE by affecting IFN-γ and IL-17 production. Splenocytes from mice that received an adoptive transfer of N1KO macrophages and EAE induction were restimulated with the MOG35–55 peptide in vitro and the levels of IL-17 and IFN-γ were measured in the culture supernatant by ELISA. We found that the level of IL-17 decreased significantly in the MOG35–55 peptide-stimulated splenocytes from animals that received activated N1KO macrophages, compared with those from the control mice (Fig. 2A). Surprisingly, IFN-γ levels were not different between the two groups (Fig. 2B). Recent evidence pointed to the pathogenic role of GM-CSF–producing CD4+ T cells in autoimmunity (31, 32). In the restimulation assay with MOG35–55 peptides of splenocytes from mice receiving control or N1KO macrophages, no differences were found in the percentages of CD4+ T cells with GM-CSF or secreted GM-CSF (Supplemental Fig. 3A, 3B).

To address whether transferred macrophages trafficked differently, we detected the appearance of macrophages labeled with fluorescent dye upon transfer for 10 h. As shown in Supplemental Fig. 3C, most transferred macrophages were found to migrate to omentum or to remain in the peritoneal fluids. More importantly, no significant differences in the appearance of transferred macrophages in tested tissues were found between control and N1KO macrophages. These data imply that the transfer of Notch1-deficient activated macrophages affects the onset and progression of EAE, possibly through its influence on the activation of a Th17-type but not a Th1-type response, and CSL/RBP-Jk in activated macrophages is dispensable for this effect.

Notch1 is dispensable for IL-12p40/70 production in IFN-γ/LPS-activated BMDMs

Notch signaling has been shown to regulate IL-12p40/70 production, and Notch1 is especially important for the regulation Il12p35 (Il12a) and p40 (Il12b) mRNA expression in LPS-activated macrophages (30). Based on the results obtained in this study in the EAE model, we asked specifically whether Notch1 is important for IL-12p40/70 production in IFN-γ/LPS-activated macrophages. BMDMs that were generated from conditional N1KO and WT mice were activated with IFN-γ and LPS, and IL-12p40/70 was detected by intracellular cytokine staining. Surprisingly, there was no significant difference in either the percentage of IL-12p40/70+ cells or the mean fluorescence intensity (MFI) between N1KO BMDMs and the control BMDMs upon activation with IFN-γ/LPS (Fig. 3A, 3B). Moreover, there was no significant difference in the level of IL-12p40/70 production detected in the culture supernatants by ELISA from IFN-γ/LPS–activated N1KO BMDMs compared with those from the control BMDMs at any time points tested (Fig. 3C). Next, we examined whether activity of the γ-secretase in N1KO macrophages could be involved in IL-12p40/70...
production in IFN-γ/LPS–activated N1KO BMDMs using GSI treatment. The reduction of cleaved Notch1 in GSI-treated WT BMDMs was confirmed by Western blot (Supplemental Fig. 2A). We found that the levels of IL-12p40/70 were similarly reduced upon GSI treatment in both N1KO and the control BMDMs, consistent with our previous results (Fig. 3D). These results implied that Notch1 is dispensable for optimal IL-12p40/70 production in IFN-γ/LPS–activated macrophages, and other γ-secretase substrates, perhaps other Notch receptors, may be essential for this regulation.

Production of IL-12p40/70 in macrophages partially depends on the canonical Notch signaling pathway

Because γ-secretase has other target substrates besides the Notch receptors, the involvement of Notch in regulating IL-12p40 production cannot be addressed by the use of GSI. To address whether canonical Notch signaling is important for IL-12p40/70 production elicited by IFN-γ/LPS stimulation, BMDMs from conditional CSL/RBP-Jκ KO mice and control mice were used and the results were compared with GSI treatment in WT BMDMs. The production of IL-12p40/70 in the WT BMDMs that were treated with GSI showed a significant reduction in both the MFI and the percentage of IL-12p40/70+ cells (Fig. 4A), compared with the vehicle control–treated BMDMs, consistent with the previous report (23). A similar trend in the reduction of IL-12p40/70 was observed in IFN-γ/LPS–activated CSL/RBP-Jκ KO BMDMs (Fig. 4B–D). These results strongly suggest that the activity of γ-secretase and the canonical Notch signaling via CSL/RBP-Jκ are required for the optimal production of IL-12p40/70 in macrophages.

Defect in c-Rel nuclear translocation in IFN-γ/LPS–activated macrophages requires CSL/RBP-Jκ but not Notch1

The production of IL-12p40/70 in activated BMDMs was shown to be partially c-Rel–dependent (33). In our previous study, we found that GSI treatment in WT BMDMs affected the nuclear translocation of c-Rel upon stimulation with IFN-γ/LPS (23). The activation of BMDMs from CSL/RBP-Jκ KO mice showed a reduction in c-Rel nuclear accumulation as detected by immunofluorescence staining whereas BMDMs from N1KO mice showed intense nuclear localization of c-Rel upon activation similar to those from the WT mice (Fig. 5). The pattern of c-Rel nuclear localization correlated well with the level of IL-12p40/70 production, which implied that the production of IL-12p40/70 in IFN-γ/LPS–activated BMDMs requires canonical Notch signaling, but not Notch1, in a c-Rel–dependent manner.

Reduced IL-6 production and CD80 expression in N1KO activated macrophages

Based on the phenotypic results from the mice in the EAE experiments described above and from in vitro restimulation with the MOG peptides, we hypothesized that other cytokines or costimulatory molecules that were produced or expressed by activated N1KO macrophages could be compromised, resulting in decreased IL-17 production in an EAE setting. We investigated IL-6 and IL-10 production and Il23p19 mRNA expression in N1KO...
BMDMs upon activation, all of which have been shown to play roles in EAE and to be involved in regulatory T cell/Th17 polarization (34, 35). As shown in Fig. 6A, a significant reduction in the percentages of IL-6–producing cells was observed upon activation, which is consistent with our previous report that Notch signaling partially regulates IL-6 (21, 22). However, no difference in IL-10 production and Il23p19 mRNA expression was found between the N1KO and WT BMDMs (Fig. 6B, 6C). We further determined the expression of costimulatory molecules that had been reported to be important in the EAE model and that are involved in T cell activation (19). Among the cell surface molecules tested, the level of a costimulatory molecule, CD80, was found to be reduced in the N1KO BMDMs (Fig. 6D).

Because transferring of RBP-Jκ KO macrophages did not affect the severity of EAE as observed in transfer of N1KO macrophages, we hypothesized that those molecules that are affected by Notch1 deficiency and play a key role in EAE severity should remain unaffected in RBP-Jκ KO macrophages. Indeed, as shown in Fig. 6E, the percentages of IL-6+ cells were similar in WT and RBP-Jκ KO macrophages. Additionally, the expression of CD86 and MHC class II increased whereas the level of CD80 was lower in RBP-Jκ KO macrophages (Fig. 6F).

To further investigate whether N1KO macrophages dictate immune response of Th cells, an in vitro coculture of activated macrophages and MOG35–55-specific TCR transgenic CD4+ T cells was performed. As shown in Supplemental Fig. 4A–C, coculture of naive CD4+ T cells with IFN-γ/LPS–activated N1KO macrophages in the presence of MOG peptide resulted in reduced IFN-γ and IL-10 production from T cells in the secondary stimulation with plate-bound anti-CD3 Ab. IL-17 was undetectable in the culture supernatant of stimulated T cells in all conditions (data not shown). Because polarization of the Th17 response requires several cytokines, including TGF-β, IL-23, together with IL-6, it is likely that an in vitro condition may be insufficient to polarize CD4+ T cells to Th17. This result indicates that Notch1 expression in activated macrophages plays an important role in dictating the optimal CD4+ T cell response. It remains to be investigated whether N1KO macrophages affect other aspects of T cell response such as migration.

Taken together, these data suggest that a reduction in IL-17 in MOG35–55–stimulated splenocytes from the mice that received activated N1KO macrophages together with the reduction in the costimulatory molecule, CD80, and IL-6 in N1KO macrophages may interfere with the optimal immune response overall and culminate in reducing the onset and progression of EAE.

Discussion
We used an animal model of EAE to evaluate the impact of Notch1 and CSL deletion in macrophages on the severity of this autoimmune inflammatory condition and showed that CSL/RBP-Jk
was detected using immunofluorescence staining. The data are representative of at least two independent experiments. Scale bars, 20 μm.

Coculture of activated macrophages with CD4+ T cells in the presence of specific Ags in vitro can skew CD4+ T cells toward Th1 or Th2, depending on the macrophage stimuli (39). In humans, LPS or LPS/IFN-γ-activated macrophages skew Th cells toward Th17 or Th1, respectively (40). In our study, coculture of LPS/IFN-γ-activated N1KO macrophages with MOG-specific TCR transgenic CD4+ T cells resulted in decreased IFN-γ and IL-10 production whereas the similar level of IL-2 was detected. More importantly, IL-17 was undetectable in this setting. Because we observed decreased IL-17 but not IFN-γ production in a MOG-restimulation assay of splenocytes from mice receiving N1KO macrophages, it is possible that in an in vivo system, transferred N1KO macrophages may influence Th cell responses by more complicated mechanisms. Therefore, it remains to be determined how Notch1 in activated macrophages is involved in determining Th1 or Th17 responses.

In the present study, endogenous macrophages were not depleted before the transfer of activated macrophages for the EAE study. Previously, a similar adoptive transfer strategy using macrophages activated by LPS and an immune complex were reported in an EAE model. This type of macrophage produced high levels of IL-10, and the decrease in disease severity was attributed to production of this anti-inflammatory cytokine (19). Because the level of IL-10 produced by N1KO macrophages was similar to that of the WT macrophages, it is unlikely that IL-10 is responsible for the decreased disease severity in our study.

Activated N1KO macrophages expressed less of the costimulatory molecule CD80. It is not clear at present how Notch signaling regulates CD80 expression. Costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) interact with CD28 on T cells; together with signaling through MHC–TCR, such interactions are important for T cell activation. Blockade of CD80 has been reported to suppress EAE (11, 41). Furthermore, blocking CD28 results in a reduction of the CD80/CD86 ratio in APCs and decreases EAE severity (10). In another autoimmune disease such as rheumatoid arthritis, CD80 is more important for Th1 cell differentiation than Th17 cells (42), whereas our data indicate that Notch1 in macrophages seems to have no impact on the Th1-type differentiation whereas IL-23 is essential for maintaining the Th17 phenotype (35). In our in vitro experiments, a deficiency in Notch1 did not have any effect on the expression of IL-12p70 and IL-23p19 mRNA, but a reduction in IL-6 and CD80 was detected. Previous data have shown that IL-23 is not essential for Th17 differentiation, whereas IL-6 and TGF-β are involved in this process (36), and the blockade of IL-6 can impair Th17 differentiation (37). In the EAE model, it has been demonstrated that IL-6 is important in this autoinflammatory disease; for example, mice that are defective in IL-6 production show a resistance phenotype to MOG peptide–induced EAE. Signaling through the A2B adenosine receptor in APCs such as dendritic cells enhances IL-6 production and its blockade helps improve the EAE phenotype (12, 38). Taken together, these data strongly implicate that IL-6 is important for Th17 cell differentiation and plays pivotal roles in EAE. We and others have previously shown that Notch1 is involved in IL-6 production in activated macrophages (20, 22). These observations, together with the findings in this study, suggest that Notch1 in activated macrophages might play an important role in regulating the outcome of T cell responses by regulating IL-6 (Th17) but not IL-12 (Th1) in the EAE model.

Coculture of activated macrophages with CD4+ T cells in the presence of specific Ags in vitro can skew CD4+ T cells toward Th1 or Th2, depending on the macrophage stimuli (39). In humans, LPS or LPS/IFN-γ-activated macrophages skew Th cells toward Th17 or Th1, respectively (40). In our study, coculture of LPS/IFN-γ-activated N1KO macrophages with MOG-specific TCR

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<th>WT</th>
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| IFNγ/LPS 4hr | DMSO (A and F) or GSI (25 μM) (B and G) before stimulation with IFNγ/LPS for 4 h. BMDMs obtained from WT mice were pretreated with vehicle control DMSO (A and F) or GSI (25 μM) (B and G) before stimulation with IFNγ/LPS for 4 h. Localization of c-Rel was detected using immunofluorescence staining. The data are representative of at least two independent experiments. Scale bars, 20 μm.

The role in regulating the outcome of T cell responses by regulating IL-6 is important for Th17 cell differentiation and plays pivotal roles in EAE. We and others have previously shown that Notch1 is involved in IL-6 production in activated macrophages. In contrast, IL-6 production was compromised only in N1KO macrophages. Mice that were adoptively transferred with activated N1KO, but not CSL/RBP-Jκ KO, macrophages showed a partial reduction in the onset and progression of EAE. We also found that IL-17 but not IFN-γ production by MOG peptide–stimulated splenocytes was compromised in mice receiving activated N1KO macrophages.}

Many reports have shown that Th1 and Th17 play important but distinct roles in this disease model. IL-12 is important for Th1 differentiation whereas IL-23 is essential for maintaining the Th17 phenotype (35). In our in vitro experiments, a deficiency in Notch1 did not have any effect on the expression of IL-12p70 and IL-23p19 mRNA, but a reduction in IL-6 and CD80 was detected. Previous data have shown that IL-23 is not essential for Th17 differentiation, whereas IL-6 and TGF-β are involved in this process (36), and the blockade of IL-6 can impair Th17 differentiation (37). In the EAE model, it has been demonstrated that IL-6 is important in this autoinflammatory disease; for example, mice that are defective in IL-6 production show a resistance phenotype to MOG peptide–induced EAE. Signaling through the A2B adenosine receptor in APCs such as dendritic cells enhances IL-6 production and its blockade helps improve the EAE phenotype (12, 38). Taken together, these data strongly implicate that IL-6 is important for Th17 cell differentiation and plays pivotal roles in EAE. We and others have previously shown that Notch1 is involved in IL-6 production in activated macrophages (20, 22). These observations, together with the findings in this study, suggest that Notch1 in activated macrophages might play an important role in regulating the outcome of T cell responses by regulating IL-6 (Th17) but not IL-12 (Th1) in the EAE model.
in decreased disease severity in our study. Additionally, N1KO macrophages may interfere with the activity of the endogenous macrophages, which may influence the outcome of the disease.

Understanding behavior of transferred macrophages in vivo will be a key piece of the puzzle to gain how adoptive transfer of N1KO macrophages reduces EAE severity. To this end, we performed a preliminary study tracking transferred macrophages in lymph nodes, omentum, peritoneal exudates, and spleen (Supplemental Fig. 3C). Transferred macrophages were found mainly in the omentum at 10 h after transferring but no difference was found between WT and N1KO macrophages. Further detailed analysis to pinpoint where the transferred macrophages migrate needs further investigation.

Previous in vitro studies have demonstrated that activation of macrophages by IFN-γ and LPS triggers cleavage of Notch receptors and the activation of Notch signaling; Notch signaling, in turn, directly or indirectly regulates production of proinflammatory cytokines such as IL-6 and IL-12p40/70. Inhibition of Notch signaling with a pharmacological inhibitor, GSI, which blocks γ-secretase activity, resulted in a reduction in IL-12p40/70. Our in vitro study revealed that the deletion of Notch1 in macrophages did not show any detectable effect on IL-12p40/70 production after activation by IFN-γ and LPS. These results imply that other Notch receptors such as Notch2, which is also highly expressed on macrophages, may play a redundant role in regulating IL-12p40 expression when Notch1 is deleted (21). In fact, we found a reduction in IL-6+ in GSI-treated N1KO macrophages, which implies that other Notch receptors may compensate for the loss of Notch1. Xu et al. (30), however, reported that macrophages from Notch1+/- mice expressed less Il12p40 than did the control WT macrophages upon LPS stimulation, and silencing Notch2 in these macrophages did not further reduce the level of Il12p40 mRNA, suggesting a dispensable role for Notch2. The discrepancy on the effect of Notch1 deletion on IL-12p40 expression between our results from N1KO macrophages and Xu et al., who used macrophages from Notch1 haploinsufficient mice, may be due to different approaches in generating the Notch1 deletion and in the use of LPS in their study versus LPS with IFN-γ in our system.
Unexpectedly, adoptive transfer of activated CSL/RBP-Jκ KO macrophages did not have any impact on the onset or severity of EAE. By comparing the cytokine production (IL-6 and IL-12p40/70) or costimulatory molecule (CD80, CD86) expression between N1KO and CSL/RBP-Jκ KO macrophages, it was interesting to note that only the amount of IL-6 produced by macrophages upon in vitro activation correlates with the disease severity.

CSL/RBP-Jκ can act as a transcriptional repressor in the absence of cleaved Notch receptors or as a transcriptional activator upon forming a multiple protein complex with Notch receptors (43). The transcriptional activation places this protein at the center of “canonical” Notch signaling where Notch receptors are cleaved upon ligand engagement. Recent evidence also emerged that suggests a noncanonical and nonnuclear mechanisms of Notch signaling, both of which are independent of the transcriptional activity of CSL/RBP-Jκ (44). Interestingly, targeted deletion of CSL/RBP-Jκ in some instances spontaneously increases the expression of genes that are actively repressed by CSL/RBP-Jκ (45). In tumor-associated macrophages, loss of canonical Notch signaling confers M2-like phenotypes (46). In our study, we found that unstimulated CSL/RBP-Jκ KO macrophages showed mixed phenotypes, as higher mRNA level of arginase 1 was observed, but not CD206 (Supplemental Fig. 4D, 4E). Because transfer of CSL/RBP-Jκ KO macrophages did not protect animals from EAE, it is unlikely that the switch from M1 to M2 plays a key role in our EAE study.

We observed a reduction in IL-12p40/70 upon the deletion of CSL/RBP-Jκ in macrophages, similar to the GSI treatment. However, the level of IL-12p40/70 production upon GSI treatment and deletion of CSL/RBP-Jκ showed only a partial reduction compared with the control, suggesting that the Notch signaling pathway may coordinate with other pathways such as NF-κB, C/EBP-β, and AP-1 in controlling the optimal production of IL-12p40/70 in IFN-γ/LPS–activated BMDMs (24). Because GSI treatment blocks both canonical and noncanonical Notch signaling, and because deletion of CSL/RBP-Jκ affects only canonical Notch signaling, it is possible that GSI treatment and deletion of CSL/RBP-Jκ may yield different outcomes. For IL-12p40/70 expression, a similar reduction was obtained from both approaches, suggesting that canonical Notch signaling may be responsible for IL-12p40/70 expression, whereas Notch1 may have a redundant role in this function. Furthermore, the expression of IL-6 may be regulated by noncanonical Notch signaling because only Notch1 deletion and GSI treatment, but not deletion of CSL/RBP-Jκ, affect its production (22).

NF-κB signaling and c-Rel, in particular, as a dimer with p50 subunit, is required for Il12p40 transcription in macrophages (27). Indeed, we observed that CSL/RBP-Jκ KO macrophages stimulated with IFN-γ/LPS exhibited reduced c-Rel nuclear accumulation, compared with the control WT macrophages. This result is consistent with that observed in the GSI-treated macrophages (23). In contrast, the activated N1KO macrophages showed a similar c-Rel pattern as the control macrophages. How CSL/RBP-Jκ and the activity of γ-secretase regulate the nuclear translocation of c-Rel upon LPS/IFN-γ treatment needs further investigation. In contrast, Xu et al. (30) reported in their system that activation of CSL/RBP-Jκ KO macrophages by LPS alone did not have any effect on the activation of the NF-κB signaling pathway. In their study, canonical Notch/RBP-Jκ signaling induced the expression of a transcription factor, IFN regulatory factor 8, which acts as a regulator of genes involved in the polarization of proinflammatory macrophages. A detailed mechanism is proposed in which CSL/RBP-Jκ selectively enhances IL-1R–associated kinase 2–dependent signaling via TLR4 to the MNK kinase. This event leads to downstream translation/initiation of control through elf4E. However, IFN regulatory factor 8 can be regulated by IFN-γ (47), and the discrepancy between the effect of CSL/RBP-Jκ deletion on the activation of NF-κB between our study and that by Xu et al. is possibly due to the difference in stimuli used, that is, LPS versus LPS/IFN-γ.

In T cells, Notch signaling functions to augment NF-κB signaling by facilitating nuclear retention of the NF-κB subunits p50 and c-Rel. This study revealed that Notch1 interacts directly with NF-κB and competes with IκBκ, resulting in the retention of NF-κB in the nucleus (48). In our study, a similar mechanism may operate in driving c-Rel nuclear retention in macrophages. This possibility needs further investigation.

Our study has reported a novel role for Notch1 in macrophages in EAE upon transfer into WT mice and for the Notch signaling pathway in regulating c-Rel activation and IL-12p40/70 expression in macrophages upon LPS/IFN-γ treatment. These findings indicate that Notch signaling in macrophages is important for the development of EAE and may have therapeutic implication for autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

References