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Autoamplification of Notch Signaling in Macrophages by TLR-Induced and RBP-J–Dependent Induction of Jagged1

Julia Foldi,*,† Allen Y. Chung,† Haixia Xu,‡ Jimmy Zhu,‡ Hasina H. Outtz,§,¶ Jan Kitajewski,§,¶ Yueming Li,‖ Xiaoyu Hu,‡,1 and Lionel B. Ivashkiv*,†,1

Several signaling pathways, including the Notch pathway, can modulate TLR activation to achieve responses most appropriate for the environment. One mechanism of TLR–Notch cross-talk is TLR-induced expression of Notch ligands Jagged and Delta that feed back to engage Notch receptors on TLR-activated cells. In this study, we investigated mechanisms by which TLRs induce Notch ligand expression in primary macrophages. TLRs induced Jagged1 expression rapidly and independently of new protein synthesis. Jagged1 induction was augmented by IFN-γ, was partially dependent on canonical TLR-activated NF-κB and MAPK signaling pathways, and elevated Jagged1 expression augmented TLR-induced IL-6 production. Strikingly, TLR-induced Jagged1 expression was strongly dependent on the Notch master transcriptional regulator RBP-J and also on upstream components of the Notch pathway γ-secretase and Notch1 and Notch2 receptors. Thus, Jagged1 is an RBP-J target gene that is activated in a binary manner by TLR and Notch pathways. Early and direct cooperation between TLR and Notch pathways leads to Jagged1-RBP-J–mediated autoamplification of Notch signaling that can modulate later phases of the TLR response. ***The Journal of Immunology*****, 2010, 185: 5023–5031.**

Macrophages are versatile cells that can respond to a wide array of environmental cues. Key roles that macrophages play include sensing of microbial pathogens, secretion of cytokines and inflammatory mediators, and presentation of Ags to T cells; thus, they are vital to the regulation of immune responses and inflammation. Macrophages express a variety of pattern recognition receptors, such as TLRs, which recognize highly conserved microbial structures and play an important role in activating innate immune responses (1). Triggering of macrophage pattern recognition receptors induces changes in gene expression that are translated into production and secretion of cytokines, which determine highly pathogen-specific immune responses. Activation of macrophages through TLRs leads to the production of inflammatory cytokines, such as TNF and IL-1, and also cytokines that regulate T cell differentiation, such as IL-6, IL-12, and IL-23; thus, macrophages help regulate the transition from innate to adaptive immunity (2). Ligands for TLR2 and TLR4 include bacterial lipopeptides and LPSs, respectively. Ligation of these receptors triggers activation of downstream signaling molecules, such as members of the MAPK family and IκB kinases (IKKs), ultimately leading to the activation of specific target genes by the transcription factors AP-1 and NF-κB (3).

The Notch pathway plays a central role in cell differentiation, proliferation, survival, and in the development of multiple tissues. The four mammalian Notch receptors (Notch1–4) and Notch ligands of the Jagged (Jagged1 and 2) and Delta-like families (DLL1, 2, and 4) are transmembrane proteins with extracellular domains important for juxtacrine ligand-receptor interactions (4). Notch signaling is activated after Notch receptor-ligand binding at the cell surface, which induces proteolytic cleavage by several proteases, including γ-secretase, which results in the release of the Notch intracellular domain (NICD) into the nucleus (5). In canonical Notch pathway activation, nuclear NICD binds the mammalian homolog of Suppressor of Hairless, recombinant-recognition-sequence-binding protein at the Jκ site (RBP-J, also known as CSL or CBF1), converting it from a transcriptional suppressor to an activator through the recruitment of coactivator proteins, such as Mastermind and CBP/p300 (6). Among the best-characterized transcriptional targets of canonical Notch signaling are members of hairy and enhancer of split (Hes) and hairy and enhancer of split with YRPW motif (Hey) families of transcriptional repressors. Hes and Hey proteins function as feedback regulators of Notch target gene expression (7). Alternative mechanisms of signaling in which the transcriptional effects of Notch are mediated in an RBP-J–independent manner have been described (8). Conversely, RBP-J also plays Notch-independent roles in development in *Drosophila* (9), as well as in mammals (10).

In the immune system, Notch has an essential role in specifying cell fate at multiple stages during T and B cell development (11), in regulatory T cell function (12), in Th cell differentiation at the APC–T cell interface (13–18), and in differentiation of $CD8^-$ splenic dendritic cells (DCs) (19). Notch ligands and receptors are induced on DCs by TLRs and other stimuli, and previous work

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Abbreviations used in this paper: BMDM, bone marrow-derived macrophage; DC, dendritic cell; GSI, gamma secretase inhibitor; Hes, hairy and enhancer of split; Hey, hairy and enhancer of split with YRPW motif; IKK, IκB kinase; NICD, Notch intracellular domain; RBP-J, recombinant-recognition-sequence-binding protein at the Jκ site; RT-PCR, real-time PCR; siRNA, small interfering RNA; Trunc, truncated; WT, wild-type.

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has demonstrated a role for DC-expressed Delta in promoting Th1 and Jagged in promoting Th2 differentiation (14). In addition to their role in development and differentiation, the Notch pathway has recently been implicated in macrophage activation. Several reports (20–26) have shown that TLRs induce macrophage expression of Notch receptors and ligands, with subsequent activation of canonical Notch signaling that contributes to cytokine production by as yet undefined mechanisms. In contrast with this indirect activation mediated by de novo expression of Notch ligands and receptors, our laboratory has demonstrated direct cooperation between the Notch and TLR pathways (i.e., independent of de novo protein synthesis) in the activation of canonical Notch target genes *Hes1* and *Hey1*, as well as inflammatory cytokine genes. NICD-mediated signals and TLR-induced p38- and IKK-mediated signals were integrated at target gene promoters in an RBP-J–dependent manner (21). This direct mechanism of Notch-TLR cooperation modulates early TLR responses, whereas indirect interactions mediated by newly expressed Notch ligands can sustain and modulate later phases of TLR-mediated macrophage activation.

Mechanisms by which TLRs induce Notch ligand expression and indirectly activate Notch signaling are not well understood. In this study, we investigated mechanisms and signaling pathways by which TLRs induce Notch ligand expression on primary macrophages. We mostly used human primary macrophages to maximize potential relevance of our findings to regulation at sites of inflammation in human diseases. However, we also used macrophages from knockout mice to take advantage of additional genetic data to support our conclusions. We found that TLRs induced expression of Notch ligands Jagged1, DLL1, and DLL4 in primary human and murine macrophages, and that induction of Jagged1 was the most robust. Induction of Jagged1 by TLRs was an early, direct event, and was augmented by IFN-γ; in contrast, induction of DLL1 and DLL4 was delayed, indirect, and suppressed by IFN-γ. Induction of Jagged1 was partially dependent on NF-κB and MAPK pathways. Strikingly, TLR-induced Jagged1 expression was strongly dependent on RBP-J and also on upstream components of the Notch pathway: γ-secretase, Notch1, and Notch2. Thus, TLR-induced expression of the Notch ligand Jagged1 is itself dependent on the Notch pathway, supporting a tight integration between TLR and Notch pathways. These findings highlight the importance of direct cooperation between TLRs and Notch, which not only contributes to the early phases of TLR-induced gene expression (21), but is important for the induction of Notch ligands that can modulate later phases of the TLR response.

Materials and Methods

Cell culture and reagents

The experiments using human and murine cells were approved by, respectively, the Hospital for Special Surgery Institutional Review Board and Institutional Animal Care and Use Committee. PBMCs were isolated by density-gradient centrifugation using Ficoll (Invitrogen, Carlsbad, CA), and monocytes were further purified by either positive magnetic separation of $CD14^+$ cells (Miltenyi Biotec, Bergisch Gladbach, Germany) or by adherence to plastic as described previously (27). Human monocytes were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) FBS (Hyclone, South Logan, UT) and M-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ) for 2 d to obtain monocyte-derived macrophages, unless otherwise noted. Murine bone marrow-derived macrophages (BMDMs) were obtained from bone marrow isolated from femurs and tibia of mice. Bone marrow was cultured in DMEM supplemented with 20% FBS and 10 ng/ml murine M-CSF (PrepoTech) for 4–5 d. Floating cells were discarded, and attached macrophages were replated in 12-well plates overnight before stimulation. Cell-culture–grade LPS, cycloheximide, SB203580, and SP600125 were purchased from Sigma-Aldrich (St. Louis, MO). Pam_3Cys was purchased from EMC Microcollections (Tuebingen, Germany). MG-132 and Bay11-7082 were obtained from EMD4Biosciences (San Diego, CA). Gamma secretase inhibitor (GSI)-34 was used as previously described (28, 29). Macrophages were primed with 100 U/ml IFN-γ overnight and preincubated with inhibitors for 1 h before treatment with TLR-L (1 ng/ml LPS or 10 ng/ml Pam_3Cys), unless otherwise indicated.

Mice

C57/BL6 and $Ticam1^{lps2/lps2}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *RBP-J*$^{flox/flox}$ mice were kindly provided by Tasuku Honjo (Department of Immunology and Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan). Mice with a myeloid-specific deletion of *RBP-J* were generated as described previously (21). Mice with an inducible deletion of RBP-J (*Rbpj*$^{flox/flox}$, *Mx1-Cre*) were generated by crossing *Rbpj*$^{flox/flox}$ animals to animals with an Mx1-driven *Cre* transgene on the C57/BL6 background (The Jackson Laboratory). Littermates with *Rbpj*$^{flox/flox}$ or *Rbpj*$^{flox/flox}$, *Cre/Cre* genotypes were i.p. injected with polyinosinic-polycytidylic acid (200 μg/mouse) three times in 5 d to induce deletion, and mice were used for experiments 2 wk later. $MyD88^{-/-}$ and $IRF3^{-/-}$ mice on C57/BL6 background were generously provided by Eric G. Pamer (Infectious Diseases Service, Department of Medicine, Immunology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY) and Erik Falck-Pedersen (Department of Microbiology and Immunology, Molecular Biology Graduate Program, Weill Medical College of Cornell University), respectively, and $Notch1^{tm1Grid}$/$Notch1^+$ mice were kindly provided by Thomas Gridley (The Jackson Laboratory).

mRNA isolation and real-time PCR

RNA was extracted from whole-cell lysates with an RNeasy Mini kit (Qiagen, Valencia, CA) and 0.5 μg total RNA was reverse transcribed with a First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). Quantitative real-time PCR (RT-PCR) was performed in triplicate wells with an iCycler IQ thermal cycler and detection system (Bio-Rad, Hercules, CA) using gene-specific primers. Threshold cycle numbers were normalized to triplicate samples amplified with primers specific for GAPDH.

Immunoblot analysis

Whole-cell lysates were prepared by direct lysis in SDS loading buffer. For immunoblot analysis, lysates were separated by 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane for probing with Ab. Polyclonal Abs against Jagged1 (C20), p38, and Shp2 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). IKKβ and Stat3 Abs were from Cell Signaling Technology (Danvers, MA).

Flow cytometry

Following stimulation, macrophages were harvested and stained with Abs specific to the extracellular portion of Jagged1 (R&D Systems, Minneapolis, MN) or an isotype control specific for mouse IgG1 (BD Biosciences, San Jose, CA), followed by a PE-conjugated secondary Ab specific for mouse IgG (Biosource International, Camarillo, CA). Cells were washed three times and analyzed on a FACScan flow cytometer (BD). Mean fluorescence intensities of individual cells was determined using Cell Quest software (BD).

Adenoviral transduction

Differentiated human macrophages were infected with an adenovirus encoding a phosphorylation-resistant superrepressor of I-kBα (Ad-I-κB-SR) or control adenovirus encoding GFP. Transduction efficiency was monitored by the fluorescence of GFP by microscopy.

RNA interference

Prevalidated IKKβ-, RBP-J–, Notch1-, and Notch2-specific small interfering RNAs (siRNAs) and nontargeting control siRNAs were purchased from Dharmacon (Lafayette, CO). siRNAs were transfected into primary human macrophages with the Amaxa Nucleofector (Lonza Cologne, Cologne, Germany) device set to program Y-001 and using the Human Monocyte Nucleofector kit (Lonza Cologne). Mouse BMDMs were transfected using the TransIT TKO transfection reagent (Mirus Bio, Madison, WI), according to the manufacturer's instructions.

Transient transfection and luciferase assay

The wild-type Jagged1 promoter, a truncated (Trunc) version of the promoter, which lacks the conserved NF-κB site at −3034 and a mutant promoter in which the NF-κB site was mutated (NF-κB mut), were cloned into the pGL3 enhancer Luciferase reporter vector as described previously (30) (generously provided by Christopher C.W. Hughes, Department of

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Molecular Biology and Biochemistry, School of Biological Sciences, Biomedical Engineering, the Henry Samueli School of Engineering, University of California, Irvine). RAW 264.7 cells were transfected in triplicate in 24-well plates with one of the reporter vectors and an expression plasmid encoding NICD1 (kindly provided by Raphael Kopan, Department of Molecular Biology and Pharmacology and Division of Dermatology, Department of Medicine, Washington University, St. Louis, MO) or equal amounts of a control empty vector and an internal control plasmid encoding Renilla luciferase (Promega, Madison, WI) using Lipofectamine LTX reagent (Invitrogen). On the next day, cell lysates were prepared and analyzed for Firefly and Renilla luciferase activity with a Dual-Luciferase Reporter Assay System (Promega).

Lentiviral expression of Jagged1

A lentivirus-based vector expressing the human Jagged1 cDNA was used to generate THP-1 (human acute monocytic leukemia cell line) cells stably expressing Jagged1 as described previously (31).

ELISA

Sandwich ELISA using paired human IL-6 Abs was performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA).

Statistical Analysis

All statistical analyses were performed by using the Student *t* test.

Results

Jagged1 is a direct TLR target gene and is superinduced by IFN-γ

TLR-induced expression of Notch ligands has been previously described in cell lines and murine DCs (14, 23, 32, 33), but underlying mechanisms are mostly unknown. To confirm and extend these results, we analyzed the expression of Notch ligands in primary human and mouse macrophages after TLR stimulation. Of the five mammalian Notch ligands, Jagged1, DLL1, and DLL4 mRNA were increased by both the TLR4 ligand LPS and the TLR2 ligand Pam_3Cys (Fig. 1*A*, Supplemental Figs. 1 and 2, and data not shown); similar results were obtained using LPS and Pam_3Cys in most experiments. Jagged1 induction was consistently observed in >40 healthy human donors (Fig. 1*A* and Supplemen-

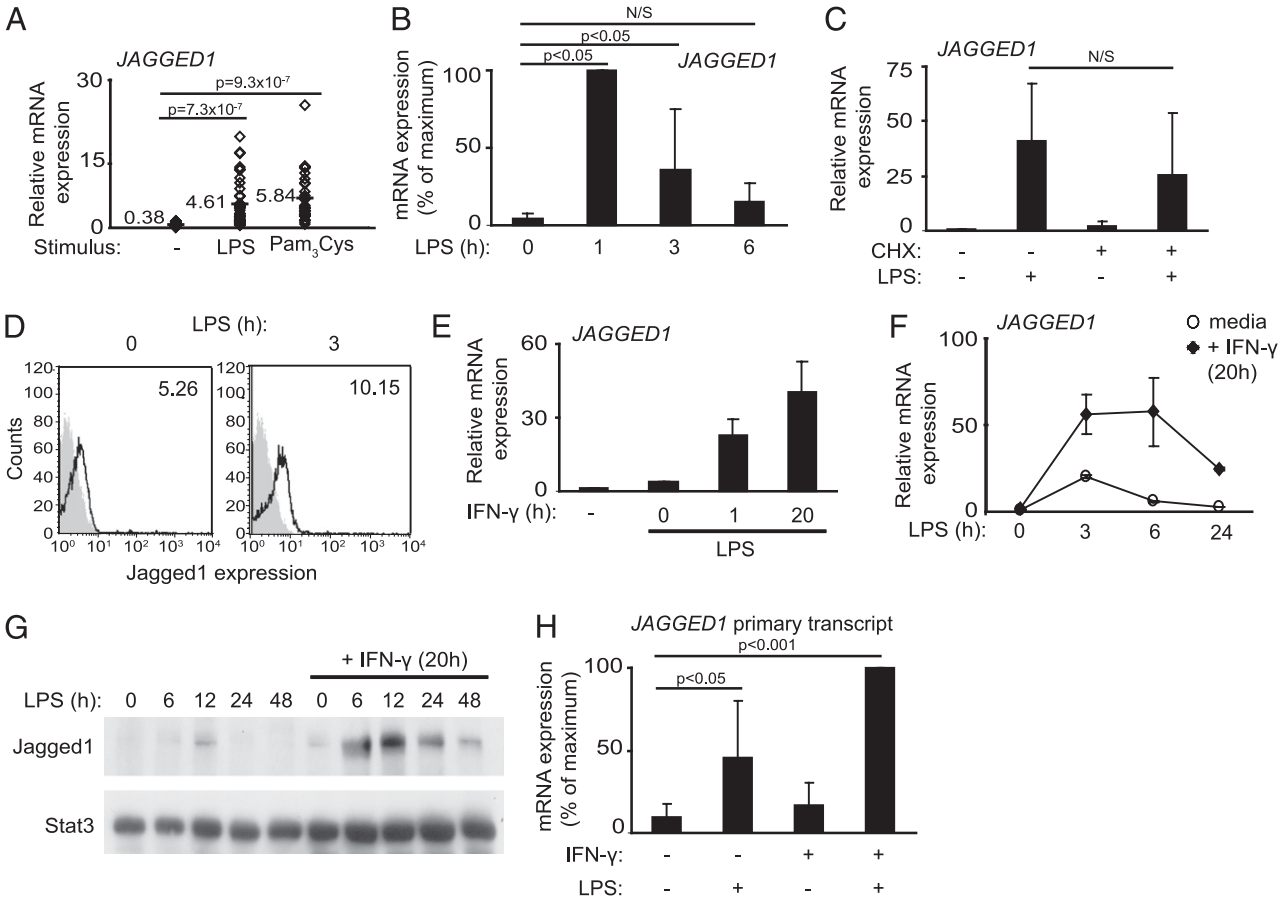

FIGURE 1. Jagged1 is a direct TLR target gene and is superinduced by IFN-γ. *A*, Primary human macrophages were stimulated with TLR ligands LPS (1 ng/ml) or Pam_3Cys (10 ng/ml) for 3 h. mRNA expression was measured by quantitative RT-PCR and normalized relative to the level of GAPDH mRNA. Relative mRNA levels are expressed as percentage of GAPDH mRNA for each human donor, and means are indicated for each condition. Data shown are pooled from 63 (untreated), 39 (LPS treatment), or 29 (Pam_3Cys treatment) independent human donors. Statistical analysis was performed using the unpaired Student *t* test. *B*, Human primary macrophages isolated from four donors were stimulated with LPS for the indicated times, and mRNA was measured. Data are graphed as the percentage of maximum mRNA expression after 1 h LPS stimulation. Statistical analysis was performed using the paired Student *t* test. *C*, Jagged1 mRNA measured from human macrophages treated with the protein synthesis inhibitor, cycloheximide (CHX; 20 μg/ml), for 30 min before stimulation with LPS for 1 h. Data are expressed as means ± SD of three independent experiments, and statistical analysis was performed using the paired Student *t* test. *D*, Flow cytometric analysis of Jagged1 surface expression from human macrophages treated with LPS for the indicated times. Data shown are from one representative donor out of more than five. Isotype control staining is shown by the shaded plot, and Jagged1 staining by the open line. Corrected mean fluorescence intensities (Jagged1-isotype) are indicated. *E*, Jagged1 mRNA from human macrophages primed with 100 U/ml IFN-γ and stimulated with LPS for 3 h. *F* and *G*, Human macrophages were primed with IFN-γ before stimulation with LPS, and mRNA (*F*) and protein (*G*) were measured. *H*, Human macrophages isolated from six donors were primed with IFN-γ before stimulation with LPS for 3 h. Primary transcript levels were measured using quantitative RT-PCR and primers designed for sequences located in the intronic regions of the *JAGGED1* gene. Data are expressed as percentage of maximum mRNA expression, which corresponds to the IFN-γ– and LPS-treated condition. Statistical analysis was performed using the paired Student *t* test.

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tal Fig. 1*A*; mean 12-fold induction). Although there was some variability among donors, differences in expression were highly statistically significant (Fig. 1*A* and Supplemental Fig. 1*A*). Induction of DLL1 and DLL4 was less robust and more variable (Supplemental Fig. 1*B*, 1*C* and data not shown). Furthermore, expression of Jagged1 and Dll4 was induced by LPS stimulation in murine BMDMs (Supplemental Fig. 2). A statistically significant induction of Jagged1 mRNA was apparent 1 h after LPS stimulation (Fig. 1*B*), which gradually returned to baseline level by ~24 h (data not shown). Induction of Jagged1 mRNA was intact in the presence of the protein synthesis inhibitor cycloheximide (Fig. 1*C* and Supplemental Fig. 1*D*), indicating that induction of Jagged1 does not require de novo protein synthesis, and thus Jagged1 is a direct primary target of TLR signaling in macrophages. TLR4-induced expression of Jagged1 was confirmed at the protein level using flow cytometry (Fig. 1*D*) and immunoblotting (Fig. 1*G*). An increase in cell surface expression of Jagged1 was consistently observed after 3 h of LPS treatment in >5 donors (Fig. 1*D*); cell surface expression at later time points was more variable, likely because of the balance between new synthesis and endocytic recycling of the ligand, which occurs during ligand-mediated activation of Notch receptors (34). Of note, we detected baseline Jagged1 expression in human macrophages (Fig. 1*D*, 0 h time point), which is consistent with low-level basal Notch signaling as we previously reported (21).

The Notch pathway contributes to induction of the inflammatory cytokines TNF and IL-6 whose expression is superinduced by IFN-γ. IFN-γ strongly enhanced TLR-induced expression of Jagged1 mRNA and protein (Fig. 1*E–G* and Supplemental Fig. 1*A*); in contrast, IFN-γ suppressed TLR-induced expression of DLL1 and DLL4 in most human donors (Supplemental Fig. 1*B*, 1*C*). TLR stimulation and IFN-γ induced Jagged1 expression, at least in part, at the level of transcription, as assessed by analysis of primary unspliced transcripts (Fig. 1*H*), a well-accepted measure of transcription rate (35). Overall, these results demonstrate robust induction of Jagged1 expression during inflammatory macrophage activation.

Regulation of Jagged1 expression by canonical TLR signaling pathways

Activation of TLR2 and TLR4 target genes is mediated by MyD88 and TRIF adaptors and the core downstream NF-κB, MAPK, and IFN regulatory factor pathways and signaling effector molecules (36). We next investigated the role of these core signaling pathways in TLR-induced expression of Jagged1. Combined inhibition of p38 and JNK had a partial suppressive effect on TLR4-induced Jagged1 expression, and MEK-ERK inhibition had minimal effect (Supplemental Fig. 3 and data not shown), suggesting a modest role for MAPK pathways in Jagged1 induction. IFN-γ augments TLR responses, in part, by increasing NF-κB activation (37), and we next investigated the role of NF-κB in TLR4- and IFN-γ–induced Jagged1 expression. Inhibition of NF-κB activation by the proteasome inhibitor MG-132 diminished Jagged1 mRNA (Fig. 2*A*) and protein (Fig. 2*B*) expression after TLR4 or IFN-γ and TLR4 stimulation. To corroborate these results, we used the well-established approach of blocking NF-κB activation using the I-κBα superrepressor (Ad-CMV-I-κB-SR) (38). Transduction of primary human macrophages with Ad-CMV-I-κB-SR, but not with control GFP-expressing adenovirus (Ad-CMV-GFP), nearly completely abrogated Jagged1 induction by TLR2 (Fig. 2*C*). As controls, I-κB-SR suppressed induction of the NF-κB–dependent gene *IL1B* but did not have a significant effect on the largely NF-κB–independent *DUSP1* gene (Fig. 2*C*). Experiments using TLR4 stimulation in macrophages in which NF-κB signaling was

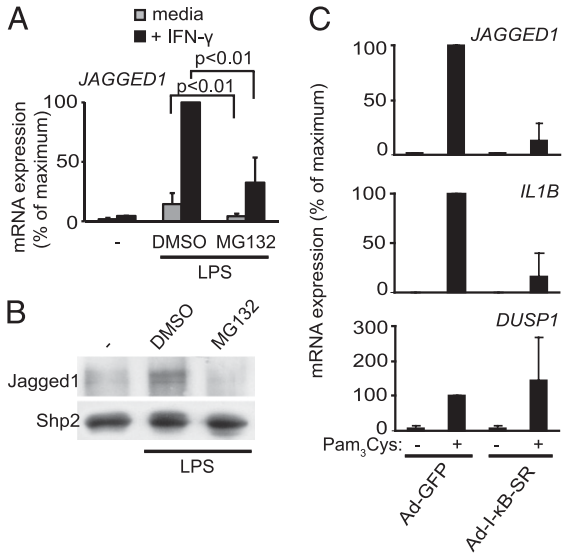

FIGURE 2. TLR induction of Jagged1 is dependent on the NF-κB pathway in human macrophages. *A*, Human primary macrophages isolated from four donors were primed with IFN-γ for 20 h. A proteasome inhibitor (MG-132, 20 μM) or vehicle control, dimethyl sulfoxide (DMSO), was added to the cells 1 h before stimulation with LPS for 3 h, and mRNA was measured. Data are expressed as means + SD of four independent experiments, and statistical analysis was performed using the paired Student *t* test. *B*, Human macrophages were treated with MG-132 for 1 h before stimulation with LPS for 6 h. Total protein lysates were blotted with Abs specific for Jagged1 and Shp2. *C*, Human macrophages isolated from two donors were infected with an adenovirus expressing a phosphorylation-resistant super repressor of I-kBα (Ad-I-κB-SR) or control adenovirus encoding GFP (Ad-GFP). Cells were then stimulated with Pam_3Cys for 3 h, and mRNA was measured.

blocked by the I-κBα superrepressor were not informative because stimulation through TLR4 led to significant cell death under these conditions. In contrast with the almost complete dependence of Jagged1 expression on NF-κB in human macrophages, inhibition of NF-κB in murine macrophages using two different approaches—inhibition of IKKs or inhibition of proteosomal processing of I-κBα—did not suppress TLR-induced expression of Jagged1, despite essentially complete inhibition of IL-6 expression (Fig. 3*A*). To further confirm this result, we used siRNA to knock down the expression of IKKβ, a kinase necessary for TLR-induced NF-κB activation, in murine BMDMs. Although we obtained an efficient knockdown of IKKβ using this method, Jagged1 induction by TLR4 stimulation remained intact (Fig. 3*B*). To exclude the possibility that the different contribution of NF-κB to TLR-induced Jagged1 expression in human and mouse macrophages could be because of the method of isolation of human monocytes by means of positive selection through the CD14 molecule, we tested LPS-induced Jagged1 expression in the presence of NF-κB inhibitors in monocytes isolated by plastic adherence. We found that similarly to monocytes isolated by positive selection, in adherence-selected monocytes, inhibition of the NF-κB pathway led to abrogated Jagged1 expression after TLR4 stimulation (Supplemental Fig. 4).

The surprising difference in the requirement for NF-κB between human and murine macrophages led us to test which upstream TLR pathways were important for Jagged1 expression. TLR2 signaling is entirely dependent MyD88, and we found that TLR2-induced Jagged1 expression was dependent on MyD88 (Fig. 3*C*). These results indicate that induction of Jagged1 is downstream of MyD88 and exclude the possibility of an unknown MyD88-independent pathway in TLR2-mediated Jagged1 expression. Pre-

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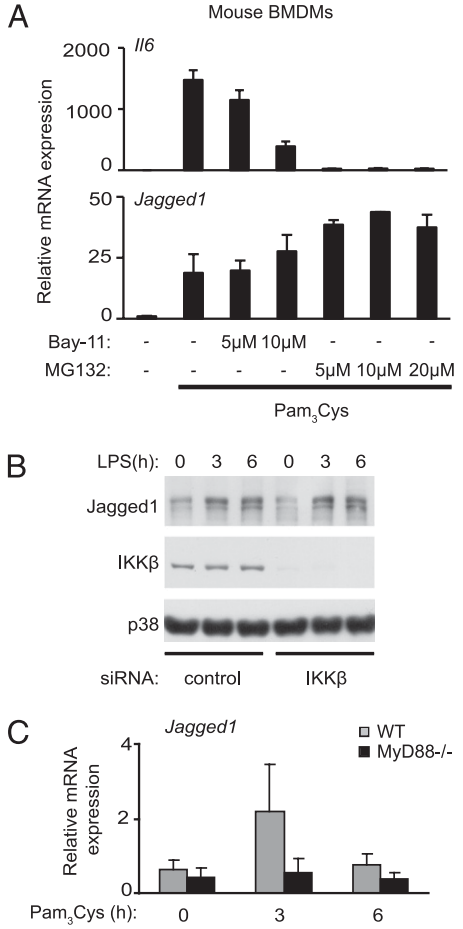

FIGURE 3. Role of canonical TLR signaling components in Jagged1 induction in murine macrophages. *A*, Mouse BMDMs from wild-type (WT) C57B/L6 mice were pretreated with the IKK inhibitor, Bay-11 (20 μM), or the proteasome inhibitor, MG-132, at different concentrations for 30 min. Cells were then stimulated with Pam_3Cys for 3 h, and mRNA was measured. Data shown are representative of three independent experiments. *B*, Mouse BMDMs were transfected with siRNA against IKKβ or control, nontargeting siRNA. Seventy-two hours posttransfection, cells were stimulated with LPS, and whole-cell lysates were immunoblotted with Abs against Jagged1, IKKβ, and p38. *C*, BMDMs from two WT and two $MyD88^{-/-}$ mice were stimulated with Pam_3Cys for 3 h, and mRNA expression of Jagged1 was measured.

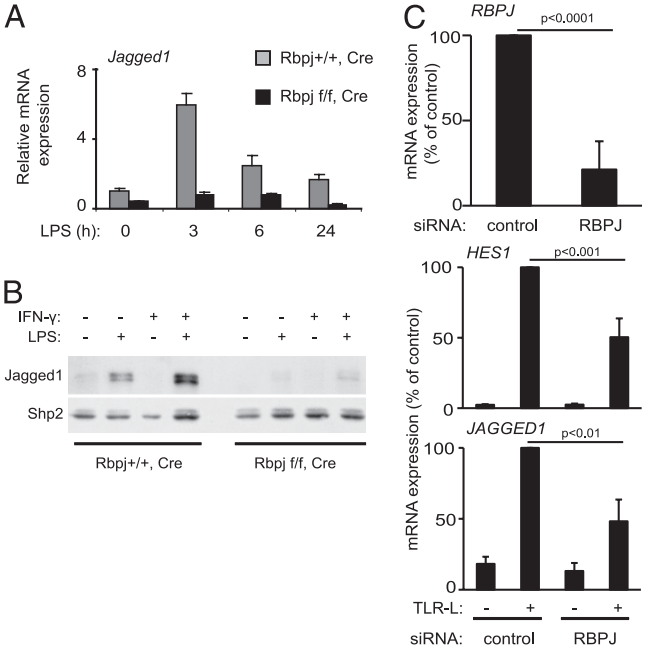

FIGURE 4. Jagged1 expression is dependent on the Notch pathway component RBP-J. *A*, Mouse BMDMs from *RBP-J$^{fl/fl}$, Cre* and *RBP-J$^{+/+}$, Cre* littermate controls were stimulated with LPS for the indicated times, and mRNA was measured. *B*, BMDMs were primed with 100 U/ml IFN-γ for 20 h before stimulation with LPS for 6 h. Total protein was immunoblotted with Abs against Jagged1 and Shp2. Data shown are representative of five experiments. *C*, Human macrophages were transfected with siRNA against RBP-J or control, nontargeting siRNA. Seventy-two hours posttransfection, cells were stimulated with TLR-L (LPS or Pam_3Cys) for 3 h, and mRNA was measured. Data are shown as percentage of maximal mRNA expression of the TLR-L–stimulated control cells and are expressed as means + SD of four independent experiments. Statistical analysis was performed using the paired Student *t* test.

vious microarray analysis showed that Jagged1 induction by TLR4 is abrogated in MyD88 and TRIF doubly deficient macrophages (39), and we found that MyD88 and TRIF played redundant roles in TLR4-induced Jagged1 expression, and IFN regulatory factor 3 was not required (Supplemental Fig. 5). Taken together, these results place Jagged1 downstream of canonical TLR adaptors MyD88 and TRIF, but exclude an important role for NF-κB in Jagged1 induction in mouse BMDMs.

Jagged1 expression is dependent on the Notch pathway component RBP-J

Because TLR-induced Jagged1 expression in murine BMDMs was independent of NF-κB and only partially dependent on MAPKs, we sought to identify the signaling pathways and molecules important for Jagged1 expression. We hypothesized that the Notch pathway might autoamplify, and thus may play a role in Jagged1 induction by TLRs. To address this, we first used a genetic approach by assessing Jagged1 expression in BMDMs deficient in RBP-J, the master regulator transcription factor of the Notch pathway. Because disruption of the *Rbpj* gene in the mouse results in early embryonic lethality (40), we used a conditional deletion approach utilizing an *Mx1-Cre* transgene to delete *Rbpj*. Macrophages obtained from the bone marrow of RBP-J–deleted animals exhibited an ~80% reduction in RBP-J expression and an ~60% reduction in baseline expression of Jagged1 mRNA (Fig. 4*A* and Supplemental Fig. 6*A*; $p < 0.05$) and protein (Fig 4*B*), suggesting that RBP-J is involved in maintaining baseline expression of Jagged1 in mice. Furthermore, TLR4-induced expression of Jagged1 mRNA and protein that was observed in control macrophages was strongly attenuated in RBP-J–deficient macrophages from genetically matched littermate control mice (Fig. 4*A*, 4*B*). We observed similar results using BMDMs from mice with a lysozyme M-Cre–mediated myeloid-specific deletion of *Rbpj* (data not shown). In contrast with Jagged1, TLR-mediated induction of Dll4 was not dependent on RBP-J (Supplemental Fig. 6*B*).

Next, we wished to corroborate the Jagged1 results in primary human macrophages. We used siRNA to knock down the expression of RBP-J: we obtained an ~70% reduction in RBP-J mRNA compared with macrophages transfected with a control, nontargeting siRNA (Fig. 4*C*, *top panel*). Although cells transfected with the control siRNA upregulated expression of the classical Notch target gene, *HES1,* in response to TLR stimulation, knockdown of RBP-J diminished this upregulation (Fig. 4*C*, *middle panel*). Similarly, knockdown of RBP-J resulted in a reduction of TLR-induced Jagged1 upregulation in human macrophages (Fig. 4*C*, *bottom panel*). Taken together, our results suggest that

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RBP-J plays an important role in TLR-induced Jagged1 expression in both mouse and human macrophages.

Notch signaling contributes to TLR-induced Jagged1 expression

Although RBP-J is best known as the master regulator transcription factor of the Notch pathway, RBP-J can also mediate signals by alternative signaling pathways (9, 10, 41–43). To investigate the requirement for the canonical Notch pathway in TLR-induced Jagged1 expression, we treated primary human macrophages with GSI-34, a chemical inhibitor of γ-secretase that prevents NICD generation. Inhibition of γ-secretase resulted in a marked reduction in TLR-induced Hes1 mRNA expression (Fig. 5*A*, *top panel*), indicating that the canonical Notch pathway was inhibited, as well as in Jagged1 expression (Fig. 5*A*, *bottom panel*, and 5*B*). In contrast with Jagged1, TLR-mediated induction of DLL4 was not dependent on γ-secretase (Supplemental Fig. 6*C*). We obtained similar results using GSI-34 to inhibit the Notch pathway in mouse BMDMs (data not shown). To more directly show a requirement for upstream Notch signaling in Jagged1 induction, we used RNAi to knock down expression of Notch1 and Notch2, the predominantly expressed Notch receptors on primary human macrophages, by ∼50 and 70%, respectively (Supplemental Fig. 7*A*). Although the efficiency of the knockdowns varied from experiment to experiment, even in the most efficient knockdown of either Notch1 or Notch2 individually, TLR-induced Jagged1 expression remained largely intact (Supplemental Fig. 7*B*), suggesting that both receptors contribute to gene induction. Corroborating this result, BMDMs heterozygous for a targeted mutation in the Notch1 gene (Notch1^{tm1Grid}/Notch1^{+}) (44) had no defect on Jagged1 induction despite an ∼80% reduction in Notch1 transcript levels (Supplemental Fig. 7*C*). Next, we knocked down expression of both Notch1 and Notch2 simultaneously using RNAi. Even though the efficiency of the knockdowns was variable among experiments, even a partial reduction in Notch1 and Notch2 expression significantly abrogated TLR-induced Jagged1 expression (Fig. 5*C*), suggesting that both Notch1 and Notch2 contribute to Jagged1 upregulation in cooperation with the TLR pathway. A role for the Notch pathway in the upregulation of Jagged1 expression was also supported by a gain-of-function approach in which forced expression of NICD1 stimulated activation of a human Jagged1 promoter-driven reporter construct in a dose-dependent manner (Fig. 5*D*). Similar to the wild-type Jagged1 promoter, a truncated version of the promoter with a previously described and highly conserved NF-κB site (30) deleted, as well as a full-length promoter with a mutation in the NF-κB binding site, were activated by increasing amounts of NICD1, suggesting that under conditions mimicking high-level Notch pathway stimulation, upregulation of

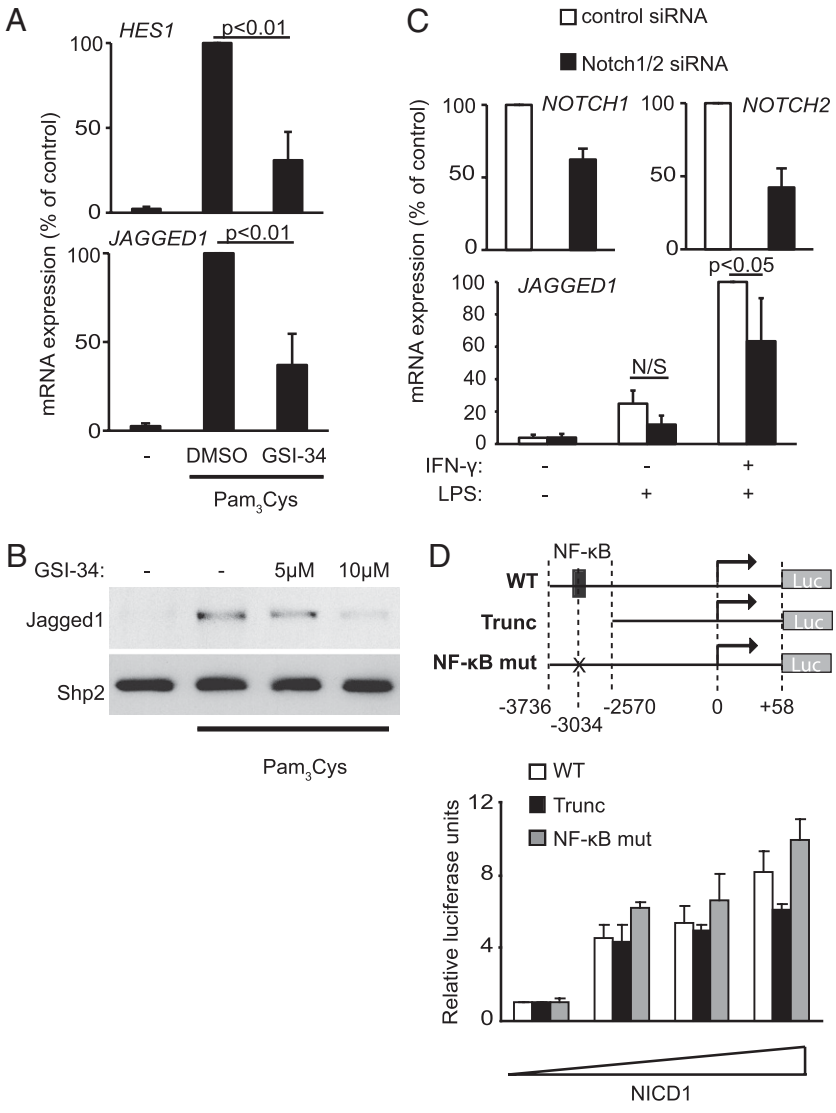

FIGURE 5. Notch signaling contributes to TLR-induced Jagged1 expression. *A* and *B*, Human primary macrophages were treated with either vehicle control or GSI-34 (10 μM) for 48 h before stimulation with Pam_3Cys for (*A*) 3 or (*B*) 6 h. Jagged1 was measured by (*A*) quantitative RT-PCR or by (*B*) immunoblot. Statistical analysis was performed using the paired Student *t* test. *C*, Human macrophages were transfected with siRNAs against both Notch1 and Notch2 (Notch1/2) or control, nontargeting siRNA. Seventy-two hours after transfection, cells were primed with IFNγ and stimulated with LPS for 3 h, and mRNA was measured. Data are shown as percentage of maximal mRNA expression of control cells, and are expressed as means + SD of three independent experiments. Statistical analysis was performed using the paired Student *t* test. *D*, The human wild-type (WT) Jagged1 promoter, a truncated (Trunc) version of the promoter, which lacks the conserved NF-κB site at −3034 and a mutant promoter in which the NF-κB site was mutated (NF-κB mut), were transfected into RAW 264.7 cells and cotransfected in triplicate with increasing amounts of an NICD1 expression plasmid or empty vector. Cell lysates were analyzed for luciferase activity 24 h after transfection. Data shown are representative of three independent experiments. There was no statistically significant difference between the induction of the three promoter constructs by NICD1.

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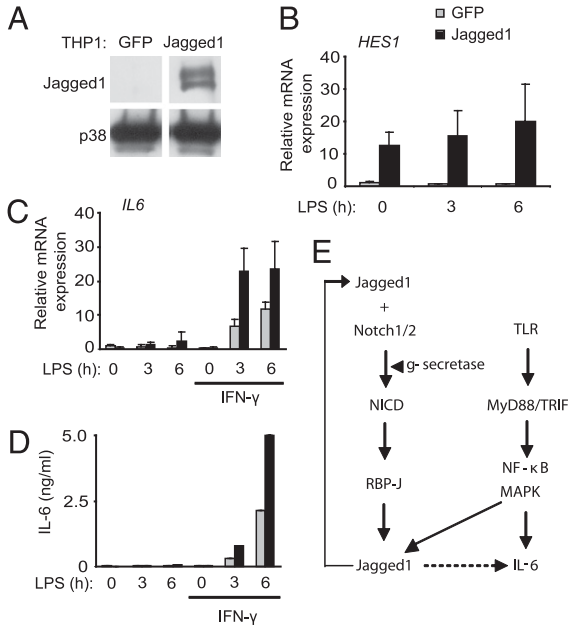

FIGURE 6. Jagged1 contributes to TLR-induced IL-6 expression. THP-1 cells were stably transduced with a lentivirus expressing full-length human Jagged1 or a control lentivirus expressing GFP. *A*, Immunoblot of whole-cell lysates from GFP- or Jagged1-expressing THP-1 cells. *B* and *C*, THP-1 cells were primed with 100 U/ml IFN-γ for 20 h followed by stimulation with 1 μg/ml LPS for the indicated times. *D*, ELISA of culture supernatants from THP-1 cells differentiated into macrophages using 0.5 μM PMA overnight followed by stimulation as in *B* and *C*. Data shown are representative of four experiments. *E*, In primary human macrophages, basal signaling through the constitutively expressed receptors, Notch1 and Notch2, as well as concomitant activation of NF-κB and MAPK by TLRs are involved in robust induction of Jagged1. Downstream of Notch, the canonical Notch signaling machinery, including cleavage of NICD by γ-secretase and activation of RBP-J, are important, whereas the signal from TLRs can be mediated by either MyD88 or TRIF, for maximal Jagged1 induction. Notch- and TLR-mediated induction of Jagged1 leads to the amplification of Notch signaling, which also contributes to the production of the cytokine, IL-6.

Jagged1 can occur independently of this upstream NF-κB site (Fig. 5*D*). Collectively, these results show that canonical Notch signaling positively regulates the expression of the Notch ligand Jagged1 in macrophages.

Jagged1 contributes to Hes1 and TLR-induced IL-6 expression

The capacity of individual Notch ligands to contribute to TLR responses is not known. To elucidate a potential role for Jagged1 in macrophage TLR responses, we expressed Jagged1 in the THP-1 human monocytic cell line using lentiviral transduction (Fig. 6*A*). In contrast with primary macrophages (21), TLR stimulation of control GFP-transduced THP-1 cells did not result in increased expression of the canonical Notch target gene *HES1* (Fig. 6*B*), likely because of the absence of basal Notch signaling in THP-1 cells (J. Foldi, unpublished data); such basal signaling is required for this induction in primary macrophages (21). In contrast, THP-1 cells transduced to express Jagged1 showed increased Hes1 expression both at baseline and after TLR stimulation (Fig. 6*B*), confirming that Jagged1 can activate the canonical Notch pathway in THP-1 cells and suggesting that Jagged1 can augment TLR responses. Furthermore, Jagged1 augmented IFN-γ– and LPS-induced expression of IL-6 mRNA and protein (Fig. 6*C*, 6*D*). These results support a role for Jagged1 in augmenting TLR-induced gene expression.

Discussion

TLRs play an important role in activating the innate immune system via recognition of microbial pathogens. Recently, the Notch pathway has been shown to cooperate with TLR signaling pathways to induce expression of Notch target genes and to regulate production of canonical TLR target genes, such as cytokines of the IL-6 and IL-12 family (21). In this study, we demonstrated that TLR and Notch pathways also cooperate to induce robust expression of the Notch ligand Jagged1. TLR-induced Jagged1 expression was strongly dependent on Notch pathway component RBP-J and, at least in part, on upstream γ-secretase and Notch1 and Notch2 receptors that are activated by basally expressed Notch ligands in our system. Canonical TLR signaling via NF-κB (in human macrophages) and MAPKs (in human and mouse macrophages) contributed to Jagged1 expression, which was superinduced by IFN-γ. Induced Jagged1 can further cooperate with TLRs at later time points after TLR stimulation to further augment expression of cytokines, such as IL-6 (Fig. 6*E*), and can also directly drive T cell differentiation (13–18). Our findings support a model of tight integration between the TLR and Notch pathways (Fig. 6*E*), in which binary activation of Jagged1 by TLRs and Notch early after TLR stimulation results in expression of Jagged1 that can then augment and sustain the expression of the subset of TLR-inducible genes that are Notch dependent. Thus, Notch cooperates with TLRs to induce an autoamplification loop that allows for sustained Notch signaling.

Activation of Notch signaling by TLRs has been previously reported (20–26). The dominant paradigm has been indirect and sequential activation, in which TLRs induce expression of Notch ligands, which then engage Notch receptors and activate canonical Notch signaling that acts in parallel with TLR signals. Our previous work has shifted this paradigm by showing direct cooperation between Notch and TLR pathways in the activation of canonical Notch targets Hes1 and Hey1 (21). We have now found that TLRs rapidly induce Jagged1 expression in a direct manner that is independent of new protein synthesis and is dependent on RBP-J and basal Notch signaling. These findings extend the concept of direct cooperation to include induction of the Notch ligand Jagged1, suggesting that indirect activation of Notch signaling by TLRs via induction of Notch ligands is at least partially dependent on initial direct cooperation leading to Jagged1 expression. Thus, TLR and Notch pathways are tightly integrated, and TLRs regulate the amplitude of Notch signaling in macrophages by modulating Notch ligand expression.

The regulation of Notch ligand expression is not well understood, and the basis for their differential expression has not been explored. In developmental systems, Notch ligands appear to be constitutively expressed and further modulated by growth factors, such as vascular endothelial growth factor. The Notch pathway itself can modulate expression of its own ligands: in some systems, Notch ligands are downregulated on cells that receive a Notch-mediated signal (45–48). In the immune system, TLRs have been shown to coordinately induce expression of Jagged and Delta ligands in mouse DCs (13, 14, 32, 33, 49). Our results extend understanding of Notch ligand expression by showing differential regulation of Delta and Jagged in macrophages activated by TLR ligands and IFN-γ. TLR-induced Delta expression was indirect (required de novo protein synthesis), was independent of Notch and RBP-J, and was suppressed by IFN-γ. In contrast, Jagged1 induction by TLRs was direct, dependent on Notch and RBP-J, and superinduced by IFN-γ. Thus, the pattern of TLR-induced Notch ligand expression (Jagged versus Delta) on activated macrophages is determined by whether cells have received prior signals from

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Notch ligands, which can be provided by macrophages themselves or in a juxtacrine manner by interaction with endothelial or stromal cells. In addition, Jagged expression will predominate over Delta expression on fully activated macrophages that have been exposed to IFN-γ. Because Jagged and Delta have different effects on endothelial and T cells (14), this differential pattern of expression has important implications for the effects of macrophages on interacting cells. In this regard, high Jagged1 expression on IFN-γ–primed and TLR-activated macrophages may contribute to the fully activated phenotype of these cells, for example, by augmenting production of inflammatory cytokines, including IL-6 and IL-12 (21, 50). In addition, in this model, suppression of TLR-induced Delta ligands by IFN-γ might ensure the specificity of macrophage activation. Furthermore, it has been suggested that Notch could cooperate with cytokines produced by APCs to optimize Th differentiation (51), and in the absence of IL-12, DLL4 expressed on $CD8^-$ DCs has been shown to direct Th1 development in vivo (18). In future work, it will be interesting to determine whether Jagged and Delta have different effects on macrophage phenotype and cytokine production, as well as their interactions with other cell types.

An emerging area of research is understanding selective expression of functionally related subsets of TLR-inducible genes. Such selective regulation of functional components of a TLR response is required to fine-tune responses to be most appropriate for specific pathogens, and to limit toxicity whereas preserving host defense. The best understood mechanisms for such gene-specific regulation involve regulation at the level of chromatin and induction of transcription factors that regulate subsets of TLR-inducible genes (52–55). An alternative mechanism for achieving selective regulation of TLR-inducible genes suggested by our work is the modulation of the core TLR response by cooperating signaling pathways that selectively target a subset of TLR-inducible genes. Notch pathway input is required to augment TLR-induced expression of Jagged1 and canonical Notch target genes, such as Hes1, and to modulate expression of IL-6; ∼10% of TLR-inducible genes is dependent on RBP-J for full induction (X. Hu and L.B. Ivashkiv, unpublished data). Thus, modulation of the Notch pathway provides a means for selectively tuning and focusing TLR responses.

An important question is how TLR and Notch pathways cooperate to induce Jagged1. Our data support a dominant role for NF-κB, downstream of TLRs, in inducing Jagged1 in primary human macrophages, which is in agreement with previous studies implicating NF-κB in Jagged1 expression downstream of TNF-α and PMA and ionomycin in other systems (30, 56). One striking finding of our study is that TLR-induced expression of Jagged1 in mouse macrophages is largely independent of NF-κB, highlighting a fundamental difference in gene regulation between the human and murine systems. Although this difference is not yet understood, our results using human *JAGGED1* promoter reporter gene assays with mutated and deleted NF-κB sites (30) suggest that even the human *JAGGED1* promoter can be activated by maximal activation of the Notch pathway (such as occurs with NICD overexpression) without the need for an NF-κB signal. Thus, it is possible that, in human cells, NF-κB is required to work in synergy with a suboptimal physiological Notch signal to the *JAGGED1* promoter, whereas in murine cells, Notch signaling is more effectively transmitted to the *Jagged1* locus, possibly because of NICD interactions with additional DNA-binding proteins or chromatin modifications, and therefore bypasses the requirement for NF-κB signaling.

Induction of Jagged1 in murine macrophages was dependent on MyD88 and TRIF (39), and thus on well-established canonical TLR signaling pathways, but the downstream signals that induce Jagged1 expression in mouse macrophages and their targets remain to be uncovered. MAPKs appear to play a partial role, possibly by targeting RBP-J or histones at the *Jagged1* locus (21), and the role of additional TLR-induced signaling pathways, such as the PI3K–Akt pathway, remains to be investigated. Recent evidence implicated inducible NO synthase in the induction of Notch pathway components by Mycobacteria (22), but we found that inducible NO synthase deficiency had no effect on TLR-induced or TLR plus IFN-γ–induced Jagged1 expression (data not shown). Overall, our data support cooperation between Notch and TLR pathways in Jagged1 induction that is mediated by RBP-J and NF-κB in human macrophages, and by RBP-J and MAPKs and additional as yet unknown TLR-induced signals in mouse macrophages.

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Disclosures

The authors have no financial conflicts of interest.

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