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Noncanonical Notch Signaling Modulates Cytokine Responses of Dendritic Cells to Inflammatory Stimuli

Madeleine E. Gentle, Anna Rose, Laurence Bugeon, and Margaret J. Dallman

Dendritic cells (DCs) act to bridge the innate and adaptive immune responses. In their immature state, DCs are highly specialized for Ag uptake and detection of pathogen-associated molecular patterns through the expression of a wide range of receptors on their surface, such as the TLR family, and form a surveillance network across virtually all tissues (1). Upon detection of a pathogenic stimulus, DCs are transformed into potent inducers of naïve T cell activation and differentiation, and further influence T cell fate via the production of polarizing cytokines (2, 3). The cytokine signature of mature DCs depends on the type of pathogen perceived and the environment it was perceived in; however, little is known about how DCs integrate different types of pathogen and environmental signals, and how these signals determine the resulting DC cytokine signature.

Notch signaling during embryogenesis provides both temporal and spatial cues that are critical for embryonic development of all animals (4). Temporal and spatial regulation of Notch activation are achieved through differential expression of Notch ligands, receptors, and modulators in a tissue and developmental stage-specific manner. In adult organisms, Notch signaling continues to play a vital role in regulating differentiation decisions in self-renewing tissues such as the hematopoietic system (5, 6), and Notch components continue to be expressed across the body in a tissue and differentiation stage-dependent manner.

Ligation of Notch at the cell surface, by a cell bearing Notch ligands, induces γ-secretase–dependent cleavage that releases the intracellular domain of Notch (N\textsuperscript{ICD}) from the plasma membrane allowing translocation to the nucleus. Notch signaling directly activates expression of target genes through γ-secretase interaction with the transcriptional switch recombination signal binding protein for immunoglobulin κ J (RBP\textsubscript{κ}J), and indirectly mediates repression of other genes through inducing expression of the transcriptional repressor Hes1 (7, 8). In addition to this canonical signaling route, alternative pathways involving RBP\textsubscript{κ}J- and γ-secretase–independent signaling have been reported (9–13). However, relatively little is known about the molecules involved in alternative signaling or their involvement in the immune system.

DCs express both Notch ligands and receptors (14, 15), and ligation with recombinant ligands has been shown to induce surface expression of MHC class II, CD80, and CD86 expression (16, 17). However, Notch ligation did not induce CD40 expression and induced a distinct cytokine profile characterized by IL-2 in the absence of more proinflammatory cytokines such as IL-6, IL-12, or IL-23 (16). These Notch conditioned DCs could sustain proliferation and suppressive activity of CD25\textsuperscript{+} regulatory T cells and induced IL-17 expression in these cultures in an IL-2–dependent manner (16). We set out to establish whether Notch signaling could influence DC maturation to proinflammatory stimuli. We demonstrate that DCs stimulated simultaneously with Notch and TLR ligands have a distinct cytokine profile compared with DCs stimulated with either stimulus alone. Modulation of DC responses to TLR ligation occurred via a noncanonical Notch signaling pathway and was dependent on PI3K activity. Further, we demonstrated that noncanonical Notch signaling can increase PI3K activity in DCs; thus, our data support a model where the Notch and TLR pathways interact in DCs to influence the cytokine profile of these cells via convergence on PI3K.
Materials and Methods

Animals

Male C57BL/6 mice (Harlan) were maintained in accordance with U.K. Home Office guidelines (Animals [Scientific Procedures] Act 1986).

Bone marrow-derived DC cultures

Bone marrow-derived DCs (BMDC) were prepared from wild-type or IL-10−/− mice (obtained from Ana Rosario, National Institute of Medical Research, London, U.K., with permission from the Mouse Genetics Cologne Foundation, Cologne, Germany) as previously described (16).

Recombinant Notch ligand rat Jagged1-humanFc fusion protein (R&D Systems) or human Hes1 (Sigma) control was immobilized onto tissue culture plates via overnight incubation (10 μg/ml). TLR ligands were added as follows: 100 ng/ml LPS from Escherichia coli O26:B6 (Sigma), 0.5 μg/ml CpG 1826, 1 μg/ml Pam3CSK4, 100 μg/ml polyinosine-polycytidylic acid (poly [I:C]) (InvivoGen). BMDC were pretreated with inhibitors for 30–60 min as specified; 2 μM BAY11-7082, 10 μM LY294002, 5 μM SB203580, 10 μM SP600125, 25 μM U0126, and 10 μM N-[3-(5-difluorophenacyl)-l-allyl]-5-phenylglycine t-butyl ester (DAPT; Calbiochem), and 10 μg/ml cycloheximide (Sigma).

Campylobacter jejuni 11168H was cultured in a variable atmosphere incubator (Don Whitley Scientific, Shipley, U.K.) under microaerobic conditions (5% O2, 15% CO2, 10% CO2) at 37°C. Initial cultures grown for 24 h on complete blood agar plates supplemented with Skirrow selective supplement (Oxoid, Basingstoke, U.K.) were used to inoculate flasks of MEM-supplemented with 20% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were either starved overnight or cultured with DAPT or DMSO (vehicle control) for 30 min at 37°C and then plated on 6-well plates precoated with Jagged1-Fc or IgG1 control with or without LPS. After 30–60 min, cells were harvested on ice and lysed in 40 μl cold lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% [v/v] Triton X-100, supplemented with 1X Complete-mini-protease inhibitors [EDTA free; Roche], 0.2 mM sodium orthovanadate, 10 mM sodium fluoride, 2 mM sodium pyrophosphate, and 100 mM calcium A) for 20 min. Protein (25 μg) was loaded onto precast 10% Tris glycine gels (Invitrogen) and, after electrophoresis, transferred onto nitrocellulose membranes (Hybond ECL; Amersham) and blocked with 5% skimmed milk (Marvel) in TBS with 0.3% Tween 20. Membranes were incubated with anti-phospho-Akt Ab (Ser473), anti-pan Akt Ab (both from New England Biolabs), or anti-actin Ab (Millipore) overnight at 4°C. Membranes were washed and then incubated with an HRP-conjugated secondary Ab (GE Healthcare) for 2 h. After washing, membranes were incubated with SuperSignal West Pico Chemiluminescence Substrate (Pierce) for 5 min and chemiluminescence was visualized on a ChemiDoc XRS+ system (BioRad).

Statistics

All analyses were carried out using GraphPad Prism (GraphPad Software). A nonparametric one-way ANOVA (Kruskal–Wallis test) was used in Fig. 1. For experiments with inhibitor, C. jejuni, or comparing wild-type and knockout, a two-way ANOVA was used instead.

Results

Notch signaling modulates DC responses to LPS

To investigate whether Notch signaling could alter the response of DCs to proinflammatory stimuli, BMDC were stimulated with immobilized recombinant Notch ligand Jagged1-Fc (or whole human IgG1 as an Fc control with similar steric constraints and m.w. to the Jagged1 fusion protein) with or without LPS. Relative mRNA transcript levels at 4 h and secreted cytokines after 24 h are shown in Fig. 1. Hes1, as a known direct target of canonical Notch signaling (18), was measured as a readout for Notch activation. As expected, Jagged1-Fc induced expression of Hes1 mRNA transcripts (Fig. 1B, see also Fig. 2 and Supplemental Fig. 1). Hey1, Hey5, and Delt1 have been reported to be Notch targets in other cell types; however, these were not present at detectable levels in BMDC after stimulation with Jagged1-Fc, LPS, or both (data not shown). The relative change in Hes1 mRNA transcripts induced by Jagged1-Fc varied considerably, ranging from 2- to 30-fold, reflecting the fact that Hes1 transcript and protein expression oscillate with a periodicity of 2 h as a result of Hes1 protein acting as a repressor at its own promoter (19), and thus relative Hes1 mRNA levels will vary with time and degree of synchronization between cell types. Addition of the protein synthesis inhibitor cycloheximide (to prevent Hes1-mediated self-repression) to these cultures confirmed that Jagged1-Fc robustly induces Hes1 transcript levels (Supplemental Fig. 1A). Furthermore, pretreatment with the γ-secretase inhibitor DAPT confirmed that expression of Hes1 mRNA in response to Jagged1-Fc requires canonical Notch signaling (Supplemental Fig. 1B), thus demonstrating that Jagged1-Fc mediates a robust canonical Notch signal.

Jagged1-Fc alone induced a significant increase in both mRNA and secreted IL-10 and IL-2, but did not significantly induce either mRNA or secreted protein levels of the more proinflammatory IL-12, IL-6, or TNF-α (Fig. 1 and Bugeon et al. [16]). LPS alone increased levels of mRNA transcripts and secreted IL-10, IL-2, IL-12, IL-6, and TNF-α compared with control (Fig. 1). IL-12p35 transcripts levels were highly variable and were not significantly induced by LPS, which may be consistent with the observation that p35 is mainly regulated at the posttranscriptional level by a series of posttranslational modifications (20, 21).

Compared with LPS alone, simultaneous stimulation with Jagged1-Fc and LPS resulted in significantly enhanced expression of IL-10 and IL-2 mRNA and protein, whereas secretion of IL-12 was significantly inhibited (Fig. 1). IL-12p35 and p40 mRNA levels were not significantly inhibited by Jagged1-Fc + LPS (compared with LPS alone), suggesting that Jagged1-Fc inhibits LPS-induced IL-12 secretion by a posttranscriptional mechanism. IL-6 and TNF-α mRNA levels were generally increased by combined Jagged1-Fc and LPS stimulation compared with LPS alone, whereas protein levels were usually reduced, but this was variable and was not statistically significant across all experiments performed. Further, as others have suggested that Hes1 may regulate IL-6 and TNF-α mRNA expression (22), we assessed transcript levels in the presence of cycloheximide and found levels unchanged by inhibition of protein translation (Supplemental Fig. 1A).

Analysis of Notch receptor transcript levels demonstrated that Notch2 is the predominant Notch receptor present in BMDC, whereas Notch1 is expressed at only relatively low levels, and Notch3 and Notch4 mRNA transcripts are barely detectable (Supplemental Fig. 2A). Notch receptor transcript levels were unaffected by stimulation with TLR ligands (data not shown).

Measurement of mRNA decay rates after stimulation with Jagged1-Fc + LPS or LPS alone for 4 h demonstrated that Jagged1-Fc does not significantly alter the stability of IL-10, IL-2, or IL-12 mRNA (data not shown), suggesting that Jagged1-Fc affects IL-12 expression either at the translational level or through regulation of posttranslational modifications, while regulating transcription of IL-10 and IL-2.

In agreement with the results obtained using recombinant Jagged1-Fc as a Notch stimulus, culturing BMDC with a cell line...
overexpressing the Notch ligand Jagged-1 also enhanced expression of IL-10 in response to LPS (Supplemental Fig. 2B). The parental cell line had no effect on LPS-induced IL-10 expression, nor did these cells make IL-10 themselves. Thus, changes in DC cytokine expression are a result of Notch signaling and not Fc-mediated effects or artifacts caused by immobilization of Notch ligand to tissue culture plastic.

In addition, Jagged1-Fc enhanced LPS-induced surface expression of CD40, increasing the percentage of CD40+ DCs from 71 to 89% and the mean fluorescence intensity from 87 to 161 (Supplemental Fig. 3). Compared with IgG1 control, Jagged1-Fc induced a small increase in CD40 expression (from 41 to 58%). However, Jagged1-Fc did not significantly increase CD80, CD86, or MHC class II surface expression compared with IgG1-stimu-

Cross talk between the Notch and TLR pathways was not limited to modulation of LPS-induced cytokine and CD40 expression; combined Jagged1-Fc and LPS stimulation significantly inhibited Hes1 mRNA transcript levels compared with cells stimulated with Jagged1-Fc alone (Fig. 1B). Suppression of Hes1-mediated oscillations with cycloheximide confirmed that LPS robustly inhibits induction of this canonical Notch target gene by Jagged1-Fc by ~4-fold (Supplemental Fig. 1A).

Our data suggest Notch and TLR4 signaling in response to Jagged1 and LPS, respectively, can interact in DCs and this interaction is bidirectional, resulting in a modulated cytokine response characterized by enhanced IL-10 and IL-2 but reduced IL-12 expression. BMDC were stimulated with plate-bound Jagged1-Fc (Jgd1) or IgG1 (Fc control) in the presence or absence of 100 ng/ml LPS. (A) mRNA transcripts were measured after 4 h by quantitative real-time PCR (qRT-PCR). (B) Accumulation of cytokines in the supernatant after 24 h was measured by ELISA. Data are mean ± SD of triplicates and are representative of at least six independent experiments. Friedman test with Dunn’s posttest was used to statistically compare each stimulus; p < 0.05 was considered significant. *p < 0.05, **p < 0.01, ***p < 0.001. ND, Not detected.
Notch MODULATES TLR SIGNALING IN DENDRITIC CELLS

FIGURE 2. Jagged1-Fc can modulate cytokine expression in response to various TLR ligands and in response to C. jejuni. (A) BMDC were stimulated with Jagged1-Fc (black bars) or IgG1 (white bars) in the presence of 100 ng/ml LPS, 0.5 μg/ml CpG, 1 μg/ml Pam3CSK4 (P3C4), 100 μg/ml poly (I:C) (PIC), or nothing (None) for 24 h, and accumulation of IL-10, IL-2, and IL-12 in the supernatant was measured by ELISA. (B) BMDC were stimulated with Jagged1-Fc or IgG1 in the presence or absence of whole, live C. jejuni at a multiplicity of infection of 100. (C) BMDC were stimulated as for (A) for 4 h, and Hes1 mRNA transcripts were measured by qRT-PCR. Data in all three panels are mean ± SD of triplicates and are representative of at least three independent experiments. Two-way ANOVA with Bonferroni posttest was used to statistically compare stimuli; p < 0.05 was considered significant. *p < 0.05, **p < 0.01, ***p < 0.001. ND, Not detected.

12 expression, as well as reduced expression of the canonical Notch target Hes1.

Notch ligation modulates DC responses to MyD88-dependent and -independent TLR agonists and to live C. jejuni

To determine whether Notch signaling in response to Jagged1-Fc could modulate the cytokine response of DCs to other agonists of the TLRs, BMDC were stimulated with CpG (TLR9 agonist), Pam3CSK4 (TLR2), or poly (I:C) (TLR3) in the presence of either Jagged1-Fc or IgG1 control. Levels of secreted IL-10, IL-2, and IL-12 are shown in Fig. 2A compared with that of BMDC stimulated with LPS (TLR4) or nothing. Similar to LPS-stimulated cells, simultaneous stimulation with Jagged1-Fc resulted in significantly enhanced levels of secreted IL-10 and IL-2 in response to CpG, Pam3CSK4, or poly (I:C). Poly (I:C) induced relatively little IL-12. In contrast, both CpG and Pam3CSK4 robustly induced IL-12 secretion, and this was significantly inhibited by Jagged1-Fc. Thus, Notch and TLR signaling can interact in DCs through both the MyD88-dependent and the Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF)-dependent TLR signaling pathways. This result was confirmed using BMDCs from either MyD88- or TRIF-deficient mice (Supplemental Fig. 4). Enhanced IL-10 and IL-12 expression in response to combined Notch and TLR2, TLR4, and TLR9 stimulation was unaffected. Conversely, TRIF deficiency had no effect on enhanced IL-10 and IL-2 secreted in response to Jagged1-Fc plus CpG or Pam3CSK4, but did prevent enhanced IL-10 and IL-2 in response to combined Notch and TLR3 signaling.

To confirm that Notch signaling can alter responses to whole pathogens, BMDC were stimulated with Jagged1-Fc (or IgG1 control) in the presence or absence of live C. jejuni. Levels of secreted IL-10 and IL-2 secreted in response to combined Notch and TLR3 signaling were unaffected. Conversely, TRIF deficiency did not inhibit Notch-induced IL-10 and IL-2 secreted in response to combined Notch and TLR3 signaling.

We next investigated how the Notch and TLR pathways interact at the molecular level to enhance expression of IL-10 and IL-2, whereas suppressing IL-12 expression. To determine whether canonical Notch signaling is required for modulation of DC responses to TLR agonists, BMDC were stimulated with Jagged1-Fc and/or LPS in the presence of the γ-secretase inhibitor DAPT (Fig. 3A). DAPT had no effect on expression of either IL-10 or IL-2, indi-
cating that modulation of LPS-induced IL-10 and IL-2 by Notch signaling occurs through a noncanonical signaling route. Although inhibition of γ-secretase activity caused a significant increase in LPS-induced IL-12 secretion, Jagged1-Fc–mediated suppression of IL-12 secretion was not abrogated, suggesting that the Notch and TLR pathways interact to modulate the DC cytokine profile through a noncanonical γ-secretase–independent route.

BMDC were stimulated with Jagged1-Fc and/or LPS in the presence of cycloheximide, and IL-10 and IL-2 mRNA transcript levels were measured at 4 h by qRT-PCR, whereas IL-12 protein concentration was measured at 24 h by ELISA. (B) BMDC were pretreated with 10 μg/ml cycloheximide or an equal volume of H2O for 30 min and then stimulated with Jagged1-Fc or IgG1 in the presence or absence of 100 ng/ml LPS for 4 h. Relative mRNA transcript levels were measured by qRT-PCR. (C) Wild-type (WT) and IL-10−/− BMDC were stimulated with Jagged1-Fc or IgG1 in the presence or absence of 100 ng/ml LPS for 24 h, and cytokine concentration was measured by ELISA. Data from all three parts are mean ± SD of triplicates and are representative of at least three independent experiments. Two-way ANOVA with Bonferroni post-test was used to statistically compare stimuli; p < 0.05 was considered significant. *p < 0.05, **p < 0.01, ***p < 0.001. ND, Not detected.

Because IL-10 can act via an autocrine mechanism to inhibit IL-12 expression (25, 26), BMDC from IL-10−/− mice were stimulated with Jagged1-Fc and LPS. As expected, IL-10 deficiency had no effect on secretion of IL-2 in response to either LPS or Jagged1-Fc, or both, whereas LPS-induced IL-12 was significantly enhanced by IL-10 deficiency (Fig. 3C). However, IL-10 deficiency did not prevent suppression of LPS-induced IL-12 by Jagged1-Fc, indicating that enhanced IL-10 expression is not sufficient to explain inhibition of LPS-induced IL-12 expression mediated by noncanonical Notch signaling.

In summary, Notch and TLR signaling interact in DCs to modulate DC responses to TLR ligands via a noncanonical Notch signaling pathway, and this interaction is dependent on de novo protein synthesis.

PI3K signaling is required for modulation of IL-10 and IL-2 secretion by Notch and TLR cross talk in DC

Because Notch and TLR signaling could interact through either the MyD88- or TRIF-dependent pathways, we focused on downstream signaling molecules that are common to both pathways, in particular, NF-κB, the MAPKs, and PI3K. To establish which of these pathways are involved in the regulation of IL-10 and IL-2.
downstream signaling molecules are required for interactions between Notch and TLR signals in DC, we stimulated BMDC with Jagged1-Fc and/or LPS in the presence or absence of inhibitors of each of these. Inhibition of NF-κB, p38, JNK, ERK, or PI3K signaling resulted in reduced IL-10 in response to LPS and in response to combined Jagged1-Fc and LPS stimulation (Fig. 4). However, compared with LPS acting alone, combined Notch and TLR4 signaling still enhanced IL-10 expression in the presence of the NF-κB, p38, JNK, or ERK inhibitors. Only inhibition of PI3K signaling with LY294002 prevented Notch-mediated enhanced expression of LPS-induced IL-10 expression (Fig. 4E), indicating that PI3K signaling is required for the interaction between the Notch and TLR pathways that leads to enhanced IL-10 expression, whereas signaling through NF-κB and the MAPKs is dispensable. Levels of Jagged1-Fc–induced IL-10 expression were too low to reliably determine the effect of these inhibitors on Jagged1-Fc–induced IL-10 expression.

Inhibition of PI3K, ERK, and JNK significantly inhibited expression of IL-2 in response to either LPS or Jagged1-Fc, or both acting together (Fig. 4C–E), demonstrating that PI3K, ERK, and JNK are all necessary for enhanced IL-2 expression in response to combined Notch and TLR signaling. Inhibition of NF-κB signaling had no effect on Jagged1-Fc–induced IL-2 expression but completely inhibited expression of IL-2 in response to LPS and Jagged1-Fc + LPS (Fig. 4A), indicating that NF-κB activity is crucial for IL-2 in response to TLR signaling. In contrast, inhibition of p38 activity led to enhanced IL-2 expression in response to either Notch or TLR4 stimulation, indicating that p38 signaling acts to limit IL-2 expression (Fig. 4B). However, the ability of combined Notch and TLR stimulation to enhance IL-2 expression by ≥2-fold was not affected by the p38 inhibitor, suggesting that p38 is not involved in cross talk between the pathways.

Because wild-type BMDC make only relatively low levels of IL-12 in response to LPS, it was not possible to reliably determine...
whether inhibition of NF-κB, p38, JNK, ERK, or PI3K signaling affected IL-12 secretion in response to combined Jagged1-Fc + LPS stimulation. Instead, BMDC derived from IL-10−/− mice were used because these DCs produce a much higher level of IL-12 after LPS stimulation. Inhibition of p38 or ERK signaling enhanced both LPS-induced and Jagged1-Fc + LPS-induced IL-12 (Fig. 5A, 5C). However, inhibition of neither p38 nor ERK altered Notch-mediated suppression of LPS-induced IL-12, suggesting these signaling molecules are dispensable for modulating IL-12 expression. Unfortunately, it was not possible to determine the effect of PI3K inhibition on IL-12 expression as IL-12 starts accumulating in the supernatant after 8 h, by which time a significant level of cell toxicity was observed in cultures containing LY294002. However, stimulating BMDC in the presence of the JNK inhibitor SP600125 mimicked the effect of Notch on LPS-induced IL-12, inhibiting IL-12 secretion by around 6-fold (Fig. 5B). Combined Jagged1-Fc + LPS stimulation was unable to further inhibit IL-12 in the presence of the JNK inhibitor, suggesting that Notch may act to inhibit LPS-induced IL-12 by inhibiting JNK signaling.

In contrast with modulation of cytokine secretion, modulation of Hes1 expression by combined Notch and TLR stimulation was not affected by inhibition of NF-κB, p38, JNK, ERK, or PI3K signaling (Supplemental Fig. 1C–G).

**PI3K is activated by Notch signaling in a γ-secretase–independent manner**

Activation of PI3K leads to phosphorylation of Akt, one of its downstream targets. To establish whether Notch signaling can activate PI3K directly, we analyzed phosphorylation of Akt in the DC-like cell line JAWSII by Western blot. Jagged1-Fc and LPS both induced IL-10 mRNA transcripts in JAWSII, and IL-10 transcript levels were greatly enhanced when both stimuli were provided simultaneously (Fig. 6A). Western blot confirmed increased phosphorylation of Akt in cells treated with the Notch ligand Jagged1-Fc (Fig. 6B) and confirmed that phosphorylation of Akt in response to Jagged1-Fc was dependent on PI3K activity (Fig. 6C). Interestingly, phosphorylation of Akt was independent of γ-secretase activity (Fig. 6D). All together, this demonstrates that noncanonical Notch signaling can activate PI3K directly (Fig. 7).

**Discussion**

DC-derived cytokines play a key role in specifying Th cell differentiation, and thus the type of immune response mounted after detection of pathogenic material. The cytokine signature of a DC depends not only on the activating pathogen-derived molecules sensed by the DC, but also on the local environment present during activation. It is not yet known how environmental cues affect DC maturation, but it is clear that immune responses show tissue specificity as pathogens in the skin elicit different immune responses compared with those present in the blood or affecting the gut. Because Notch signaling is known to influence cell fate decisions and Notch ligands are expressed in a tissue-specific manner all over the body, we were interested in whether Notch signaling can act as an endogenous environmental signal and influence DC maturation to pathogen-derived stimuli. We demonstrate in this article that the Notch and TLR pathways interact in DCs, and as a result of this interaction, DCs stimulated simultaneously with Notch and TLR ligands have a distinct cytokine profile, characterized by enhanced IL-10 and IL-2 and reduced IL-12 expression, compared with DCs stimulated with either Notch or TLR ligands alone. This interaction between Notch and TLR signaling occurs through a noncanonical γ-secretase–independent Notch signaling pathway and is dependent on PI3K signaling. PI3K can be activated downstream of TLR signaling via direct interaction with either MyD88 or TRIF (27, 28). PI3K activity leads to downstream signaling through activation of Akt and Bruton’s tyrosine kinase. Both Akt and Bruton’s tyrosine kinase enhance NF-κB activity via phosphorylation of the p65 subunit (29, 30). However, inhibitor and knockout (p85α) studies have demonstrated that PI3K signaling also acts to limit expression of proinflammatory modulators in response to TLR activation. In particular, PI3K signaling is associated with altering TLR responses to enhance IL-10 and reduce IL-12 expression, both IL-10–dependent and -independent mechanisms (31, 32). Modulation of IL-10 and IL-12 expression by PI3K signaling is likely to involve Akt and modulation of MAPK activity via interactions with the MAPK kinase kinases (32, 33). Because concurrent Notch and TLR signals lead to enhanced IL-10 and reduced IL-12 through IL-10–dependent and -independent mechanisms, we speculated that PI3K could be involved in the interaction between
from Notch1, and Perumalsamy et al. (12) demonstrated that Notch and PI3K could physically interact in Jurkats overexpressing NIC cell lines (12, 34–38). Furthermore, Sade et al. (38) showed Notch signaling activates the PI3K-Akt pathway in a variety of cell lines. Using phosphorylation of Akt as a readout for PI3K activity, we show that increased Akt phosphorylation in response to Jagged1-Fc corresponds to increased PI3K activity. Inhibition of PI3K with LY294002 further confirmed that these two pathways. Using phosphorylation of Akt as a readout for PI3K activity, we show that γ-secretase–independent Notch signaling in DCs activates PI3K. Inhibition of PI3K with LY294002 further confirmed that increased Akt phosphorylation in response to Jagged1-Fc corresponds to increased PI3K activity.

Consistent with our findings, a number of studies have shown that Notch signaling activates the PI3K-Akt pathway in a variety of cell lines (12, 34–38). Furthermore, Sade et al. (38) showed Notch and PI3K could physically interact in Jurkats overexpressing N<sup>IC</sup> from Notch1, and Perumalsamy et al. (12) demonstrated that Notch1 promoted Akt activation in Hela cells via a noncanonical route, independent of RBPj. It was not clear whether γ-secretase cleavage was required for activation of Akt; however, the authors did show that Akt activation required a cytoplasmic localization for Notch, and that a membrane tethered N<sup>IC</sup> fragment was sufficient to induce phosphorylation of Akt, suggesting that activation of the PI3K-Akt pathway may occur in the absence of γ-secretase–mediated cleavage. Thus, combined with our findings that Notch acts to modulate TLR-induced cytokine expression and that this critically requires PI3K activity, we propose that Notch alters the DC cytokine profile by activating PI3K and thus Akt, which modulates the activity of NF-κB and the MAPKs (Fig. 7).

Modulation of IL-10, IL-2, and IL-12 expression occurs through three distinct routes. Enhanced IL-10 and IL-2 expression occurs at the transcriptional level and relies on de novo synthesis of at least one unknown protein, whereas inhibition of IL-12 occurs posttranscriptionally, most likely by preventing the posttranslational modifications required for IL-12 secretion. Inhibition of JNK activity mimicked Notch-mediated repression of IL-12 secretion, suggesting Notch signaling via PI3K-Akt may interfere with posttranscriptional regulation of IL-12 by regulating JNK activity. Although NF-κB, p38, JNK, ERK, and PI3K activity are all required for optimal IL-10 expression in response to a TLR stimulus, only PI3K activity was required for enhanced IL-10 expression in response to simultaneous Notch and TLR ligation. Enhanced IL-2 expression, however, required ERK, JNK, and NF-κB activity in addition to PI3K activity. Because Akt signaling can modulate the activity of ERK, JNK, and NF-κB, it is likely that noncanonical Notch signaling enhances TLR-induced IL-2 expression through activation of PI3K and Akt (Fig. 7).

Interaction between the Notch and TLR pathways was bidirectional: in addition to modulation of TLR-induced cytokines, mRNA transcript levels of the canonical Notch target gene Hes1 were suppressed. Suppression of Hes1 mRNA after simultaneous Notch and TLR stimulation did not require NF-κB, ERK, JNK, p38, or PI3K activity, suggesting that inhibition of Hes1 may reflect competition between the canonical and alternative Notch signaling pathways rather than direct inhibition by TLR signaling. Studies reporting putative pathways for noncanonical Notch signaling support a model where canonical and alternative Notch signaling pathways compete with one another as the protein–protein interactions and/or localizations required are often mutually exclusive. For example, Song et al. (39) showed that Akt can phosphorylate N<sup>IC</sup>, which promotes localization to the cytoplasm and results in reduced expression of canonical Notch targets as less N<sup>IC</sup> is available to bind to RBPj in the nucleus. These data combined with the finding that Notch requires a cytoplasmic localization to active Akt (12) suggest that as Notch enhances Akt activation, Akt, in turn, promotes a cytoplasmic localization for Notch that further enhances this alternative signaling pathway through activation of Akt to the detriment of canonical Notch target expression in the nucleus. Thus, the reduction in Hes1 expression reported in this article may simply indicate that in the presence of a TLR stimulus, noncanonical Notch pathway predominate over canonical signaling. Competition between canonical and alternative Notch pathways may help explain apparently contradictory data published by Hu et al. (22) suggesting that TLR signaling promotes expression of Hes1 in human monocytes incubated with M-CSF for 24 h in a γ-secretase– and RBPj–dependent manner, because whether the canonical or the alternative pathway predominate will depend not only on TLR signaling but also on the cell type and differentiation stage. Indeed, two microarray studies following differentiation of human monocytes to macrophages showed that Hes1 is relatively highly expressed...
in monocytes but is downregulated during differentiation and is expressed only at low levels in mature macrophages (40, 41). This suggests that canonical Notch signaling is strong in monocytes relative to that in fully differentiated macrophages or DCs. We hypothesize that under conditions of strong canonical Notch signaling, such as in monocytes, combined TLR and Notch signaling is insufficient to favor the alternative Notch pathway and allow it to outcompete the dominant canonical pathway. In mature cells, by contrast, where canonical signaling appears to be reduced, the canonical and alternative pathways are more evenly balanced and the addition of TLR signaling allows the alternative pathway to predominate. In support of our data with DCs, other studies have suggested Hes1 expression is inhibited by TLR signaling in mature primary alveolar macrophages and rat microglial cells, as well as in the macrophage cell line RAW264.7 (42–44).

Competition between pathways could suggest the involvement of different Notch receptors. However, whereas APCs predominantly express the Notch2 receptor and a lower level of Notch1 mRNA transcripts (Supplemental Fig. 2), activation with LPS has only a relatively modest effect on overall Notch receptor transcript levels (14, 42, 44–47, and data not shown).

Notch ligation has been shown to induce DC maturation, although these Notch conditioned DCs exhibit a distinct cytokine profile and altered T cell stimulatory capacities when compared with DCs induced by TLR ligands (16, 17, 48). However, little is known about how Notch signaling in DCs may affect responses to TLR ligands. Other studies have suggested that Notch and TLR pathways may interact in macrophages; however, contradictory data have been published regarding the outcome of any such interactions. In two studies, Notch signaling in response to recombinant Jagged1–Fc or overexpressed N1IC in microglial cells or RAW267.4 resulted in reduced inducible NO synthase and proinflammatory cytokine expression in response to LPS (42, 44). Other studies using a γ-secretase inhibitor and overexpressed N1IC suggested that canonical Notch signaling promoted TNF-α, IL-6, and inducible NO synthase expression in RAW264.7 cells by promoting activation of NF-κB (46, 49). However, these data must be interpreted carefully because overexpression of N1IC results in an intracellular concentration of N1IC many orders of magnitude greater than required for signaling, and thus may allow interactions that could not be possible under physiological conditions, and γ-secretase inhibitors prevent cleavage of >25 proteins in addition to Notch (50). We demonstrate that there are two routes for Notch signaling in DC, and that these two pathways compete. Thus, although our data do not exclude the possibility that canonical Notch signaling may interact with TLR-induced NF-κB activity, as suggested earlier, it may explain these otherwise contradictory data because, if the alternative and canonical pathways each have opposing effects on TLR signaling, differences in culture conditions and method for inducing or inhibiting Notch signaling will affect which pathway predominates, and thus the outcome of combined Notch and TLR signaling. Differential roles for canonical versus noncanonical Notch signaling in DCs could be further investigated using the recently generated conditional Nicastrin knockout (51). Nicastrin is a critical component of the γ-secretase complex; thus, the conditional knockout would allow study of the noncanonical Notch signaling pathway in isolation.

Notch signaling is known to influence cell fate decisions in response to external stimuli, and this work demonstrates that a concurrent Notch signal can modulate the functional maturation...
of DCs to stimulation with a TLR ligand, in terms of the secretion of immunostimulatory cytokines. This altered cytokine profile will affect the outcome of DC–T cell interactions and the resulting adaptive immune response. Jagged1 is expressed on the surface of many different cell types, but it is not currently known what cells are capable of delivering a Notch signal to DC, and thus it is not known where and to what purpose Notch signaling may regulate DC maturation to TLR ligation in vivo. Because Jagged1 knock-out is embryonic lethal and it is not possible to reliably administer immobilized Notch ligands to mice in a fashion that can be shown to induce Notch signaling in the relevant cell type, we in vitro data cannot be supported by in vivo evidence at this time. Further investigation will be required to confirm the biological relevance of these findings in the whole organism. However, we hypothesize that expression of Jagged ligands may act as an endogenous environmental cue that acts to fine-tune the functional maturation of DCs to complement the environment in which TLR ligation occurred. Differential expression of Jagged ligands may help explain why different tissues can induce subtly different adaptive responses to the same microbial stimulus. The intestine is an example of a tissue or organ where immune responses must be tightly regulated to prevent development of excessive inflammation. Both regulatory T cells and expression of IL-10 and IL-2, whereas limiting IL-12, Notch signaling may play an important role in modulating immune responses to TLR stimuli to maintain gut integrity and homeostasis. Although Notch and TLR cross talk in DCs may be beneficial in some tissues, this interaction may lead to or facilitate disease when Jagged ligands are overexpressed out of context. For example, a relatively high level of Jagged1 expression is associated with aggressive breast and prostate cancers (52, 53), and Jagged1 may act to facilitate tumor progression through altering the functional maturation of DCs and subsequent immune responses.

Understanding how Notch and TLR signaling pathways interact to modulate the functional maturation of DCs to inflammatory stimuli may guide the development of new approaches for manipulating DCs in the treatment of diseases resulting from a disproportional immune response such as in autoimmunity, cancer, and infectious disease.

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Disclosures
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References


Supplementary Figure S1  Hes1 is directly induced by γ-secretase dependent Notch signaling and suppression of Jagged1-Fc induced Hes1 by LPS is not significantly altered by inhibition of NFκB, MAPK, or PI3K activity. BMDC were pretreated with (A) 10 μg/ml cycloheximide, (B) 10 μM of DAPT, (C) BAY11-
7082, (D) SB203580, (E) SP600125, (F) U0126, (G) LY294002 or a corresponding quantity of vehicle control (DMSO) for 1 hour and then stimulated with Jagged1-Fc or IgG1 in the presence or absence of 100 ng/ml LPS for 4 h. Relative levels of Hes1, IL-6 or TNFα mRNA transcripts were measured after 4 h by qrt-PCR. Data in all parts are mean ± SD of triplicates and are representative of at least 3 independent experiments. Two-way ANOVA with Bonferroni post-test was used to statistically compare stimuli; P<0.05 was considered significant. * = P<0.05, ** = P<0.01, *** = P<0.001, ns = not significant.
Supplementary Figure S2  BMDC express Notch1 and Notch2 mRNA and Notch ligation of DC augments LPS induced IL-10.  (A) Relative steady state expression levels of Notch receptor mRNA transcripts present in BMDC.  (B) BMDC were co-cultured with the parental L cell line or L cells expressing Delta-like1 (L-ΔL1) or Jagged1 (L-Jgd1) at a ratio of 1:1 in the presence or absence of 100 ng/ml LPS. IL-10
present in the supernatant after culture for 24 h was measured by ELISA and data shown are representative of 3 independent experiments.

**Supplementary Figure S3**  Jagged1-Fc enhances surface CD40 expression.

BMDC were stimulated with plate bound IgG1 or Jagged1-Fc in the presence or absence of 100 ng/ml LPS for 24 h. Surface CD40, CD80, CD86 and MHC Class II
expression was measured by flow cytometry. Histograms are shown in A, representative dot-plots for CD40 expression are shown in B, while the percentage of CD40⁺, CD80⁺, CD86⁺, and MHC class II⁺ cells are given in B. Data are representative of two independent experiments.

Supplementary Figure S4  MyD88 is required for Notch modulation of CpG and Pam₃CSK₄ induced cytokines while TRIF is required for Notch modulation of Poly [I:C] induced cytokine expression. BMDC from MyD88⁻/⁻ (A) or TRIF⁻/⁻ (B)
mice were stimulated with plate bound IgG1 or Jagged1-Fc in the absence (none) or presence of 100 ng/ml LPS, 0.5 μg/ml CpG, 1 μg/ml Pam3CSK4 (P3C4) or 100 μg/ml Poly [I:C] (PIC) for 24 hours. Accumulation of IL-10, and IL-2 protein in the supernatant was measured by ELISA. Data shown are mean ± standard deviation of cell culture triplicates from a representative example of data obtained from two mice per genotype. Two-way ANOVA with Bonferroni post-test was used to judge whether observed differences were statistically significant; P<0.05 was considered significant. Black stars indicate a statistically significant difference between cells stimulated with IgG1 and Jagged1-Fc. Grey stars indicate a statistically significant difference between wild-type and MyD88−/− or wild-type and TRIF−/− respectively. Absence of stars indicates that the difference is not significant. * = P<0.05, ** = P<0.01, *** = P<0.001. BD = below the detection limit of the assay.