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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 (NICΔ) from the plasma membrane allowing translocation to the nucleus. Notch signaling directly activates expression of target genes through NγC interaction with the transcriptional switch recombination signal binding protein for immunoglobulin κ J (RBPj), 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 RBPj- 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+ 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.

Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; DAPT, N-acetyl-N-[3,5-difluorophenacyl]-l-salanilyl)-L-phenylglycine t-butyl ester; DC, dendritic cell; NICΔ, intracellular domain of Notch; poly (I:C), polynucleoside-polyacrylic acid; qRT-PCR, quantitative real-time PCR; RBPj, recombination signal binding protein for immunoglobulin κ J; TRIF, Toll/IL-1R domain-containing adapter-inducing IFN-β.

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Materials and Methods

Animals

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

Bone marrow-derived DC cultures

Bone marrow-derived DCs (BMDCs) 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 Col- ogne 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). BMDCs were pretreated with inhibitors for 30–60 min as specified: 2 μM BAY11-7082, 10 μM LY294002, 5 μM SB20358, 10 μM SP600125, 25 μM U0126, and 10 μM N'-(3,5-difluorophenacyl)-l-allyl]-S-phenylglycine t-buty1 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, 85% N2, 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-aMEM (supplemented with 20 μM-serine and 40 μM-galactosamine mixed with rabbit anti-C. jejuni serum) and allowed to grow overnight before being diluted to a level at which C. jejuni were no longer visible to the naked eye.

Statistics

A nonparametric one-way ANOVA (Kruskal–Wallis test) was used in Fig. 1. Measurement of mRNA decay rates 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 mRNA levels are regulated to a lesser extent compared with Hes1 protein levels.

ELISA

ELISA sets were purchased from BD Biosciences.

Quantitative real-time PCR

Total RNA was prepared as previously described (16). Results were normalized to 18S rRNA content and relative mRNA levels are expressed as fold change compared with the IgG1 control or, when no expression was detected, calculated relative to the limit of detection.

Western blot

JAWSII DC-like cell line was maintained in RPMI supplemented with 20% FCS, 2mM glutamine, 50 μM penicillin, and 50 μg/ml streptomycin. Cells were grown in 10-cm2 tissue culture flasks at 37°C in a humidified 5% CO2 incubator. JAWSII cells were starved overnight and pretreated 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, and RNA was extracted as described for BMDCs. Hes1 mRNA levels will vary with time and degree of synchronization between 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 mRNA levels are regulated to a lesser extent compared with Hes1 protein levels.

Results

Notch signaling modulates DC responses to LPS

To investigate whether Notch signaling could alter the response of DCs to proinflammatory stimuli, BMDCs 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 Deltix1 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 mRNA levels are regulated to a lesser extent compared with Hes1 protein levels.

Jagged1-Fc 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 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).

Comparison 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 and IL-2, whereas inhibiting 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.

FIGURE 1. Simultaneous Jagged1-Fc and LPS stimulation of DCs preferentially enhances expression of IL-10 and IL-2, whereas inhibiting 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.

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-
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-2 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 whole, live C. jejuni at a multiplicity of infection of 100. Jagged1-Fc–induced Hes1 expression was significantly inhibited by CpG, Pam3CSK4, and LPS (Fig. 2C). In contrast, poly (I:C) did not inhibit Notch-induced Hes1 mRNA transcript levels. Modulation of the DC cytokine profile by interaction between the Notch and TLR pathways is dependent on de novo protein synthesis, but canonical Notch signaling is dispensable.

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 quantified after 4 h to determine whether IL-10 and IL-2 are direct targets of this noncanonical Notch signaling pathway (Fig. 3B). Only Jagged1-Fc–induced IL-10 was insensitive to cycloheximide, indicating that Notch signaling in response to Jagged1-Fc can directly induce expression of IL-10 transcripts. LPS-induced IL-2 was prevented by cycloheximide, indicating that TLR-induced IL-2 expression is dependent on de novo protein synthesis. Similarly, Jagged1-Fc–induced IL-2, as well as IL-10 and IL-2 transcripts induced by combined Jagged1-Fc and LPS stimulation, was completely abolished by cycloheximide, suggesting that interaction between the Notch and TLR signaling pathways requires de novo protein synthesis for enhanced IL-10 and IL-2 expression.

Because IL-10 can act via an autocrine mechanism to inhibit IL-12 expression (25, 26), BMDC from IL-10–deficient 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

![FIGURE 3. Modulation of TLR-induced cytokine expression depends on de novo protein synthesis but is independent of canonical, γ-secretase–mediated Notch signaling and enhanced IL-10 expression.](http://www.jimmunol.org/)

(A) BMDC were pretreated with 10 μM DAPT or an equal volume of DMSO for 30 min and then stimulated with Jagged1-Fc or IgG1 in the presence or absence of 100 ng/ml LPS. 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.
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 enhancement 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

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** PI3K activity is required for Jagged1-Fc–mediated enhancement of both LPS-induced IL-10 and IL-2. BMDC were pretreated with (A) BAY11-7082, (B) SB203580, (C) SP600125, (D) U0126, (E) LY294002, or a corresponding quantity of vehicle control (DMSO) for 1 h, and then stimulated with Jagged1-Fc or IgG1 in the presence or absence of 100 ng/ml LPS. Accumulation of IL-10 and IL-2 in the supernatant after 4–24 h was measured by ELISA (A, 8 h; B, C, 24 h; D, 8 h; E, 4 h). Data in all parts 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 < 0.001. ND, Not detected.
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, via 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 experimental conditions and how this might impact DC maturation.

**FIGURE 5.** LPS-induced IL-12 expression requires JNK activity. BMDC from wild-type (WT) or IL-10−/− mice were pretreated with (A) SB203580, (B) SP600125, (C) U0126, or a corresponding quantity of vehicle control (DMSO) for 1 h and then stimulated with Jagged1-Fc or IgG1 in the presence or absence of 100 ng/ml LPS. Concentration of IL-12 in the supernatant after 8 h (A, B) was measured by ELISA. Data 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 < 0.001. ND, Not detected.
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 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 predominates 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 the presence of 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.

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 NRC, which promotes localization to the cytoplasm and results in reduced expression of canonical Notch targets as less NRC 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, thus dependency, because whether the canonical or the alternative pathway predominates 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 tumor antigen presenting cells in mice. By the negative feedback loop. Cell. Mol. Life Sci. 67: 1653–1664.


