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The Notch Ligand Jagged-1 Is Able to Induce Maturation of Monocyte-Derived Human Dendritic Cells

Sanne Weijzen,* Markwin P. Velders,† Amira G. Elmishad, Patricia E. Bacon,‡ Jeffrey R. Panella, Brian J. Nickoloff,† Lucio Miele,‡ and W. Martin Kast2*

Notch receptors play a key role in several cellular processes including differentiation, proliferation, and apoptosis. This study investigated whether the activation of Notch signaling would affect the maturation of dendritic cells (DCs). Direct stimulation of Notch signaling in DCs with a peptide ligand induced DC maturation, similar to LPS: DCs up-regulated maturation markers, produced IL-12, lost endocytosis capacity, and became able to activate allogeneic T cells. Furthermore, coculture of DCs with cells expressing Notch ligand Jagged-1 induced up-regulation of maturation markers, IL-12 production, T cell proliferative responses, and IFN-γ production. Our data suggest that activation of Notch by Jagged-1 plays an important role in maturation of human DCs. Additionally, they reveal a novel role for Notch signaling in cell maturation events distal to the cell fate decision fork. These data may have important medical implications, since they provide new reagents to induce DC activity, which may be beneficial as adjuvants in situations where an immune response needs to be elicited, such as tumor immunotherapy. The Journal of Immunology, 2002, 169: 4273–4278.

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dendritic cells (DCs)3 are a family of professional APCs that play a key role in the induction of T cell responses. Many subsets of DCs have been identified in both mice and humans (i.e., plasmacytoid DCs, lymphoid DCs, myeloid DCs) (1–4). All these subsets undergo a similar developmental differentiation program, called DC maturation. Immature DCs are mostly located in peripheral tissues and are highly efficient at Ag uptake by phagocytosis, receptor-mediated endocytosis, and macrophagocytosis. After Ag uptake, DCs become specialized in Ag presentation, and migrate to local lymph nodes to initiate immune responses. Maturation can be induced by diverse signals, such as tissue damage or infection. It can be triggered by components of the bacterial wall (i.e., LPS), inflammatory cytokines (TNF-α or IL-1), or viral components (i.e., dsRNA, virus-like particle) (see review in Ref. 5). Signal transduction events following maturation signals depend largely on the specific signaling receptor that is activated (TNFR, Toll-like receptors (TLR)), but all signals seem to route primarily through NF-κB (6).

Notch receptors are a family of highly conserved heterodimeric transmembrane proteins involved in various cell fate decisions, such as proliferation, differentiation, and apoptosis (7). Mammals have four known Notch receptors, which vary in tissue and cellular distributions. Activation of the Notch pathway is triggered by binding of ligands that are generally cell membrane associated. Engagement of the receptor induces cleavage of the transmembrane subunit (N^{TM}), releasing an intracellular domain (N^{IC}) that translocates to the nucleus. Nuclear N^{IC} exerts transcriptional control of its many target genes, including basic helix-loop-helix transcription factors, primarily through the transcriptional regulator C promoter binding factor-1/recombination signal sequence binding protein-Jk (8). Notch signaling regulates differentiation of numerous cell types (9–12). Among cells of the hemopoietic lineage, Notch mRNA and protein expression can be detected in human immature CD34+ hemopoietic progenitors; lymphoid, myeloid, and erythroid precursors; as well as B and T cells, monocytes, and neutrophils (13, 14). Notch signaling is involved in lineage decisions and differentiation at several stages during hemopoietic development (15–17). One study reported that inducible Notch-1-deficient mice have normal DC development. Neither peripheral DCs nor Langerhans cells were affected by deficient Notch-1 signaling (18). However, that study did not address the functionality of the Notch-1-deficient DCs and only investigated immature DCs. Therefore, we investigated whether activation of Notch-1 signaling would influence the maturation of human monocyte-derived dendritic cells. We show that direct engagement of the Notch receptor by a soluble Notch ligand or native Jagged-1 naturally expressed on primary keratinocytes or transfected into Jagged-negative fibroblasts induces phenotypical and functional DC maturation. These data indicate that activation of Notch-1 signaling by Jagged-1 can induce maturation of human DC. Moreover, they identify a new class of reagents that can mature DCs in vitro through the Notch signaling pathway.

Materials and Methods

Cells and plasmids

Human DCs were generated from PBL obtained from healthy donors by leukapheresis. Leukocytes were purified by Lymphoprep (Nycomed, Oslo, Norway) and were stored until further use. Frozen PBL were thawed and washed in RPMI 1640 containing 2 mM glutamine (Mediatech, Herndon, VA) 10 mM pyruvic acid (Life Technologies, Gaithersburg, MD), 10 mM nonessential amino acids (Life Technologies), 100 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO), 10% FCS (HyClone Laboratories, Logan UT), and plastic-adherent cells were selected after incubation for 2 h at 37°C in 5% CO2. Nonadherent cells were washed away, and adherent cells were
cultured for 3 days in 1000 U/ml recombinant human GM-CSF (Intergen, Purchase, NY) and 1000 U/ml recombinant human IL-4 (Intergen). On day 3 nonadherent cells were collected, washed in PBS, and incubated for 1 h in 1 ml in the presence of one of the following activation stimuli: 10 μg/ml LPS (Sigma-Aldrich), TNF-α (PeproTech, Rocky Hill, NJ), 50 μM Jagged peptide, 50 μM Scrambled peptide, human keratinocytes, fibroblasts transfected with human Jagged-1 or empty vector, or PBS alone as a negative control. DCs were then cultured for an additional 48 h before use in phenotypical or functional assays. In the case of coculture with keratinocytes or fibroblasts, DCs were gently collected after 48 h, leaving the monolayer of keratinocytes or fibroblasts intact. This guaranteed a minimal contamination of DCs with keratinocytes or fibroblasts. To ensure that the expression of measured activation markers was not the result of contamination, the expression of MHC class I or II, CD80, CD83, CD86, CD40, and CCR7 on keratinocytes and fibroblasts alone was determined.

Normal human keratinocytes were isolated from neonatal foreskins and grown in low calcium (0.07 mM), serum-free medium (Clonetics, San Diego, CA) on plastic dishes as previously described (19). Normal fibroblasts were isolated from a 4-mm skin biopsy that was incubated overnight at 4 °C in dispase II (Roche, Indianapolis, IN) and RPMI 1640 (Life Technologies). Dermis and epidermis were separated, dermis was minced, and the small pieces were incubated in trypsin/EDTA (Life Technologies) for 4 h at 37 °C, after which IMDM containing 10% FCS was added. Human Jagged-1 cDNA was provided by Dr. S. Artavanis-Tsakonas (Harvard Medical School, Boston, MA) and subcloned into NolI in pLZRS. The Phoenix-Ampho packing cell line (American Type Culture Collection, Manassas, VA, with permission of Dr. G. Nolan, Stanford University Medical Center, Stanford, CA) was transfected as previously described (20). Fibroblasts were infected with Jagged or empty vector as previously described (20).

**Western blot**

Immunoblotting of cellular extracts was performed after SDS-PAGE on 7 or 10% Tris-acetate gels (Invitrogen, Carlsbad, CA). Equal protein loading was verified by Bradford protein assay and by stripping and reprobing membranes with anti-GAPDH.

**Abs and reagents**

Abs to human Notch-1, Jagged-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (Chemicon, Temecula, CA) and secondary Abs anti-goat IgG-HRP (Vector, Burlingame, CA) and anti-mouse/rabbit F(ab)2; IgG-HRP (Roche, Mannheim, Germany) were used for Western blotting. Abs to human CCR7, CD40, CD80-FITC, CD86-FITC, CD83, isotype controls, streptavidin-allophycocyanin, and HLA-DR, -DQ, and -DP-FITC were purchased from BD PharMingen (San Diego, CA), and GAPDH (Chemicon, Temecula, CA) and secondary Abs anti-fluorescent signal, neither LPS nor Scrambled-treated immature DCs internalized and degraded BSA at 37°C. Immature DCs were also functionally active, the production of bioactive IL-12(p70) (24, 25) and T cell activation was determined. Both LPS- and Jagged-treated DCs produced IL-12(p70), whereas immature DCs and Scrambled-treated immature DCs rapidly internalized and degraded BSA at 37°C; as indicated by a high fluorescent signal, neither LPS-treated nor Jagged-treated DCs internalized the green fluorescent BSA. The maturation status of these DCs was confirmed by high expression of CD83, which was low in the immature DCs (Fig. 1C).

**DCs matured by direct stimulation of Notch are functionally active**

Up-regulation of maturation markers after stimulation of the Notch receptor is an indication that immature DCs have differentiated into their mature form. To determine whether these Jagged-treated DCs are also functionally active, the production of bioactive IL-12(p70) (24, 25) and T cell activation was determined. Both LPS- and Jagged-treated DCs produced IL-12(p70), whereas immature DCs and Scrambled-treated immature DCs did not (control vs Jagged, p < 0.05). However, IL-12(p70) production by LPS-treated DCs was significantly higher than that in Jagged-treated DCs (Fig. 2A).

To establish T cell activation, differently treated DCs were cocultured with allogeneic PBL in an MLR. After 4 days, supernatant was collected for cytokine analysis, and [3 H]thymidine was added (Microscint-20; Packard Instrument). The proliferation of PBL was determined by a liquid scintillation counter (Packard Instrument).

**Results**

**DCs gain a mature phenotype after direct stimulation of the Notch signaling pathway**

To determine whether activation of Notch-1 in DCs in the absence of a known DC maturation signal results in DC maturation, Notch signaling was stimulated in immature DCs in the absence of LPS. For this purpose, a synthetic peptide (Jagged), corresponding to the ΔSerrate/LAG-2 domain of Jagged1, was used. The 17-mer peptide binds to the extracellular portion of the Notch receptor and initiates the signaling cascade (21). As a negative control, a synthetic peptide (Scrambled) with the scrambled sequence of the Jagged peptide was used (22). These reagents were tested for the presence of endotoxin to rule out DC activation as a result of endotoxin contamination. The concentration of endotoxin was < 0.015 endotoxin units/ml, which is a concentration of endotoxin far below the level needed for DC activation (23). Exposure of immature DCs to Jagged peptide at the time when they are normally exposed to LPS resulted in appearance of active Notch (N0), in contrast to exposure of DCs to Scrambled or control DCs (Fig. 1A).

To assess maturation of DCs by Jagged, the expression of maturation markers was determined by flow cytometry (Fig. 1B). A significant up-regulation of MHC class I and II, CD80, CD83, and CD86 surface expression on Jagged-treated DCs was detected. DCs exposed to LPS showed similar expression levels as DCs treated with Jagged. Incubation of DCs with Scrambled peptide did not affect the expression levels of these maturation markers, which were similar to those of immature DCs. The level of up-regulation of maturation markers after Jagged treatment was equal to or higher than up-regulation measured after treatment with CD40L (0.5 μg/ml; data not shown).

An important property of immature DCs is their ability to internalize and process Ags. To examine Ag uptake by DCs after exposure to Jagged, DCs were incubated with self-quenching dye conjugates of BSA that gains green fluorescence only upon internalization and subsequent degradation. While control or Scrambled-treated, immature DCs rapidly internalized and degraded BSA at 37°C, as indicated by a high fluorescent signal, neither LPS-treated nor Jagged-treated DCs internalized the green fluorescent BSA. The maturation status of these DCs was confirmed by high expression of CD83, which was low in the immature DCs (Fig. 1C).
IFN-γ production is another indicator of T cell activation. Significant IFN-γ production by T cells was measured by ELISA in response to LPS- or Jagged-stimulated DCs (control vs Jagged, \( p < 0.01 \)). Stimulation of PBL by immature DCs or DCs exposed to Scrambled peptide induced a significantly lower IFN-γ production (Fig. 2C). In summary, these data show that DCs stimulated through the Notch signaling pathway, not only exhibit a mature DC phenotype, but are functionally active as well.

**Cell-bound Notch ligand induces DC maturation**

Exposure of DCs to the synthetic Jagged peptide to simulate ligand-induced activation of the Notch signaling pathway may be considered nonphysiological. To determine whether contact between cell-bound Notch ligand and Notch-1 on a DC would result in maturation of DCs as well, we studied two models. Human keratinocytes endogenously express Jagged-1 under physiological conditions, as shown by Western blot analysis (Fig. 3A). Immunohistochemical staining showed that Jagged-1 was localized on the cell surface (22). Keratinocytes lack CD40 ligand expression, as determined by FACS analysis (data not shown). Coculture of immature DCs with human keratinocytes resulted in up-regulation of MHC class II, CD80, CD83, and CD86 (Fig. 3B and C). Moreover, these DCs were able to induce T cell proliferation and IFN-γ production by allogeneic T cells (control vs keratinocytes, \( p < 0.001 \); Fig. 3, C and D). This indicates that cells physiologically expressing Jagged-1 can induce DC maturation in vitro.

However, this observation per se does not prove that the observed DC maturation is mediated by Jagged-1 expressed on keratinocytes. To further investigate the role of cell-associated Jagged-1, fibroblasts were transfected with human Jagged-1 (MVJ) or empty vector (MVV). Fibroblasts have low endogenous expression of Jagged-1, and transfection of Jagged-1 cDNA significantly increased this expression (Fig. 3A). Jagged-transfected fibroblasts were cocultured with immature DCs, and the expression of activation markers was assessed (Fig. 4A). Expression of CD80, CD83, and CD86 on DCs cultured with Jagged-transfected fibroblasts was similar to the expression of these maturation markers on LPS-treated DCs. In contrast, DCs cultured with vector-transfected fibroblasts showed low expression of these markers, similar to control DCs. IL-12 production by DCs cultured with Jagged-transfected fibroblasts was determined 24 h after the start of the coculture and compared with IL-12 production...
Notch signaling is involved in multiple lineage decisions in the hemopoietic system. In the lymphoid lineage several developmental choices are thought to be regulated by Notch. The T vs B cell lineage choice (26–28), the αβ vs γδ T cell choice (29), and the CD4 vs CD8 T cell decision (16, 30, 31) are all thought to be regulated by Notch signaling. In the myeloid lineage Notch signaling has been implicated in the early transition from a granulocyte-monocyte progenitor to differentiated granulocyte, although there is controversy on the exact function of Notch in this cell lineage (32–35). Evidence obtained in conditional Notch-1 knock-out mice suggested that Notch-1 is dispensable during the differentiation from this granulocyte-monocyte progenitor to immature DCs (18). However, in antisense Notch-1 transgenic mice, reduced Notch-1 expression resulted in significantly lower NF-κB activity (36), which is known to play a major role in DC maturation. This suggested that Notch-1 might be involved in the maturation process of DCs rather than in their development. Here, we show that direct stimulation of the Notch signaling pathway with a soluble or a cell-bound Notch ligand induced DC maturation similar to LPS: Jagged-treated DCs expressed maturation markers were no longer capable of Ag uptake, produced IL-12 (p70), and induced T cell proliferation and IFN-γ production. Thus, although Notch-1 is dispensable for DC development, our data provide ample evidence that activation of Notch-1 signaling promotes the maturation of human DCs.

According to a commonly accepted model for Notch signaling, high levels of activated Notch cause transcriptional down-regulation of lineage-specific genes. This results in a delay of cell differentiation and continuation of an uncommitted state until a second differentiation signal determines a different cell fate (37–41). Our data suggest that Notch activation can also lead to the opposite: transcription of lineage-specific genes and terminal differentiation into mature DCs. It is quite possible that in some mammalian cell lineages a timed activation of one or more Notch receptors controls the timing of cell maturation. Similar observations were made in murine keratinocytes (42) and more recently in human keratinocytes (22). Human keratinocytes express Notch-1, -2, -3, and -4 depending on their maturation state. Treatment with the Jagged peptide triggers terminal differentiation, culminating in cornification. Additionally, Notch-1 signaling is necessary for the differentiation of preadipocytes (43), murine erythroleukemia cells (9), and CD8 single-positive thymocytes (30). The studies mentioned above, like the present one, do not rely on transfected, overexpressed forms of constitutively active Notch receptors, which may have nonphysiological effects. As a separate study has shown that Notch signaling does not affect DC development (18), our data indicate that in DCs, Notch-1 signaling modulates relatively late stages of differentiation, even terminal differentiation, rather than initial cell fate commitment. Therefore, it appears that in several instances, physiologically regulated expression of endogenous Notch receptors controls the timing of cell differentiation and can positively affect differentiation in several cell types, including DCs (44, 45).

Our data indicate that it is possible to induce complete maturation of human DC by activating the Notch signaling pathway. This raises the obvious question of whether DCs are exposed to Notch ligands in the microenvironments where they reside. Knowledge of the tissue expression of Notch ligands is relatively limited. Jagged-1 expression was observed in endothelial cells (46, 47), thymus (48), bone marrow stromal cells (21), and suprabasal keratinocytes. Jagged-2 is expressed in thymus (49). Expression of Delta-3 has been detected in brain (50), and Delta-4 is located in arterial endothelium (51). It is not likely that DCs are induced to mature upon the mere encounter of a Notch ligand expressed on a cell in its immediate environment, such as in bone marrow, skin, thymus, or blood. In skin, keratinocytes constitutively express high levels of Jagged-1. Skin DCs, such as Langerhans cells, that express Notch-3 and -4 (B. Nickoloff, unpublished observations) are not constitutively induced to mature even though our data show that it is possible to induce monocyte-derived DC maturation by keratinocytes or Jagged-1-transfected fibroblasts. Therefore, to prevent constitutive maturation of DCs, Notch signaling must be tightly regulated. It is conceivable that Jagged-Notch signaling is inhibited until physiological conditions require DC maturation. It
should be noted that the activity of Notch receptors is modulated by multiple mechanisms that control the maturation of Notch proteins, including the amount of Notch exposed on the membrane through membrane recycling, the activation of transmembrane Notch, and the half-life of intracellular Notch (for a review, see Ref. 52). Potential negative regulators of Notch signaling include the endocytic mediator Numb (53, 54), the Notch-binding protein Notchless (55), and E3 ubiquitin-ligases such as SEL-10 (56, 57).

A more detailed analysis of the downstream targets of the LPS signaling pathway needs to be performed to determine whether and how signaling triggered by TLR receptors and/or CD14 cross-talks with Notch signaling. Based on our data and previous reports (36, 59), it is likely that NF-κB cross-talks with the Notch signaling pathway. NF-κB plays a major role in signaling cascades that lead to maturation of DCs. Most maturation signals, such as CD40 (60), TNF-α (6), and TLR (6), involve NF-κB. Recent data indicate that Notch-1 is required for the expression of several NF-κB subunits in murine hematopoietic precursors (36). Moreover, we have observed that Jagged-1 peptide treatment of human keratinocytes leads to rapid activation of NF-κB (22). Additionally, NF-κB has been proposed to trans-activate Jagged-1, one of the Notch ligands (61). Taken together, these data suggest that Notch-1 may enhance NF-κB-dependent signals and, in turn, NF-κB may amplify the effect by up-regulating Notch ligand Jagged-1. The latter may act cell-autonomously or trans-cellularly to further activate Notch-1.

The present observations have potentially important medical implications. Activation of Notch-1 may be a viable strategy for in vivo and ex vivo immunomodulation in applications such as DC-based tumor vaccines or vaccines against infectious agents or toxins.


