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Ligation of Notch Receptors in Human Conventional and Plasmacytoid Dendritic Cells Differentially Regulates Cytokine and Chemokine Secretion and Modulates Th Cell Polarization

Begoña Pérez-Cabezas,* Mar Naranjo-Gómez,* Patricia Bastos-Amador,* Gerard Requena-Fernández,† Ricardo Pujol-Borrell,* and Francesc E. Borras*†

Notch signaling is involved in multiple cellular processes. Recent data also support the prominent role of Notch signaling in the regulation of the immune response. In this study, we analyzed the expression and function of Notch receptors and ligands on both human blood conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs). The expression and modulation upon TLR activation of Notch molecules partially differed between cDCs and pDCs, but functional involvement of the Notch pathway in both cell types was clearly revealed by specific inhibition using DAPT. Beyond the induction of Notch target genes and modulation of maturation markers, Notch pathway was also involved in a differential secretion of some specific cytokines/chemokines by DC subsets. Whereas Notch ligation induced IL-10 and CCL19 secretion in cDCs, Notch inhibition resulted in a diminished production of these proteins. With regard to pDCs, Notch activation induced TNF-α whereas Notch inhibition significantly abrogated the secretion of CCL19, CXCL9, CXCL10, and TNF-α. Additionally, Notch modulation of DC subsets differentially affected Th polarization of allostimulated T cells. Our results suggest that the Notch pathway may function as an additional mechanism controlling human DC responses, with differential activity on cDCs and pDCs. This control mechanism may ultimately contribute to define the local milieu promoted by these cells under the particular conditions of the immune response. The Journal of Immunology, 2011, 186: 000–000.

D endritic cells (DCs) are professional APCs that play essential roles in mediating immunity in front of invading pathogens and also in tolerance to self and innocuous Ags. These apparently opposed functions are performed by DCs due to their plasticity and adaptability to respond to different physiological and pathological stimuli. Various DC subsets differ in their anatomic distribution, cell surface marker expression, and function (1, 2). Human peripheral blood contains two main sub-

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Abbreviations used in this article: Cbt, cord blood T; cDC, conventional dendritic cell; DAPT, N-[N-(3,5-difluorophenacetyl)-(L)-alanyl]-S-phenylglycine t-buty1 ester; DC, dendritic cell; DI, Delta-like ligand; Jag, Jagged; MHC-II, MHC class II; pDC, plasmacytoid dendritic cell; R484, resiquimod.

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target genes in mammals are Hes and Hey (12). Other targets include CD25 (13), NF-κB (14), cyclin D (15), and p21 (16).

In the immune system, Notch signaling is involved in T and B cell development (17–19) and in myeloid lineage commitment (20, 21). Notch also plays an important role in mature cells participating in peripheral T cell and B cell activation (22, 23), T cell regulatory function (24), and Th cell differentiation (25–28). Regarding DCs, Notch signaling has an important role in their development (29–31). The presence of Notch receptors and ligands has been widely described in mice (macrophages, thymic DCs, splenic DCs, bone marrow-derived DCs) and human myeloid cells (macrophages and monocyte-derived DCs) (reviewed in Ref. 32) and in mouse splenic pDCs (33). Only one study has reported the expression of Notch molecules in human BDCA1+ peripheral blood cDCs (34). Cellular activation alters the expression of Notch molecules on DCs, which modifies Th cell polarization (32, 35, 36). Additionally, Notch signaling itself is also involved in activation of DCs, as ligand binding induced upregulation of MHC class II (MHC-II), CD80, CD83, and CD86, production of different cytokines, and higher levels of T cell proliferation (37–39). Interestingly, TLR and Notch pathways presented integrated regulation and autoamplification (40–42).

In this study, we describe the expression of functional Notch molecules in both conventional and plasmacytoid human peripheral blood DCs and reveal a role for the Notch pathway in a differential regulation of cytokine/chemokine responses and Th polarization induced by cDCs and pDCs, thus modulating the immunological function of these cells.

Materials and Methods

Culture media and reagents

Culture media was composed of RPMI 1640 (Life Technologies/Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated FCS (Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin (CEPA, Madrid, Spain), and 100 μg/ml streptomycin (Laboratorios Normon, Madrid, Spain). Recombinant human IL-3 (R&D Systems, Minneapolis, MN) was added at 10 ng/ml to all pDC cultures. Resiquimod (R848) (Alexis Biochemicals, San Diego, CA) and LPS (Sigma-Aldrich) were used at 5 μM and 1 μg/ml, respectively, at the indicated times. The γ-secretase inhibitor IX N-[3(5-difluorophenacetyl)-L- t-alanyl]-S-phenylglycine t-butyler (DAPT; Calbiochem/Merck, Darmstadt, Germany) was present during the whole time of the indicated cultures at a concentration of 10 μM. DAPT inhibits γ-secretase, preventing the release of the intracellular domain of Notch and its translocation to the nucleus, and thus it is considered the most selective inhibitor of the Notch pathway (43). DAPT was tested for the absence of endotoxin using the TLR and Notch pathways presented integrated regulation and autoamplification (40–42).

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Antibodies

The following murine mAbs were used: FITC-labeled mAbs: CD86 (BD Biosciences, San Jose, CA), CD4 (ImmuNoools, Friesoythe, Germany), CD123 (Miltenyi Biotec, Bergisch Gladbach, Germany); PE-labeled mAbs: CD3, CD40, CD86 (BD Biosciences), CD25 (R&D Systems), CD14 (ImmuNoools), BDCA2 (Miltenyi Biotec); PE-cyanine dye 5-labeled mAbs: CD4 (BD Biosciences); allophycocyanin-Cy7-labeled mAbs: HLA-DR (BD Biosciences); isotype-matched Abs were used as controls. The following purified polyclonal Abs were used: goat anti-human Notch-1 (C-20), goat anti-mouse Notch-2 (M-20), goat anti-human Notch-3 (M-20), goat anti-human Jag1 (C-20), rabbit anti-human Jag2 (H-143), goat anti-human Dll (F-15), rabbit anti-human Dll1 (H-70) (Santa Cruz Biotechnology, Santa Cruz, CA). As secondary developing Abs, rabbit anti-IgG goat and goat anti-IgG rabbit Alexa 488-labeled Abs were used at 1:100 dilution (Molecular Probes, Eugene, OR).

Immunostaining and flow cytometry

For Notch receptor and ligand detection, cDCs and pDCs were maintained overnight in control conditions or activated with LPS or R848, respectively. After that, cells were washed and fixed during 15 min following the manufacturer’s instructions (IntraStain fixation and permeabilization kit; Dako, Glostrup, Denmark). Then, cells were washed again and labeled with the primary Ab at 1:50 dilution in PBS for surface labeling (Jag2, Dll4) or in kit solution B for intracellular detection (Notch-1, Notch-2, Jag1, Dll1) during 30 min. Finally, cells were washed and incubated with the corresponding secondary developing Ab at a final concentration of 1:100 during 30 min. All procedures were performed at room temperature. For phenotype analysis, cells were washed, resuspended in 50 μl PBS, and incubated with mAbs for 15 min at room temperature. Acquisition was performed in a FACS Canto II flow cytometer using the standard FACS Diva software (BD Biosciences). Subsequent analyses were performed using FlowJo software version 7 (Tree Star, Ashland, OR). Samples were gated using forward and side scatter to exclude dead cells and debris.

DC isolation

Buffy coats, provided by our Blood Bank Department, were obtained from healthy blood donors following the Institutional Standard Operating Procedures for blood donation and processing. Samples were processed as described before (44). Briefly, PBMCs were isolated by Ficoll-Paque density gradient centrifugation (400 × g, 25 min) (Lymphoprep; Axis- shield, Oslo, Norway), and CD3+ cells were depleted by RosetteSep human CD3 depletion cocktail (StemCell Technologies, Seattle, WA). Recovered cells were washed twice in PBS and counted using PerfectCount (Biomerieux, Lyon, France). Preparations were examined in an Axio Observer microscope using the Axiowvision software (Carl Zeiss).

Coated Notch ligand cultures

Ninety-six–well plates were coated at 4°C overnight using 10 μl recombinant mouse Dll1 Fc chimera or recombinant human Jag1 Fc chimera (R&D Systems) at a concentration of 10 μg/ml in PBS. Human IgGs were used as control at the same concentration. After that, PBS was removed and mAbs or cDCs were plated at 100,000 cells in 100 μl medium per well. Cells were incubated during 5 (for gene expression analysis) or 24 h (for phenotype studies and protein detection).

RNA isolation and quantitative real-time RT-PCR

RNA was extracted from DCs using the Quagen RNeasy Micro kit (Quagen, Hilden, Germany). Quality, purity, and concentration were assessed using the Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA). RNA integrity was >7 in all samples included in the assay. Total RNA from each sample was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. cDNA obtained was preamplified with the TaqMan PreAmp Master Mix kit (Applied Biosystems) for each gene-specific target using primers of TaqMan gene expression assays as a source of primers. This preamplification reaction generated ∼1,000–16,000-fold amplification of each gene-specific target without inducing any bias. The resulting preamplified material was diluted and used as the starting material for the subsequent singleplex real-time PCR with each of the following individual TaqMan gene expression assays (Applied Biosystems) represented in the assay pool: BATE; Hs00232390_m1; HES1, Hs00172878_m1; HEY1, Hs01141131_m1; GAPDH, Hs00999905_m1. GAPDH cDNA levels were used as the endogenous control to normalize cDNA quantities. The relative cDNA levels of each gene were calculated as follows: relative cDNA expression = 2−ΔΔCt, where Ct is the cycle threshold (45). To
The GraphPad Prism v4.00 software (GraphPad Software, San Diego, CA).

Measurement of cytokine and chemokine production
IL-10, IL-12p70, and IFN-γ were detected by ELISA following the manufacturer’s instructions (eBioscience, San Diego, CA). IL-6, TNF-α, IFN-α, CCL19, CXCL9, CXCL10, and CXCL11 were analyzed using Milliplex multi-analyte profiling (Millipore, Bedford, MA) following the overnight protocol described by the manufacturer. The minimum detectable concentration (pg/ml) of each protein was 0.3 for IL-6, 2.0 for IL-10, 4.0 for IL-12p70, 0.1 for TNF-α, 24.5 for IFN-α, 4.0 for IFN-γ, 2.1 for CCL19, 10.3 for CXCL9, 1.2 for CXCL10, and 0.4 for CXCL11. All the cytokines measured exceeded the detection limit mentioned before, otherwise stated in Results. As in RNA data, results were normalized to control situation to diminish interindividual variability.

Cord blood T cell isolation and allostimulatory assays
Cord blood T (CbT) cells were obtained from umbilical cord blood samples supplied by the Cord Bank of Barcelona with donor consent. Cord blood mononuclear cells were collected in PBMCs, and CD3+ T lymphocytes were purified by negative selection using the EasySep human T cell enrichment kit (StemCell Technologies) following the manufacturer’s instructions. Purity was >95% in all experiments. Enriched CbT cells were plated (5 × 10^5 cells/well) in 96-well round-bottom plates. CbT cells were cocultured, at 1:20 ratio (DC/CbT cells), with cDCs or pDCs differently treated as indicated in Results. After 4 d, supernatants were partially recovered for subsequent analyses and cells were pulsed with 1 μCi/well [3H]thymidine (Amersham Pharmacia Biotech, Freiburg, Germany) for an additional 16 h to determine cell proliferation. Cells were harvested (Harvester 96; Tomtec, Hamden, CT) and analyzed using a scintillation counter (1450 MicroBeta TriLux; Wallac, Turku, Finland).

Statistical analyses
Results are expressed as the mean ± SD unless otherwise stated. To minimize the effect of interindividual variability (inherent to human samples), the results are expressed in some experiments as relative values to the control situation. Comparison among groups was conducted using the paired t test for paired observations. Analyses were performed using the GraphPad Prism v4.00 software (GraphPad Software, San Diego, CA). A p value < 0.05 was considered significant.

Results
Human peripheral blood DCs express Notch receptors and ligands
The expression of Notch receptors and ligands was explored in resting and activated (LPS-stimulated) cDCs. Data obtained from eight individuals tested revealed that cDCs expressed Notch-1 (range, 73–89%) and Notch-2 (range, 64–90%) at similar levels in resting conditions (Fig. 1). LPS activation induced a significant decrease in Notch-1 (47–88% of positive cells, p = 0.0265) but did not induce major changes in Notch-2 expression (51–88%). Notch-3 was not detected in any condition (data not shown). The expression of Notch ligands exhibited a high level of interindividual variation. Jag1 was expressed by 28–86% of resting cells. In three of eight individuals studied, the percentage of Jag1-positive cDCs increased after LPS activation (from 46 to 58% and to 76%, and from 67 to 91%), remained unaltered in four other samples (range, 29–87%), and was reduced in one particular donor. In contrast, Jag2 and Dll4 diminished, although not significantly, after activation (2–83% versus 5–61% for Jag2; 3–84% versus 2–64% for Dll4) in six of eight donors. Dll1 was the molecule showing lower levels (0–41%) and without remarkable differences between resting and activating conditions (Fig. 1). In summary, cDCs expressed high levels of Notch-1 and Notch-2, and a variable proportion of the cells was positive for Jag1, Jag2, Dll1, and Dll4. Activation by LPS significantly diminished the expression of Notch-1 in cDCs, whereas a reduced (but not statistically significant) expression of Jag2 and Dll4 was observed in most donors.

As in cDCs, the expression of Notch ligands and receptors was explored in resting (IL-3–maintained) and activated (R848-stimulated) pDCs. Data obtained from five individuals revealed that resting pDCs expressed Notch-1 (range of positive cells, 62–86%) and Notch-2 (50–91%) (Fig. 2). In contrast to activated cDCs, Notch-1 on pDCs significantly increased after R848 activation, being expressed by almost all cells (86–97%, p = 0.0032), whereas Notch-2 had variable behavior following activation. Again, Notch-3 was not detected in any of the conditions tested (data not shown). Regarding the expression of Notch ligands, and as shown before in cDCs, a variable proportion of pDCs expressed Jag1 and Jag2 (range, 39–83% and 60–93%, respectively). Three of five donors increased the proportion of positive pDCs for Jag1 and Jag2 after R848 activation, whereas the other two individuals showed no changes (45–87% and 69–94%, respectively). As in cDCs, pDCs moderately expressed Dll1 (11–44%), with no major changes after activation. Finally, Dll4 was expressed by 47–84% of resting pDCs, and that expression was maintained after activation in three of five donors analyzed and increased in two of them (47–67% and 56–81%) (Fig. 2). Thus, a high proportion of resting pDCs expressed Notch-1, Notch-2, Jag1, Jag2, Dll1, and Dll4 at different levels. TLR activation using R848 significantly increased the expression of Notch-1.

The pattern of expression of Notch receptors and ligands was also studied on control and activated cDCs and pDCs by immunofluorescence (Fig. 3). In general, staining of Notch receptors and ligands exhibited a dotted pattern. Overall, the expression of Notch molecules was homogeneously distributed on the cell membrane in both DC subsets and the molecules showed no changes in their distribution upon activation.

Human peripheral blood DCs respond to Notch ligands Jag1 and Dll1
To verify the functional activity of Notch receptors in cDCs, the expression of Notch-response genes such as Hes1, Hey1, and BATF was analyzed in cDCs cultured during 5 h on Jag1- or Dll1-coated culture plates in the presence or absence of DAPT, as the most selective γ-secretase inhibitor. IgG-coated plates were used as control conditions. As positive activation controls, cells were cultured with LPS or R848. Conventional DCs maintained in control conditions expressed basal levels of Notch target genes, probably reflecting the interaction between cDCs through ligation of Notch receptors by ligands expressed in nearby cells. However, cDCs responded to Notch ligands inducing the expression of the three Notch target genes studied, Hes1, Hey1, and to a lesser extent BATF (Fig. 4A). Comparatively, Dll1 ligation induced a higher response than Jag1 (Hes1, 1.8× for cDCs plus Dll1 versus 1.3 for cDCs plus Jag1 compared with control situation; Hey1, 1.6 versus 0.93; BATF, 1.35 versus 1.2). In most of the situations, the increased expression was significantly abrogated by DAPT. Activation of cDCs by TLR ligands such as LPS or R848 also induced the expression of the three Notch target genes. Interestingly, R848 stimulation resulted in a stronger induction of Hes1 and BATF compared with LPS. As before, the addition of DAPT to the culture media blocked the induction of Notch target genes induced by TLR ligation (Fig. 4A). Thus, both Notch and TLR ligation induced the transcription of Notch target genes in cDCs. This induction was blocked by the specific γ-secretase inhibitor DAPT.

The functional activity of Notch receptors in pDCs was also studied. Cells were cultured during 5 h on Jag1- or Dll1-coated culture plates in the presence or absence of DAPT. As before, R848 activation was used as positive control. As seen in cDCs, pDCs maintained in control conditions already expressed basal...
levels of Hes1. The presence of ligands in culture induced a similar increase in the expression of Hes1 (mean, 1.40 ± 1.41 for pDCs plus Jag1 compared with control situation). In all cases, this expression was significantly abrogated in the presence of DAPT (Fig. 4B). However, neither Jag1 nor Dll1 induced the expression of the two other Notch target genes, Hey1 and BATF, on pDCs. However, TLR stimulation by R848 caused the increase of the three Notch target genes tested, and all three were blocked by DAPT (Fig. 4B). In short, Notch ligation increased the expression of Hes1 in pDCs, but TLR activation induced the transcription of the three genes studied. As in cDCs, activation was blocked by DAPT.

FIGURE 1. cDCs express Notch receptors and ligands, which are modified by TLR stimulation. To verify the expression of Notch molecules by cDCs and its alteration by TLR activation, cells were maintained in control conditions or activated with LPS during 16 h and then labeled using the corresponding Ab and isotype control. A, Each panel shows the percentage of positive cells of the eight individuals tested for the indicated molecule, in the absence (−) or presence (+) of LPS (+). *p < 0.05. B, Dot plots show the results obtained from one representative individual for control (cDC CTRL) and activated cDCs (cDC LPS). Percentages indicate the proportion of positive cells for the corresponding molecule.

FIGURE 2. pDCs express Notch receptors and ligands, which are modified by TLR stimulation. To verify the expression of Notch molecules by pDCs and its alteration by TLR activation, cells were maintained in control conditions or activated with R848 during 16 h and then labeled using the corresponding Ab and isotype control. A, Each panel shows the percentage of positive cells of the five individuals tested for the indicated molecule (R848). **p < 0.01. B, Dot plots show the results obtained from one representative individual for control (pDC CTRL) and activated pDCs (pDC R848). Percentages indicate the proportion of positive cells for the corresponding molecule.
Phenotype changes on DC subsets are modulated by Notch

To verify whether ligation of Notch receptors had any effect on DC phenotype, pDCs and cDCs were cultured as before during 24 h (n = 5). Under these conditions, Dll1 significantly increased the expression of CD86 on cDCs. The same ligand also increased, although not significantly, the expression of CD25 on these cells. These markers were faintly reduced on DAPT-treated cDCs. Surprisingly, inhibition of the Notch pathway by DAPT induced the expression of CD83 on cDCs in all the conditions tested, reaching statistical significance in some cases (Fig. 5A). The CD83 expression on LPS- or R848-activated cDCs also showed a faint increase in the presence of DAPT (Fig. 5A).

Regarding pDCs, Notch ligation did not induce a clear increase in phenotype markers, but the presence of DAPT significantly decreased the expression of CD25, CD86, and, in contrast to cDCs, also CD83. Accordingly, the increase in CD25, CD83, and CD86 induced by R848 was inhibited by DAPT. The expression of HLA-DR on cDCs and pDCs was not altered either by Notch ligation or Notch inhibition (Fig. 5B).

Notch pathway is involved in cytokine production and Th polarization capacity of cDCs and pDCs

To further evaluate Notch activation, the secretion of several cytokines and chemokines, including IL-6, IL-10, IL-12p70, TNF-α, IFN-α, CCL19, CXCL9, CXCL10, and CXCL11, was studied on cDCs and pDCs cultured 24 h over Dll1- and Jag1-coated plates or activated with LPS or R848 in the presence or absence of DAPT. The results are depicted in Fig. 6. Raw values of protein secretion are also available in Supplemental Table I.

Overall, Notch ligation on cDCs did not affect IL-6, IL-12p70, IFN-α (data not shown), TNF-α, and CXCL9/10/11 secretion (Fig. 6A). Interestingly, Dll1 significantly induced the production of CCL19 and IL10, which were inhibited by DAPT. When using a potent TLR stimulation as positive control, the presence of DAPT significantly diminished the production of CCL19 (Fig. 6A).

Regarding pDCs, Notch ligation did not induce IFN-α, IL-6, IL-10, IL-12p70, and CXCL11 production (data not shown). In sharp contrast to that observed in cDCs, Notch ligation in pDCs significantly induced the secretion of TNF-α, an effect clearly blocked by DAPT (Fig. 6B). Notch ligation also induced, although the results did not reach statistical significance, the secretion of CXCL9 and CXCL10. Again, the production of these chemokines was significantly reduced in presence of DAPT. Additionally, although Notch ligation failed to induce the secretion of CCL19, the presence of DAPT significantly reduced the basal levels observed. Finally, when using TLR-activated pDCs as positive control, Notch activity was revealed by the inhibitory effect of DAPT, reducing the secretion of CCL19 and CXCL9 (Fig. 6B).

To study whether Notch ligation had additional implications in other key functional properties of DCs, alloproliferation experiments were used as a model to study Ag presentation and Th polarization capabilities of human cDCs and pDCs. DC subsets

FIGURE 3. Immunofluorescence staining of cDCs and pDCs revealing the pattern of expression of Notch receptors and ligands on resting and activated cells. Green fluorescence (Alexa 488) shows pattern for the indicated protein in each condition. Nuclei are shown in blue (Hoescht 33342). Original magnification ×630. Images are representative of three individuals.
obtained from different donors were cultured as before and were mixed with CbT cells ($n = 4$). After 4 d culture, allogeneic T cells that were cultured with DAPT-treated cDCs produced significantly lower levels of IFN-γ than did T cells cultured with control cDCs, whereas IL-10 production was not altered (Fig. 7A). In contrast, Notch inhibition on pDCs did not modify IFN-γ production but significantly diminished IL-10 secretion by allostimulated T cells (Fig. 7B, Supplemental Table II).

**FIGURE 4.** Notch ligation on DCs upregulates the transcription of Notch target genes. cDCs (A) and pDCs (B) cultured over coated IgG (control conditions), Dll1, or Jag1 or activated with LPS or R848 during 5 h in absence (−, filled symbols) or presence of DAPT (+, open symbols). Cells were recovered and the expression of Hes1, Hey1, and BATF cDNA was analyzed by real time RT-PCR. Results were normalized to control condition cDC/pDC plus IgG (basal value = 1). Each symbol represents an independent experiment. Bar indicates the mean of the three experiments. *$p < 0.05$, **$p < 0.01$.

**FIGURE 5.** Notch ligation alters the phenotype of DCs and is involved in TLR activation processes. The expression of CD25, CD83, CD86, and HLA-DR was studied by flow cytometry on cDCs (A) and pDCs (B) after Dll1 or Jag1 ligation and TLR (LPS or R848) activation, in the absence (−) or presence (+) of DAPT. Results were normalized to control condition cDC/pDC plus IgG (basal value = 1). Symbols and bars indicate the mean ± SD of the five experiments performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 

6 NOTCH EXPRESSION AND FUNCTION IN HUMAN DCs
**Discussion**

The Notch signaling pathway has been mainly involved in cell development and differentiation. Recent data also support a prominent role of Notch receptors and ligands in the regulation of the immune response. Several studies suggest that Notch activation may help in the promotion and maintenance of the inflammatory environment, contributing to the fully activated phenotype of macrophages by increasing the production of inflammatory cytokines (42). Different Notch ligands could modulate macrophage function depending on the immunological environment where these cells develop their function (46) and determine the M1 versus M2 macrophage polarization (47, 48). Additionally, RBP-J–deficient DCs showed diminished expression of CXCR4 and CCR7, critical chemokine receptors for DC differentiation and migration (39, 49). Also, Notch molecules have been involved in other important functions such as the communication between DCs and NK cells (50), protection from apoptosis (51), and adhesion between mast cells and stromal cells (52).

To date, data on Notch molecules expressed by human peripheral blood DCs have been partially defined by Liotta et al. (34) only in cDCs. To our knowledge, the present study is the first to largely describe the expression of Notch receptors and ligands in human peripheral blood cDCs and pDCs and to provide evidence of their functional activity. The results revealed that human peripheral blood DCs highly expressed Notch receptors and showed a more variable expression of Notch ligands among all individuals tested. This expression may change upon activation depending on the cell type. Whereas LPS activation induced a downregulation of Notch-1 in cDCs, R848-activated pDCs significantly increased the expression of Notch-1. Different reports in human macrophages or mice macrophages/DCs described the expression of Notch molecules in control and activated conditions. Human macrophages constitutively express Notch-1, Notch-2, Jag1, and Dll1 (40). Human and mice macrophages upregulated Notch-1 (46, 53) and also Jag1 and Dll4 upon TLR4 ligation (35, 42, 46, 54–56). LPS-induced upregulation of Notch-1 and Jag1 seems to be dependent on p38 MAPK and NF-κB (46), and Dll4 increase depended on MyD88 (28). Foldi et al. (42) demonstrated that TLR induction of Jag-1 in human macrophages was directed, dependent on Notch and RBP-J, and superinduced by IFN-γ, whereas TLR-induced Dll expression was indirect (required de novo protein synthesis), independent of Notch and RBP-J and suppressed by IFN-γ. Other stimuli, such as PGE2, cholera toxin, and TNF, seem to promote Jag2 expression (35, 55, 56). The different behavior of Notch

**FIGURE 6.** Notch pathway is implicated in cytokine and chemokine secretion by DCs. cDCs (A) and pDCs (B) maintained in control conditions were cultured during 24 h over coated IgG, Dll1, or Jag1 or activated with LPS or R848 in the absence (–, filled columns) or presence (+, open columns) of DAPT. Cells were recovered and the secretion of the indicated proteins was measured on supernatants by ELISA or Luminex assays. Results were normalized to control condition cDC/pDC plus IgG (basal value = 1). Bar indicates the mean ± SD of the six experiments performed. *p < 0.05, **p < 0.01, ***p < 0.001.
molecules observed in this and other studies may be attributed to the different stimuli, species (human), organ (blood), and cell lineage (cDCs) that may have different regulatory mechanisms controlling the expression of Notch molecules.

Regarding pDCs, to date, only a single study reported the expression of Notch receptors and ligands in mouse splenic pDCs (33), and one recent study showed the expression of Notch-1 in human pDCs (57). Mouse splenic pDCs were found to express Dll1 and Jag1 but not Jag1 and Jag2, both highly expressed by human pDCs in our results. In mice, the expression of Dll4 increased after immunization and CpG administration as observed in some human samples. Also, human pDCs increased Notch-1 after R848 stimulation, a process that could be related to cell differentiation and maturation, as has been described for monocytes and B cells (58, 59). Then, TLR-induced pDC maturation may promote changes in the expression levels of Notch molecules, which could contribute to the cellular response to a particular stimulus.

Demonstration of the functional activity of Notch molecules in human blood DCs has been directly shown by induction of Notch target genes (Hes1, Hey1, and BATF) upon ligation and, mostly, by using the γ-secretase inhibitor DAPT. In our study, both cDCs and pDCs presented a basal Notch activity most probably due to the recognition of ligands expressed by nearby cells during culture. This would undoubtedly hamper the level of activation induced by soluble ligands. Even so, cDCs responded notoriously to Notch ligands, as indicated by the increase in the transcripts of Hes1, Hey1, and BATF. TLR ligation (LPS or R848) also induced the expression of the Notch target genes studied as described before on macrophages (40, 42). In human macrophages, basal Notch activity and Dll1 or Jag1 treatment resulted in a moderate induction of Hes1 and Hey1 (40), and similar results have been reported in mice bone marrow-derived DCs cultured with Jag1, Dll1, or Dll4 (38). Studies using ligands suggested that Dll1 may have a more potent effect than does Jag. This has been reported in CD34⁺ cord blood progenitor cells (60), in CD34⁺CD1a⁺ thymic precursors (61), and in hematopoietic progenitor cells (62). In this last study, whereas Dll1 induced DC differentiation, Jag1 inhibited DC differentiation and promoted the accumulation of immature myeloid cells. The distinct effect of the two ligands may reflect a particular physiological role.

As in cDCs, pDCs maintained in control conditions also had a basal Notch activity, revealed by the basal levels of Hes1. In pDCs, Hes1 transcription was induced in a similar way by Dll1 and Jag1 and was abrogated by DAPT. However, Notch ligation did not promote the expression of Hey1 and BATF in pDCs, perhaps revealing a weaker capacity of pDCs to directly respond to Notch ligation in the absence of further stimulation. However, a potent TLR stimulation using R848 induced the transcription of the three genes tested. DAPT treatment also blocked this induction, thus showing an interplay of Notch and TLR pathways in pDCs as well, as observed on HSV-2-stimulated human pDCs (57).

Phenotypically, human monocyte-derived DCs and mouse bone marrow-derived DCs were described to increase MHC-II, CD80, and CD86 after Notch ligation by Jag (37, 38). Interestingly, Notch activation seems to promote macrophage maturation and differentiation to DCs, driving them to a more prominent role as APCs by increasing ICAM-1 and MHC-II and by limiting their cytotoxic capacity (46). Moreover, RBP-J–deficient DCs show less dendrites, lower levels of MHC-II, and reduced capacity of migration and Ag presentation both in vitro and in vivo (39). Our results confirmed that Notch ligation on cDCs also induced the upregulation of CD86, but the expression of HLA-DR was not modified either in cDCs or pDCs. In fact, Notch ligation did not induce phenotype changes on pDCs, probably as a result of their weaker response to Notch-soluble ligands. However, the role of the Notch pathway in pDCs is still revealed by the significantly lower expression of CD25 and CD86 observed in cells cultured in the presence of DAPT, CD25, a maturation marker on human pDCs (63), is also considered a Notch target gene (13). Notch-1 activity seems to play a role in maintenance of the high-affinity IL-2R complex on the cell surface of CD4⁺ T cells (64). Accordingly, activated CD4 T cells cultured in the presence of γ-secretase inhibitors reduced IL-2 secretion and CD25 expression (35), as we observed in our experiments. Finally, an intriguing observation was the inversely regulated expression of CD83 in pDCs and cDCs. Whereas inhibition of the Notch pathway resulted in the reduction of CD83 in pDCs, the same conditions promoted the expression of this molecule on cDCs. Further experiments are needed to clarify the role played by the Notch pathway in the regulation of CD83 expression by DC subsets.

Beyond changes in the phenotype and induction of Notch target genes, we sought to study the role of Notch activation in other functions of human DCs. Cytokines and chemokines play a central role in the orchestration of the immune response. Several reports

FIGURE 7. DAPT treatment of cDCs and pDCs modifies cytokine production by allostimulated T cells. cDCs (A) and pDCs (B) obtained from four donors and maintained in control conditions were cultured during 24 h over coated IgG, Dll1, or Jag1 or activated with LPS or R848 in the absence (−, filled columns) or presence (+, open columns) of DAPT. Then, DCs were extensively washed, counted, and cultured with CbT cells at a 1:20 ratio (DC/CbT cells). After 4 d, supernatants were harvested and the presence of IFN-γ and IL-10 was measured by ELISA. Results were normalized to control condition cDC/pDC plus IgG (basal value = 1). Bar indicates the mean ± SD of the four experiments performed. *p < 0.05, **p < 0.01.
suggest a role for Notch and Notch ligands in the modulation of cytokine secretion (65, 66). Transcription factors, such as NF-kB, are key regulators of cytokine production, and Notch signaling may exert some effects on DC maturation through NF-kB. In fact, NF-kB has been largely considered a putative Notch target gene, regulated by Notch signaling through RBP-J (14), and several reports have revealed the role of Notch ligation in NF-kB activation (41, 53, 67, 68). Interestingly, a report suggested that Jag1/Notch signaling supports the inflammation mainly through NF-kB and also JAK/STAT/STAT6 pathways in inflamed CNS (69). Among others, NF-kB is implicated in the induction of key cytokines produced by activated DCs such as IL-6 (70) and TNF-α (71), as well as CCL19 (72), a chemokine that regulates the migration of mature DCs and recruits naïve T cells close to mature DCs (73–75). Overall, our results point to cytokines such as IFN-γ, IL-6, IL-12, and CXCL11 not being modified by Notch in peripheral blood DCs. Other cytokines, such as CCL19, are regulated in a similar way by the Notch signaling pathway in both cDCs and pDCs. Interestingly, as found for the CD83 expression, we have also identified a differential effect of the Notch pathway on some cytokines/chemokines secreted by cDCs and pDCs. In particular, Notch ligation induced TNF-α, and Notch inhibition significantly hampered TNF-α and CXCL11/10 secretion by pDCs, whereas in cDCs it had no effect. Conversely, IL-10 production was significantly induced by Notch ligation (and reduced by DAPT) in cDCs, but no effect was observed in pDCs. Perhaps reflecting this differential regulation by Notch pathway on DC subsets, Th polarization of CbT cells was also modified by each cell type. Notch inhibition on cDCs resulted in a reduced secretion of IFN-γ but no changes in IL-10 by allostimulated T cells. Conversely, DAPT-treated pDCs did not affect IFN-γ secretion but drastically reduced IL-10 secretion by allostimulated T cells. Whether these observations are directly related to the cytokine/chemokine profile or phenotype changes regulated by Notch on DC subsets is still unclear. However, these results suggest a differential role of the Notch pathway in cDCs and pDCs, and the mechanisms underlying these effects merit further investigation.

In summary, our study is the first, to our knowledge, to fully describe Notch receptors and ligands in human peripheral blood DCs. Functionally, peripheral blood DCs respond to ligands Dll1 and Jag1, showing cDCs a higher capacity to respond to Notch ligation compared with pDCs. Notch ligation/inhibition promote changes in phenotype, cytokine and chemokine secretion, and Th polarization induced by cDCs and pDCs. Importantly, Notch–Notch ligand interactions have been related to cellular infiltration in vascular inflammation (76), to secretion of proinflammatory cytokines in autoimmunity (69), and also to Kaposi sarcoma herpesvirus-associated suppression of genes related to cell cycle in adjacent endothelial cells (77). Additionally, Notch molecules may also be involved in communication between immune adjacent cells as shown between DCs and NK cells (50). Our study suggests a possible role for the Notch pathway as an additional mechanism controlling human DC responses. It is therefore conceivable that changes in Notch and Notch ligand expression on DCs and nearby cells will ultimately contribute to define the local milieu found under the particular conditions of the immune response.

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Disclosures

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