The Notch ligand delta-1 is a hematopoietic development co-factor for plasmacytoid dendritic cells.

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Abstract

Plasmacytoid dendritic cells (pDC) play an important role in innate and adaptive immunity, prompting interest in mechanisms controlling the production of this lineage of cells. Notch signaling via one of the Notch ligands, delta-like 1 (delta-1), influences the hematopoietic development of several lymphoid and myeloid lineages but whether or not delta-1 affects the formation of pDC is unknown and was tested here. Human CD34+ progenitor cells were cultured onto delta-1-expressing OP9 stroma in the presence of flt-3 ligand and IL-7, and this efficiently generated BDCA2+ CD123+ CD4+ CD11c- cells with the characteristic morphology of pDC, expressing TLR9, pre-Tα mRNAs and secreting CpG-induced IFN-α. Delta-1 augmented the numbers of BDCA2+ cells produced, without affecting their proliferation, and the effect was blocked by gamma secretase inhibition. The development of pDC was stroma-, delta-1- and cytokine-dependent and could be induced from committed lymphoid progenitor cells which responded to delta-1 by opposite changes in pDC and B cell production. Our results identify delta-1 as a novel factor enhancing pDC hematopoiesis and delineate a new role for Notch signaling in lymphopoiesis by showing its opposite effect on pDC and B lineage determination.
Introduction

Dendritic cells (DC) constitute a complex system of antigen presenting cells (APC) providing the immune system with multiple possibilities for anti-microbial and anti-viral host defenses (recently reviewed in 1). Within this system, plasmacytoid DC (pDC) are distinguished from so-called conventional DC by the expression of specific microbial pattern recognition receptors and by the unique ability to secrete high levels of type I interferon (IFN) in response to viruses. Being immature precursor cells, pDC poorly stimulate T cells and may be tolerogenic but once activated they can direct various types of T cell responses depending on associated pathogenic or inflammatory signals. In humans, pDC are recognized by expression of the C-type lectin BDCA-2, BDCA-4/neuropilin-1, CD4 and high levels of the IL-3Rα chain (CD123). They have low levels of MHC class II antigens and lack expression of CD1a or CD11c which are found on conventional DC 2. Several lymphoid transcripts are expressed in pDC such as pre-TCRα chain, λ5, IgH D-J gene rearrangements and Spi-B, and not found in most conventional DC 3. Altogether, pDC constitute a distinct and important component of innate and adaptive immunity. They have been implicated not only in viral defenses but also in autoimmunity as well as immune regulations occurring in solid tissue and bone marrow transplantation 1,4. Delineating the molecular mechanisms that contribute to the production of pDC could therefore provide new insights into the physio-pathology and therapies of such conditions.

The hematopoietic development of DC lineages seems strikingly flexible compared to other leukocytes. Recent studies have formally established that pDC, like conventional DC, have diverse origins and can arise from progenitor cells engaged towards lymphoid or myeloid differentiation programs 5-8. One challenge is to identify the soluble factors and cell contact-dependent signals that regulate the development of specific DC progenitor
The cytokine flt-3 ligand (FL) has emerged as a major regulator of pDC development in vivo and in vitro. Cell to cell interactions are generally important during developmental processes particularly for lymphoid cells. Interactions between Notch receptors/Notch ligands constitute an evolutionarily conserved example of interactions between precursor cells and their surrounding microenvironment. Notch receptors (Notch 1-4) and their ligands (classified into two families of proteins: Jagged/serrate (Jagged 1 and 2) and delta (delta-like 1 (delta-1), 3 and 4)) are widely expressed in the hematopoietic system (recently reviewed in 11). Notch signaling is a prominent regulator of the immune system essentially through marked effects on T/B lymphopoiesis and terminal T cell differentiation. One of the Notch ligands, delta-1 is a major inducer of Notch signaling in lymphoid cells. Stromal cell lines expressing delta-1 support T cell differentiation, facilitate T/NK precursor development and block B lymphopoiesis. In contrast to lymphoid cells, much less is known about the effects of delta-1 or Notch signaling in DC development. In humans, delta-1 acts as a negative regulator of monocyte/macrophage differentiation and induces monocyte apoptosis specifically in the presence of M-CSF. Delta-1 does not seem to affect the differentiation of myeloid precursor cells into conventional DC. Whether delta-1 has a role on pDC development is not known. Because delta-1 affects lymphoid progenitor cells, and because pDC have some lymphoid characteristics we hypothesized that delta-1 may regulate pDC formation. We adapted a culture system used for lymphoid differentiation based on M-CSF-deficient OP9 stromal cells and the cytokines FL and IL-7, to test if human delta-1 affected human pDC hematopoiesis. Our results show that delta-1 supports pDC formation from hematopoietic progenitor cells and is particularly efficient on committed lymphoid progenitor cells which differentiate into pDC while B lymphopoiesis is blocked. Our results therefore delineate a new role for this Notch ligand in lymphopoiesis.
Materials and methods

Hematopoietic progenitor cells

Samples of umbilical cord blood (UCB) and adult bone marrow (BM) were obtained in accordance with the French bioethics laws and the French National Bioethics Committee and with approval from the "Banque de Tissus pour la Recherche" scientific review committee. For BM samples, informed consent was provided according to the Declaration of Helsinki. Mononuclear cells (MNC) were isolated by centrifugation over Ficoll (Lymphoprep, Abcys, Veyrier du lac, France). UCB CD34+ cells were purified using magnetic activated cell sorting and microbead-conjugated CD34 antibodies according to the manufacturer’s recommendations (Miltenyi Biotec, Bergisch-Gladbach, Germany) and generally cryopreserved before use. BM lymphoid progenitor cells were prepared from cryopreserved BM-MNC using flow cytometry cell sorting (MoFlo, Cytomation) with the following antibodies: APC-conjugated CD34 (clone 581, BD biosciences, Le pont de Claix, France), FITC-conjugated lineage (Lin) markers (TCRαβ, clone T10B9.1A-31, BD biosciences, CD14, clone 3C10E12 prepared in the laboratory, CD40 clone 5C3, BD biosciences, CD19, clone SJ25-C1, Caltag Laboratories, Burlingame, CA, CD20, clone H147, Caltag Laboratories) and PE-conjugated CD10, clone SJ-1B4, Caltag Laboratories.

Generation of OP9 stroma cell lines

The cDNA encoding human delta-1 (delta-like-1 accession AF003522 - gi 10518496) was kindly provided by S. Artavanis-Taskonas (Harvard Medical School, Charlestown, MA). The ORF (EcoRV-Xbal 2897 bp fragment) was cloned into the BamH1 site of the multiple cloning site of the self-inactivating pWPIReGFP bi-cistronic HIV-1-derived lentiviral transfer
vector, kindly provided by P. Salmon (University of Geneva, Switzerland), and allowing concomitant expression of human delta-1 under the control of the EF1-α promoter and of the green fluorescent protein (GFP) through an ECMV IRES sequence. The vector encoding only GFP was used as control. VSV-G-pseudotyped lentiviral vector stocks were produced by quadritransfection of 293T cells as described 25. The same parental stock of OP9 cells 26 was transduced either with delta-1/GFP-encoding lentiviral vector to produce OP9-Del1 cells or with the control GFP vector to produce OP9-C control cells. Lines expressing high levels of transgene were enriched by flow cytometry cell sorting based on GFP expression. OP9 cells are known to lack expression of murine delta-1 22. High levels of human delta-like 1 expression were confirmed in OP9-Del1 cells, but absent in OP9-C cells, by real time PCR (Primers and probes sequences were hDel1 F CTCCTGAGGTCCTCGACGC, hDel1 R CGACGTCACGGAAGGCAG, hDel1 P ACAGCCTGTCCGGCCCG, expression of target gene was normalized using the endogenous gene β2M (Taqman PDAR reagent; Applied Biosystems), data not shown).

Co-culture assay

Cocultures were initiated by seeding 2 - 3 X 10^4 CD34+ cells per well of a 24 well-plate into which OP9-Del1 or OP9-C cells were seeded the day before at 28 000 cells / cm^2. Culture medium (0.5 ml / well of R10 medium) consisted of RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), L-glutamine, penicillin/streptomycin (Gibco BRL, Life technologies Ltd, Paisley, Scotland) that was supplemented with recombinant human Flt-3-ligand (FL) and human interleukin-7 (IL-7) (5 ng / ml each, R&D Systems, Minneapolis, MN) unless otherwise indicated. In some experiments, γ-secretase inhibitor (N-{N-(3,5-difluorophenylacetyl-1-alanyl)}-S-phenylglycine t-butylerster (DAPT)) was added every 3-4 days to culture medium (10μg/ml, Calbiochem, EMD biosciences, San Diego, CA) and
controls consisted in treating cultures with same concentration of DMSO carrier. Cells were collected at different time points after forceful pipetting to disrupt stroma, counted and used for subsequent analyses and experimental assays. For transwell experiments, CD34+ cells were separated from the OP9 stroma by 0.4μm pore size membrane inserts (Corning B.V, life Sciences, Schiphol-Rijk, The Netherlands).

Flow cytometric analysis

Directly conjugated mouse anti human mAbs, including APC-conjugated CD19 (clone SJ25-C1, Caltag Laboratories), HLA-DR (clone TU36, Caltag Laboratories), CD14 (clone TuK4, Caltag Laboratories), PE-conjugated CD56 (clone B159, BD biosciences), CD1a (clone VIT6B, Caltag Laboratories), CD11c (clone BU15, Caltag Laboratories), CD4 (clone S3.5, Caltag Laboratories), FITC conjugated CD3 (clone S4.1, Caltag Laboratories) and isotypes control mAbs. BDCA-2 FITC, CD123 PE, BDCA-4 APC and BDCA-1 FITC were from Miltenyi Biotech, Gladbach, Germany. Cells were stained with mAbs for 30 minutes on ice and washed twice with PBS, 0.2% BSA, 0.02% NaN3. Cellular staining was measured on a FACSCalibur instrument (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software, with results expressed as percentages of cells staining above background staining obtained with irrelevant mAbs. Stromal cells and dead cells were excluded from analysis. For cell cycle experiments, DNA content was detected using Hoechst 33342 DNA binding dye (Molecular probes, Eugene, OR). Cells were resuspended at 1X10^6/ml in HBSS, 10% FCS, 1g/L glucose, Hepes 2mM and Hoechst 33342 10μg/ml during 90 mn at 37°C. Cells were washed and labeled with directly conjugated BDCA-2 FITC antibodies. Simultaneous analysis of DNA content and surface phenotype were performed on a dual laser LSR instrument (UV 488nm, BD Biosciences).
RT-PCR

Total RNA was extracted from $5 \times 10^5$ total cocultured cells using SV total RNA isolation system (Promega, Madison, WI) and was reverse transcribed using random hexamers according to the manufacturer's instructions (superscript first strand synthesis system for RT-PCR, invitrogen, Paisley, Scotland).

PCR primers sequences were as follows: TLR9 F: TTATGGACTTCTGCTGGAGGTGC, TLR9 R : CTGCGTTTTGTGAAGACCA, Pre-Tα F: GGCACACCTTTTCTTCT, Pre- Tα R: GCAAGTCTGTGGCTTAAAGGC, TLR4 F : CTGCAATGGATCAAGGACCA, TLR4 R : TCCCACTCCAGTAAATGTTT, HPRT F: TATGGACTGACTGAACGTCTTGC, HPRT R : GACACAAAACATGATTCAAATCCCTGA. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

In vitro activation

For in vitro activation, 2 - 5 $\times 10^5$ total cocultured cells were transferred to a well of a 24-well tissue culture plate in R10 medium supplemented or not with a mixture of recombinant human CD40L trimer (1 $\mu$g/ml, a kind gift from Immunex) and IL-1β (10 ng/ml; R&D Systems), CpG oligodeoxynucleotide type A (ODN 2216 2μM), Poly I:C (50μg/ml) or peptidoglycan (PGN) (10μg/ml) (Invivogene, San Diego, CA). After 24 to 48 h., cell supernatant fluids were collected and stored frozen at -20°C until used to measure IL-8 (BD PharMingen) and IFN-α (Biosource, Camarillo, CA) contents by ELISA according to manufacturer's instructions. Purified pDC (BDCA2+CD123+ cells or BDCA4+CD123+ cells) obtained from co-culture by flow cytometry cell sorting were activated in culture medium supplemented with IL-3 (25 ng/ml) and for 72 hours as described 27
Statistical analysis

The statistical analysis of data was performed by paired t test using 95% confidence interval for significance.
Results

Delta-1 induces human CD34+ cells to differentiate into pDC.

To examine the effects of human delta-1 on pDC hematopoiesis, we compared how M-CSF-deficient OP9 stromal cells expressing or not this molecule, affected growth and differentiation of multipotent hematopoietic progenitor cells in cultures supplemented with FL and IL-7. In this system, UCB CD34+ cells grew and differentiated over time. In 20 days, total cell numbers expanded 98 +/- 57 fold on OP9-Del1 cells which was higher than 61 +/- 52 fold on OP9-C stroma (n=17 experiments, p=0.0001). Flow cytometric analyses were performed at different time points to monitor hematopoietic differentiation through the expression of mature cell markers on cultured cells. Representative kinetics studies showed that BDCA-2, a marker of pDC, was readily detected after one week in both OP9-Del1 and OP9-C cultures and was expressed, as expected, on a well-defined population lacking the CD14 antigen (Figure 1-A). The percentage of BDCA-2+ cells was significantly higher in OP9-Del1 cultures than in control cultures as measured at day 10 (15 ± 6% vs. 7 ± 2%) and at day 20 (14 ± 10% vs. 9 ± 8%) in a total of 13 separate experiments (Figure 1-B). Over time, higher total numbers of BDCA-2+ cells were generated in OP9-Del1 cultures compared to control (Figure 1-B). In approximately 3 weeks, we calculated that one single CD34+ cell yielded in theory 18 BDCA-2+ cells in the OP9-Del1 system, compared to 6 BDCA-2+ cells in control cultures.

As CD34+ cells decreased similarly over time in both OP9-Del1 and OP9-C cultures, the results suggested that delta-1 enhanced the differentiation of hematopoietic progenitor cells into BDCA-2+ cells or alternatively, that delta-1 expanded committed cells. Comparisons of cell cycle analyses on BDCA-2+ cells produced in OP9-Del1 or OP9-C cultures showed identical distribution of cells in the various phases of the cell cycle and in
particular no augmentation of cells in G2-M in cells produced on OP9-Del1 stroma (Figure 2). These results argue against an effect of delta-1 on BDCA-2+ cell expansion and support the interpretation of enhanced hematopoietic differentiation of CD34+ cells into the BDCA-2+ cell lineage.

**BDCA2+ cells generated onto OP9-Del1 displayed characteristic pDC features.**

The entire population of cells expressing BDCA2 that was produced onto OP9-Del1 stroma, also displayed high levels of CD123 as well as BDCA-4 and CD4, intermediate levels of HLA-DR and lacked expression of CD11c, CD1a and CD14 antigens (Figure 1-A and Figure 3-A). Overall, this is a cell surface phenotype that defines so-called circulating precursors of pDC 1,2. As a confirmation, and to exclude trivial artefacts from dying OP9 cells, we purified cells co-expressing the human-specific CD45 marker with BDCA2 and CD123 from OP9-Del1 co-cultures. We found that such cells displayed the characteristic morphology of immature pDC, with small size and a high nucleus/cytoplasm ratio (Figure 3-B). The molecular signature of pDC was evident in OP9-Del1 co-cultures with the detection of transcripts for pre-TCRα chain and TLR9 mRNAs (Figure 3-C). These transcripts were more abundantly expressed in the presence of delta-1, consistent with the augmented numbers of BDCA2+ cells.

pDC express specific microbial pattern recognition receptors such as TLR9 for recognition of DNA with high CpG content. Conventional DC express TLR 2 - 5 for recognition of bacterial products such as peptidoglycan or of poly I:C that mimicks viral double-stranded RNA 28. Activation of the bulk of cultured cells with various stimuli consisting of TLR ligands or of inflammatory stimuli confirmed the existence of a functional response via TLR9 since CpG oligonucleotides induced the secretion of high levels of IFN-α (Table 1). Such production of IFN-α was statistically higher in OP9-Del1 cultures than in controls, in a
range that was compatible with the enhancement of pDC numbers. In such bulk cultures, delta-1 did not seem to affect the cellular capacity for IFN-α production since the calculated production of IFN-α per BDCA2+ cell was 0.19 ± 0.15 pg/cell in OP9-Del1 cultures versus 0.06 ± 0.06 pg/cell in OP9-C cultures (n=5), which was statistically not significantly different. The induction of IFN-α was specific to CpG stimulation as expected from published studies. We also directly confirmed that OP9-Del1-generated pDC could produce IFN-α after CpG stimulation. Homogeneous populations of purified BDCA-2+ CD123+ or BDCA-4+ CD123+ cells were isolated by flow cytometry cell sorting, stimulated with CpG and IL-3 (IL-3 was used to maintain viability) and IFN-α was produced in all experiments (n=3) averaging 0.05 ± 0.02 pg/cell, which is in the range of calculated values from bulk cultures. These values are also consistent with the literature since pDC produced in stroma-free cultures and activated with herpes virus produce an estimated 0.1 pg/cell IFN-α. Using these sorted cells we also confirmed the expression of pre-Tα and TLR9 mRNA (Figure 3-D) which further demonstrated their pDC characteristics. In addition, the cells produced on OP9-Del-1 were activated via CpG or CD40L+IL-1 and both of these stimuli augmented the levels of CD86 cell surface expression on the BDCA-2+ cell population (Table 2) providing evidence that phenotypic maturation could be induced on these cells. Thus, our data showed that delta-1 was an effective signal that enhanced the differentiation of hematopoietic progenitor cells into functional pDC.

Effects of delta-1 on other hematopoietic lineages of cells.

Multiple types of hematopoietic cells were generated in the OP9 co-culture system, including cells of lymphoid lineages. Delta-1 significantly decreased CD19+ CD10+ B cell differentiation since at day 20, the percentages of CD19+ B cells in OP9-Del1 cultures were 1 ± 1% versus 6 ± 3 % in OP9-C cultures (p=0.02, n=5) and overall, approximately 3
times less CD19\(^+\) cells were produced in OP9-Del1 cultures compared to controls (p=0.05) (Figure 4). In opposition, the production of CD56\(^+\) cells, putatively NK cells, was significantly augmented by delta-1 both in proportion \((11 \pm 5 \% \text{ versus } 4 \pm 1\%, p=0.04, n=5)\) and total numbers of cells, since approximately 3.5 times more CD56\(^+\) cells were produced in 3 weeks onto OP9-Del1 stroma compared to controls \((p=0.03, n=5)\) (Figure 4). T lymphocytes were not examined in these experiments. However separate studies in our laboratory showed that our OP9-Del1 cells support the differentiation of murine lineage-negative bone marrow progenitor cells into CD3\(^+\) CD8\(^+\) T cells in 3 weeks as expected from prior reports \(^{18}\). Furthermore, our OP9Del1 cells support the differentiation of human CD34\(^+\) CD38\(^-\) umbilical cord blood progenitor cells into CD7\(^+\) CD3\(^+\) human T cells after more than 3 weeks of culture, as reported\(^{19,20}\) (Supplemental Figure S1).

As for myeloid cells, monocytic cells expressing CD14 were produced in small proportions which were significantly reduced by delta-1, as shown in Figure 1-A, 1-B and Figure 4. For instance at day 20, CD14\(^+\) cells represented on average 2 +/- 1\% of cells in OP9-Del1 cultures versus 11 +/- 7\% in OP9-C cultures, \(p=0.0007, n=13\) experiments). We also identified populations of CD1a\(^+\) cells that were generated in these cultures (Figure 4). In the absence of other T cell markers, the population of CD1a\(^+\) CD14\(^-\) cells most likely represented conventional DC rather than immature T cells. Indeed, such CD1a\(^+\) cells also co-expressed BDCA1, CD11c and high levels of HLA-DR antigens which are characteristic DC markers (data not shown). Besides, we also observed CD1a\(^+\) CD14\(^+\) cells, resembling the intermediate cells generated during DC differentiation from CD14\(^+\) cells \(^{29}\) There was no statistically significant effect of delta-1 on the numbers of total CD1a\(^+\) cell produced \((n=5)\). However, further analysis of subtypes of CD1a\(^+\) cells should be performed to determine if an effect of delta-1 exists on subsets of conventional DC. At the functional level, cytokine secretion profiles confirmed that monocytes and conventional DC were also generated with pDC in the culture system. Stimulation of the cultures with the pro-
inflammatory stimuli CD40L+IL-1 or with the TLR2 ligand peptidoglycan induced the secretion of respectively IL-12 and IL-8 cytokines which were found at similar levels in OP9-Del1 and OP9-C cultures (Table 1). Both stimuli are known to activate cytokine secretion in CD1a⁺ interstitial DC or Langerhans cells 28,30. The expression of mRNA for TLR4, a receptor known to be present on CD1a⁺ DC and monocytes, appeared to be similar in both cultures (Figure 3-C). Altogether, Delta-1 significantly modulated the differentiation of hematopoietic progenitor cells into pDC, CD14⁺ cells, B and CD56⁺ lymphoid lineages in the same time frame. However, at the functional level, delta-1 did not appear to be a strong modulator of activities attributed to conventional DC, in this particular experimental set-up.

**Hematopoiesis induced by Delta-1 is Notch-, contact- and cytokine-dependent.**

To confirm that delta-1 affected hematopoietic progenitor cell differentiation into pDC through Notch signaling, we tested the effects of DAPT, an inhibitor of the γ-secretase responsible for the cleavage of Notch induced by ligand binding and a well-accepted inhibitor of the Notch signaling pathway 31. Treatment of cultures with DAPT significantly reduced the percentages and numbers of BDCA2⁺ CD123⁺ cells produced in culture (Figure 5-A and 5-B). Concomitantly, and as expected, DAPT enhanced the levels of CD14⁺ cells, while reducing that of CD56⁺ cells, thus validating the system (Figure 5-B). To characterize the mode of action of delta-1, we compared co-cultures performed by direct cell contact or through a transwell insert that physically separated progenitor cells and stroma. Results showed that direct contact between CD34⁺ cells and stroma was essential for the efficient production of BDCA-2⁺ pDC (data not shown). The presence of stroma was confirmed to be essential since culturing CD34⁺ cells in the presence of FL+IL-7 cytokines but without OP9-Del1 cells did not generate pDC (data not shown). While FL was known to
be essential for the production of pDC. The importance of IL-7, a lymphoid growth factor, in pDC differentiation was not known. Results showed that removal of either IL-7 or FL compromised the production of BDCA2+ cells (Figure 6) demonstrating that a cooperation is needed between these 2 signals and delta-1 to efficiently support pDC formation.

The production of pDC is induced from lymphoid progenitor cells.

Since delta-1 significantly modulated pDC and lymphoid cell fates, we wondered if this signalling pathway could directly target a population of lymphoid progenitor cells. Several developmental origins of pDC have been demonstrated in the murine system. In particular, BM lymphoid progenitor cells constitute one population capable of producing pDC in vivo and in vitro in the presence of FL. In humans, a BM committed lymphoid progenitor cell population (CLP) that displays T, B, DC and NK cell potential can be isolated on the basis of expression of CD34 and CD10 and lack of CD19 and other lineage markers. This lymphoid progenitor cell is more abundantly found in BM than UCB and therefore BM CLP were tested here. The co-culture of this CLP onto OP9-C in the presence of FL and IL-7 rapidly generated large proportions of CD19+ B cells (Figure 7) thus confirming its strong B lymphoid commitment. CLP were able to produce pDC. The presence of delta-1 strongly enhanced the proportion of BDCA-2+ CD123++ pDC in CLP cultures (average 43 +/- 7 % vs. 20 +/- 17 % of cells in OP9-Del1 vs. OP9-C cultures, n=3 experiments) while consistently reducing B lymphoid differentiation (average 19 +/- 13 % vs. 41 +/- 23 % of cells in OP9-Del1 vs. OP9-C cultures, n=3 experiments) (Figure 7). There was little expansion and total cell numbers produced could not be determined reliably in these experiments. These results demonstrate that pDC can be produced directly and rapidly from a population of committed human BM lymphoid progenitor cells. Depending on the
presence of delta-1, the CLP were able to differentiate into B cells or into pDC within the same time frame and in comparable proportions. The near complete lack of CD14+ cell production confirmed the peculiar lymphoid-restricted developmental potential of the CLP contrasting with the control population of CD34+ Lin- CD10- CD19- progenitor cells that comprise myeloid and multipotent hematopoietic progenitor cells (Figure 7). On such cells and at the time points examined, delta-1 had a modest effect on pDC or B cell formation. Therefore, at present, our results essentially show that delta-1 acts on lymphoid committed cells to regulate pDC and B cell lineage differentiation in opposite fashion.
Discussion

This study describes the Notch ligand delta-1 as a pDC development co-factor and shows that this signal is particularly efficient at triggering the differentiation of human lymphoid progenitor cells into pDC. Delta-1 provides a positive signal for hematopoietic development into the pDC lineage by augmenting the proportions and the absolute numbers of pDC produced from progenitor cells without having a demonstrable effect on expansion of committed pDC. The induction of differentiation is highly likely however we cannot exclude that delta-1 acts in part through enhanced survival of pDC precursors. The term co-factor is used because the efficiency of the delta-1 signal is dependent upon the presence of both FL and IL-7 cytokines.

The effects of delta-1 are blocked by $\gamma$-secretase inhibition which strongly implicates the Notch signaling pathway in the control of human pDC development. The genes and signals resulting from Notch activation and susceptible to control pDC lineage determination are not defined yet. It is commonly accepted that ligands binding to the Notch receptors engage a core signaling pathway which results in the nuclear translocation of a signal-transducing Notch intracellular domain (NICD) that heterodimerizes with CBF-1 (also known as CSL or RBP-Jk) converting this repressor into an activator which subsequently regulates several target genes $^{35}$. One known Notch target gene is the pre-TCR$\alpha$ gene $^{36}$ which is relevant to pDC, thus providing indirect evidence that Notch signalling can occur in this lineage. Further evidence that delta-1 has induced Notch signaling in our system, comes from observing the modulation of lineages other than pDC as expected from the literature $^{21,22}$. The production of CD19$^+$ B cells and of CD14$^+$ cells was reduced while that of CD56$^+$ cells was enhanced by delta-1 and the reversal of these effects by $\gamma$-secretase inhibition is documented here on the NK and monocyte lineages. The modulation of B, NK and monocyte production is known to be
Notch-dependent, being reproduced with enforced Notch signaling via NICD expression or via enforced expression of the Notch target genes HES1 and HES5 $^{15,37}$. The relevance of delta-1 in pDC development \emph{in vivo} has yet to be evaluated. These cells were not examined in mice conditionally ablated for delta-1 which otherwise lacked splenic marginal zone B cells but had normal T cell development $^{38}$. The lack of effect of delta-1 ablation on T cells was explained by a redundant activity of delta-4. Thus, by analogy, Notch ligands other than delta-1 could regulate pDC development, although this is unknown at the moment. The inactivation of Notch 1 through the Mx-Cre recombinase strategy suggests that this receptor is not essential for DC formation, neither for interstitial DC nor Langerhans DC nor pDC $^{39}$. However, mutant models using such IFN-mediated conditional deletion may be ill-adapted to the study of pDC since the Cre-inducing agent, poly I:C, has a major effect on this lineage of cells $^{40}$. Thus alternative methods of investigations of Notch signaling on pDC development \emph{in vivo} seem required.

Several recent studies have established that pDC, like conventional DC, have complex developmental origins. A lymphoid origin, initially postulated on the basis of lymphoid transcripts in pDC $^{3}$, is now clearly established in the murine and human systems as pDC can be directly obtained by \emph{in vitro} culture or by \emph{in vivo} transplantation of lymphoid lineage-restricted progenitor cells $^{5-7}$. We confirm and extend these findings by showing that human CLP effectively produce pDC and identify delta-1 as a signal of differentiation for these cells. Delta-1 controlled a symmetrical process of CLP differentiation towards pDC or B cells that occurred in a similar context and time frame and from a relatively homogeneous progenitor cell population. Stromal contact and soluble factors played a determining role in pDC formation. The cytokine FL is known to be a major regulator of human or murine pDC development \emph{in vivo} $^{8}$ and \emph{in vitro} $^{9,10,27}$. Flt3 receptor is found on lymphoid and myeloid pDC progenitor cells, further supporting the relevance of using FL to generate pDC $^{7,41}$. While the effects of FL on pDC are well-established, the
enhancing activity of IL-7 had not been appreciated before. The effects of IL-7 on pDC production could be context-dependent or progenitor-specific. Indeed, prior studies have shown that IL-7 reduces the effects of FL on pDC development from primitive hematopoietic progenitor cells. The identification of human lymphoid progenitor cells as efficient precursor cells for pDC may be an important aspect of the IL-7 response. Indeed, CLP express the IL-7Rα chain and high levels of IL-7Rα mRNA are detected in CLP and in their pDC progeny contrary to myeloid progenitor cells and their pDC descendence. However, a positive effect of delta-1 on IL-7R expression is unlikely as we found no difference in IL-7R expression in OP9-C or OP9-Del1 cultures (data not shown). There is also an important myeloid differentiation pathway for pDC production that has been shown both in vitro with human cells and in vivo in mice. In mice, spleen and liver pDC populations were more effectively reconstituted after transplantation of committed myeloid progenitor cells (CMP) than CLP. However, this was not the case in the thymus where CLP-derived pDC reconstitution was 9 fold greater than for CMP. Being present on murine MHC class II+ thymic epithelium, delta-1 may provide a physiologically relevant signal regulating the fate of CLPs entering the thymus. Through the reciprocal control of pDC versus B cell development, the presence or the absence of delta-1 may constitute an essential aspect of the niche in which lymphoid progenitor cells develop. A recent study in mice seems to contradict our findings, reporting an inhibitory effects of delta-1 on the production of pDC from early lymphoid progenitor cells of bone marrow. However, these murine BM lymphoid cells were cultured for short periods of time (8 days) using FL only and pDC were produced from IL-7Rα-deficient cells which contrasts with our observations of an essential need for IL-7 in the human system. One confounding factor in the murine study is that pDC were identified by cell surface markers which happen to be shared by lymphoid progenitor cells making it difficult to segregate the effects of delta-1 on these two cell types. Furthermore, those putative pDC did not produce high levels of IFN-
 unlike in our study. It is therefore possible that delta-1 could have predominantly affected an undifferentiated progenitor cell compartment in this murine study. Another element of variability seems to be the amount of delta-1 that is provided to the progenitor cells in the initial steps of culture. In our hands, we could abolish the enhancing effect of delta-1 by plating the same numbers of CD34+ cells onto much fewer OP9-Del1 cells compared to our usual conditions (A. Galy, unpublished preliminary observations). Thus, future experiments should determine what are the level of delta-1/Notch signalling that are required to modulate pDC development. In addition, future studies should investigate the possibility that delta-1 regulates pDC progenitor cell pathways in addition to the lymphoid progenitor cell pathway that we document.

The OP9-Del1 culture system, constitutes a practical tool to efficiently produce various types of human DC, which is useful for developmental and immune studies. So-called conventional DC are produced in similar amounts as in control cultures based on CD1a+ cell surface phenotype, TLR4-expression or IL-12-production. This is consistent with prior studies showing little effect of delta-1 on this type of DC 23. A distinguishing feature of the OP9-Del1 system is its efficiency for production of pDC based on phenotype and functional activity as it is superior to previously reported systems. Starting with total UCB CD34+ cells, each CD34+ cell yields 18 pDC in 3 weeks, which is three times the numbers obtained in optimized liquid cultures supplemented with thrombopoietin and FL that reportedly generate 6 pDC per CD34+ cell in the same time frame 27. The pDC produced onto OP9-Del1 appear to be functional as determined essentially by their cytokine production which constitutes an underpinning mechanism for the anti-viral and immuno-modulatory effects of pDC 4. However more studies are needed to fully evaluate the immune activities induced by pDC developed onto delta-1. This system provides an opportunity to study the role played by Notch ligands in immune responses through effects
on pDC, in addition to their well-recognized roles in the regulation of lymphopoiesis and Th1 T cell differentiation 17.
Acknowledgements

The authors are grateful to Graziella Griffith for help with vector construction, to Philippe Rameau for help with flow cytometry, to Sonia Poirault for help with cell cycle experiments and to Roseline Yao for help with pDC cultures. We also acknowledge Drs. A. Turhan (Institut Gustave Roussy, Villejuif), P. Salmon (University of Geneva), S. Artavanis-Tsakonas (Harvard Medical School, Charlestown, MA) and Immunex (Seattle, WA) for the kind gifts or reagents. The generosity and help from the Obstetrics staff at Hopital Louise Michel (Evry, France), AFM-Tissue bank and Genethon tissue processing personnel is also acknowledged.
References


27. Chen W, Antonenko S, Sederstrom JM, Liang X, Chan AS, Kanzler H, Blom B, Blazar BR, Liu YJ: Thrombopoietin cooperates with FLT3-ligand in the generation of
plasmacytoid dendritic cell precursors from human hematopoietic progenitors. Blood 103:2547, 2004


**Abbreviations:** pDC (plasmacytoid dendritic cells), FL (flt3-ligand), CD40L (CD40 ligand), GFP (green fluorescent protein), NICD (Notch intracellular domain), PGN (peptidoglycan).
### Table 1. Cytokine production induced by various stimuli

<table>
<thead>
<tr>
<th></th>
<th>Exp.</th>
<th>None Del1</th>
<th>None Control</th>
<th>CpG Del1</th>
<th>CpG Control</th>
<th>CD40L+IL-1 Del1</th>
<th>CD40L+IL-1 Control</th>
<th>PGN Del1</th>
<th>PGN Control</th>
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<tr>
<td><strong>IFN-α pg/ml</strong></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>15 238</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<tr>
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<td>N.D.</td>
<td>18 163</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>20 475</td>
<td>60</td>
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<td>&lt;10</td>
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<td>7 700</td>
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<td>&lt;10</td>
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<td>&lt;10</td>
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<td><strong>Average n = 6</strong></td>
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<tr>
<td></td>
<td></td>
<td>16 493</td>
<td>5 766</td>
<td></td>
<td>± 4 934</td>
<td>± 5 028</td>
<td></td>
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<td></td>
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<tr>
<td><strong>IL-8 pg/ml</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
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<td>313</td>
<td>514</td>
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<td>292</td>
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<td>3 821</td>
<td>2 276</td>
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<tr>
<td><strong>Average n = 3</strong></td>
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<tr>
<td></td>
<td></td>
<td>573</td>
<td>218</td>
<td>1 130</td>
<td>1 793</td>
<td>± 179</td>
<td>± 1 356</td>
<td>± 1 760</td>
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<td>9</td>
<td>19</td>
<td>15</td>
<td>9</td>
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<td>37</td>
<td>356</td>
<td>466</td>
<td>N.D.</td>
<td>N.D.</td>
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</table>

N.D. = not done

Cells produced on OP9-Del1 (Del1) or OP9-C stroma (Control) after 3 weeks of culture from CD34⁺ cells, were collected and stimulated for 24-48 hours with the indicated agents to induce the production of cytokines in the culture medium. IFN-α, IL-8 and IL-12 were measured by ELISA.
Table 2: Evidence of phenotypic maturation of pDC produced on OP9-Del1

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>None</th>
<th>CpG</th>
<th>CD40L+IL-1</th>
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<tbody>
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<td>Exp.</td>
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<tr>
<td>1</td>
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<td>428</td>
<td>741</td>
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<tr>
<td>3</td>
<td>88</td>
<td>138</td>
<td>220</td>
</tr>
<tr>
<td>4</td>
<td>219</td>
<td>562</td>
<td>not done</td>
</tr>
</tbody>
</table>

Cells produced on OP9-Del1 stroma after 20 days, were stimulated for 72 hours with the indicated agents and the expression levels of CD86 were measured by multi-color flow cytometry after gating on BDCA2+ cells.
Figure Legends

Figure 1. Differentiation of human CD34+ cells into BDCA-2+ cells. UCB CD34+ cells were cultured on either OP9-Del1 or OP9-C stroma for up to 3 weeks in the presence of FL + IL-7 cytokines. A. Flow cytometry analysis of the expression of BDCA2 and CD14 on the cultured cells at 1, 2 and 3 weeks (W) of culture. These plots represent one representative kinetic experiment out of two. Numbers indicate the percentage of cells in each quadrant. Irrelevant Ig controls provided less than 2% background staining. B. Percentages and numbers of BDCA-2+ and CD14+ cells produced after 10 and 20 days of culture. The data are from 13 different experiments, each represented by a symbol. Average values are indicated by an bar with representation of standard deviation values. Statistical analysis compared the Control and Del1 groups at each time point and the p values of the paired t test are indicated.

Figure 2. Cell cycle analysis. UCB CD34+ cells were cultured on either OP9-Del1 (black bars) or OP9-C stroma (grey bars) and after 7-14 days, cells were stained with BDCA2 and Hoescht to measure cell cycle by flow cytometry. Results represent the means ± SD values of gated BDCA2+ cells in the different phases of cell cycle obtained on from 4 independent experiments. A representative histogram of the cell cycle of BDCA2+ cells cultured onto OP9-Del1 stroma is inserted.

Figure 3: BDCA2+ pDC that develop onto OP9-Del1 stromal cells have characteristic markers of pDC. A: Flow cytometric analysis on the cells produced in OP9-Del1 co-culture, representative of 3 experiments. B. Purified pDC were obtained by flow cytometry isolation of CD45+ CD123+ BDCA2+ cells from OP9-Del1 co-culture. Cells were then spun on glass slides and stained with Wright Giemsa. C. Analysis of mRNA transcripts
expressed by $1 \times 10^6$ cells obtained at day 20 of culture of CD34$^+$ cells onto OP9Del1 or OP9-C. D. mRNA transcripts expressed by sorted BDCA4$^+$ CD123$^+$ cells.

Figure 4. Various hematopoietic lineages are produced on OP9-del1 and OP9-C co-culture. Cultures on OP9-Del1 (black dots) or OP9-C (open square) were analyzed for expression of CD1a/CD14, CD19/10 and CD3/CD56 markers to identify monocytes, DC, B cells and NK cells. On the right are represented the yields of CD19$^+$, CD56$^+$ and CD1a$^+$ cells normalized to the input of a single CD34$^+$ cell after 10 and 20 days of culture. Data are from 5 different experiments and average values are indicated by an X symbol with representation of standard deviation values.

Figure 5. pDC hematopoiesis is blocked by $\gamma$-secretase inhibition. A. Representative FACS analysis showing the modulation of BDCA-2 and CD14 expression by the $\gamma$-secretase inhibitor DAPT (gsi) or DMSO control. B. Average percentages and total numbers of BDCA2$^+$CD123$^+$ cells ± SD produced in 3 independent experiments in the presence of not of gamma secretase inhibition with concomitant analysis of the changes in percentages of CD56$^+$ NK cells and CD14$^+$ monocytes in these cultures.

Figure 6. pDC hematopoiesis is FL and IL-7- dependent. UCB CD34$^+$ cells were cultured onto OP9-Del1 (black bars) or OP9-C (grey bars) in the presence of FL alone (F), IL-7 alone (7) or both FL+IL-7 (F7). Results show the average ± SD of the number of BDCA2$^+$ CD123$^+$ cells produced at day 20 per well, from 3 independent experiments.

Figure 7. Delta-1 enhances the development of bone marrow-derived committed lymphoid progenitor cells into pDC. Common lymphoid progenitor cells were purified from bone marrow using flow cytometric sorting of Lin$^-$CD34$^+$CD10$^+$ cells. The control Lin$^-$
CD34<sup>+</sup>CD10<sup>-</sup> cells were also obtained and tested. Cells were seeded onto the indicated OP9 stroma cells lines in the presence of FL+IL-7. The progeny was harvested at day 14 and analyzed by flow cytometric analysis for expression of CD19, CD14 and BDCA2 markers. Numbers indicate the percentage of each subset in quadrants.
Figure 4

CD19

W1 | W2 | W3
---|---|---
2  | 2  | 2
7  | 7  | 20

Del1

Control

CD10

Del1

Control

CD3

W1 | W2 | W3
---|---|---
6  | 6  | 16
3  | 3  | 6

Del1

Control

CD56

CD14

W1 | W2 | W3
---|---|---
5  | 4  | 16
2  | 3  | 7
13 | 4  | 6
0.6| 0.3| 1

Del1

Control

CD1a

CD1a

W1 | W2 | W3
---|---|---
2  | 3  | 4
7  | 3  | 2

Del1

Control

CD1a

Days of coculture

Days of coculture

Days of coculture

Days of coculture

CD19

Diel

Control

Days of coculture

Diel

Control

Days of coculture

Diel

Control

Days of coculture

Diel

Control

Days of coculture
Figure 7
The Notch ligand delta-1 is a hematopoietic development co-factor for plasmacytoid dendritic cells

Aurelie Olivier, Evelyne Lauret, Patrick Gonin and Anne Galy