Large-Scale Human Dendritic Cell Differentiation Revealing Notch-Dependent Lineage Bifurcation and Heterogeneity

Graphical Abstract

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In Brief
Balan et al. report a protocol to simultaneously generate large numbers of human pDCs, cDC1s, and cDC2s from cord blood and non-mobilized CD34+ progenitors. This culture system will enable experimental testing of mechanisms controlling the differentiation or functions of human DC types and their translational application to treat cancer.

Highlights
- A CD34+ cell culture protocol yields large numbers of human pDCs and cDC1/2s
- Notch signaling is critical for cDC1 generation and GM-CSF has a synergistic effect
- scRNAseq confirms homology of in-vitro-derived DC types to their blood counterparts
- CLEC9A-positive XCR1-negative cells were identified as immediate precursors of cDC1s

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Large-Scale Human Dendritic Cell Differentiation Revealing Notch-Dependent Lineage Bifurcation and Heterogeneity

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SUMMARY

The ability to generate large numbers of distinct types of human dendritic cells (DCs) in vitro is critical for accelerating our understanding of DC biology and harnessing them clinically. We developed a DC differentiation method from human CD34+ precursors leading to high yields of plasmacytoid DCs (pDCs) and both types of conventional DCs (cDC1s and cDC2s). The identity of the cells generated in vitro and their strong homology to their blood counterparts were demonstrated by phenotypic, functional, and single-cell RNA-sequencing analyses. This culture system revealed a critical role of Notch signaling and GM-CSF for promoting cDC1 generation. Moreover, we discovered a pre-terminal differentiation state for each DC type, characterized by high expression of cell-cycle genes and lack of XCR1 in the case of cDC1. Our culture system will greatly facilitate the simultaneous and comprehensive study of primary, otherwise rare human DC types, including their mutual interactions.

INTRODUCTION

Dendritic cells (DCs) are a heterogeneous family of rare antigen-presenting cells, which sense danger signals and convey them to lymphocytes for the orchestration of adaptive immunity. Although widely used, immunotherapies using human-monocyte-derived DCs (MoDCs) showed limited efficacy due to poor recirculation of MoDCs to lymph nodes (Adema et al., 2005; Plantinga et al., 2013) and other differences with lymphoid tissue-resident DCs (LT-DCs) (Balan et al., 2014). Hence, efforts are ongoing to better characterize human LT-DCs (Heidkamp et al., 2016; See et al., 2017; Villani et al., 2017; Vu Manh et al., 2015a).

Human LT-DCs encompass three major cell types: CD141(BDCA3)*CLEC9A* classical DCs (cDC1s), CD1c(BDCA1)* classical DCs (cDC2s), and CLEC4Q(BDCA2)* CD123* plasmacytoid DCs (pDCs) (Ziegler-Heitbrock et al., 2010). Human and mouse LT-DCs are homologous, based on comparative transcriptomics and functional studies (Robbins et al., 2008; Vu Manh et al., 2015a). Human and mouse cDC1s excel in cross-presentation of cell-associated antigens, as well as of particulate antigen when delivered in combination with polyinosinic-polycytidylic acid (poly(I:C)) stimulation or to lysosomes through Fcγ receptors (Vu Manh et al., 2015a). cDC1s express XCR1, a chemokine receptor whose ligands are mostly produced by natural killer and CD8+ T cells (Vu Manh et al., 2015a). They also strongly express Toll-like receptor 3 (TLR3), which induces high levels of interferon λ (IFN-λ) when triggered by poly(I:C) or certain viruses and enhances cross-presentation (Vu Manh et al., 2015a). Hence, cDC1s are key candidate cells for the development of innovative immunotherapies against cancer or chronic infections by intracellular pathogens (Saxena et al., 2018). Committed cDC precursors (pre-cDCs) giving rise only to cDC1s or cDC2s exist in both mice and humans and can be mobilized in vivo using FLT3L (Breton et al., 2015, 2016; Lee et al., 2015; Maraskovsky et al., 1996; Schlitzer et al., 2015; See et al., 2017). Nevertheless, the mechanisms controlling human cDC1 development and functions are not well understood, which hampers their clinical targeting.

pDCs are prominent producers of type I (α/β) and III (λ) IFNs, which mediate potent antiviral effects (Tomassello et al., 2014) and can promote protective immunity to cancer (Saxena et al., 2018). Favorable immune responses were observed in melanoma patients treated by adoptive transfer of autologous pDCs loaded ex vivo with antigen and matured with an attenuated virus vaccine (Tel et al., 2013). The crosstalk between cDC1s and pDCs promotes the induction of optimal, protective, adaptive immune responses to viral infections and cancer in mice (James et al., 2014; Nierkens et al., 2011; Zhang et al., 2015) and likely in humans as well (Sluijter et al., 2015). Hence, targeting cDC1s and their crosstalk with pDCs for the design of innovative immunotherapies is very promising.
DCs are rare cells in blood and most tissues, which complicates not only their clinical application, including for adoptive transfer immunotherapy against cancers (Bol et al., 2013), but also basic studies aiming at deciphering their biology. This problem could be solved by developing methods to generate all three DC types in vitro from cultures of hematopoietic stem cells (Lee et al., 2015; Thordardottir et al., 2014). However, further studies are required to rigorously demonstrate the identity of in vitro CD34+ stem cell-derived DC types and the extent of their homology to their blood counterparts (Villani et al., 2017). Moreover, yields were very low in these studies, emphasizing an unmet need to further develop optimal protocols to generate these cells in vitro in larger numbers and enable their manipulation. The present study was designed to overcome this bottleneck.

RESULTS

Development of an In Vitro Culture System to Differentiate Large Numbers of Human cDC1s and pDCs

Human pDCs can develop from CD34+ progenitors cultured on OP9 stromal cells with FLT3L and interleukin-7 (IL-7). Contradictory results were reported on the role of Notch signaling in this process (Dontje et al., 2006; Olivier et al., 2006). The differentiation of cDC1s was not examined in these culture systems. Thus, we investigated whether OP9 stromal cells allow simultaneous differentiation of both pDCs and cDC1s from human CD34+ cord blood (CB) progenitors and whether Notch signaling affects this process. We developed an in vitro model of human DC differentiation (Figure 1A). It was built by combining two previously published protocols, ours for cDC1 generation in the absence of a feeder layer (Balan et al., 2014) with one using OP9 stromal cells for pDC development (Dontje et al., 2006), with additional key adaptations. Specifically, CD34+ CB cells were first expanded with FLT3L, SCF, TPO, and IL-7 (FST7) for 7 days. Cells were then differentiated with FLT3L, TPO, and IL-7 (FT7) for 14–21 days. (B) On day 18 of differentiation, within the live-cell gate, pDCs were identified as CD206−/CD14−/CD123+BDCA2+ cells and cDC1s as CD206−/CD14−/CLEC9A+ cells positive for BDCA3 or CADM1. Plots show one experiment using CB204 donor CD34+ cells for parallel differentiation on OP9+OP9_DLL1 feeder layers. Data are representative of 5 experiments each performed with cells from one or two different donors. Pie charts show mean percentage of pDCs or cDC1s within the live-cell gate from the 5 experiments. (C and D) Frequencies (C) and numbers per well (i.e., per 10^4 expanded CD34+ cells) (D) of cDC1s (top) and pDCs (bottom) among total live cells on day 18 after differentiation on the 3 different feeder layers. Graphs show individual results for each of the 6 donors. See also Figures S1 and S2 and Table S1.
Notch ligand Delta-like 1 (DLL1) or on a combination of these cells (OP9+OP9_DLL1) for 18–21 days (Figures 1A and S1A). At the end of the culture, pDCs and cDC1s were identified by flow cytometry (Figure 1B). OP9 cells allowed efficient generation of pDCs, consistent with an earlier report (Dontje et al., 2006). This culture condition yielded only very low frequencies of cDC1s (Figures 1B and 1C). In contrast, in the presence of OP9_DLL1, a much higher frequency of cDC1s was found (7.8% ± 5.3 versus 0.3% ± 0.3; p = 0.03), with significantly lower pDC frequencies (8.4% ± 9.3 versus 17.4% ± 7.4; p = 0.03) (Figures 1B and 1C). Differentiating the expanded CD34+ CB precursors on a mixed (OP9+OP9_DLL1) feeder layer yielded maximal frequencies for both DC types within the same culture (Figures 1B and 1C). Large numbers of cells were generated on OP9+OP9_DLL1 for both cDC1s and pDCs, whereas this was the case on OP9_DLL1 only for the former population and on OP9 only for the latter (Figure 1D). On a mixed OP9+OP9_DLL1 feeder layer, 10^4 expanded input cells yielded 1.1 ± 0.62 x 10^5 cDC1s and 4.1 ± 2.12 x 10^5 pDCs (Figure 1D). FLT3L was critical for cDC1 generation and strongly synergized with IL-7 and TPO to increase yields of both DC types (Figures S1B–S1E).

Recently, the CD123+ BDCA2+ gate commonly used to identify blood pDCs was shown to encompass a newly identified population of AXL+SIGLEC6+ DCs that failed to produce type I/III IFNs and was proposed to account for the T cell-activating functions previously attributed to pDCs (See et al., 2017; Villani et al., 2017). Our FT7 cultures contained a very low frequency of these cells (Figure S2). Since CD34+ CB cell numbers increased 2.9-fold ± 1.4-fold under FST7 expansion conditions, our culture system on OP9+OP9_DLL1 gave cDC1 and pDC yields 3 to 20 times higher than those previously reported (Lee et al., 2015; Thordardottir et al., 2014)(Table S1). In addition, it identified DLL1-dependent Notch signaling as a critical factor to promote cDC1 differentiation in vitro.

**Inhibition of Notch Signaling Blocks cDC1 Development In Vitro**

To further evaluate the dependence of cDC1 development on Notch signaling, we assessed the effect of treating our cultures with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT). This inhibitor blocks the γ-secretase, an enzyme required for Notch activation downstream of its engagement by its ligands. When FT7 cultures were treated with DAPT weekly during the differentiation phase (Figure 2A), both cDC1 frequency and numbers dropped dramatically, whereas pDC frequency and numbers remained high (Figures 2B–2D). To assess when during differentiation Notch signaling is required the most,
we added DAPT at different time points (Figure 3A). The inhibition of cDC1 development by DAPT was particularly strong when it was added at the beginning of differentiation (w1 or w1+2). Delayed treatment had a much lower (w2+3) or even no (w3) impact (Figures 3B and 3C). By contrast, pDC development was rather increased upon DAPT addition to the cultures (Figures 3B and 3C). Hence, in our culture system, Notch signaling is required at early time points for efficient in vitro differentiation of cDC1s, whereas it can inhibit pDC development.

**In-Vitro-Generated pDCs and cDC1s Share Key Functional Characteristics with Their In Vivo Equivalents**

To characterize in vitro generated pDCs and cDC1s functionally, we assessed their activation upon stimulation with synthetic TLR agonists. All TLR agonists tested increased cDC1 expression of HLA-DR, CD80, CD83, and CD86 (Figure 4A). By contrast, pDCs mainly upregulated HLA-DR, CD80, and CD86 upon TLR7 stimulation (R848 or R848+poly(I:C)) and CD83 upon TLR9 stimulation (CpG2216) (Figure 4A). A high proportion of in-vitro-derived cDC1s produced IFN-λ, but not IFN-α, specifically upon TLR3 triggering (poly(I:C) or R848+poly(I:C)) (Figure 4B). IL-12 was induced strongly only in cDC1s, specifically upon TLR8 triggering (R848 or R848+poly(I:C)) (Figure 4C). Tumor necrosis factor (TNF) production was induced in cDC1s both by TLR3 and TLR8 activation and very weakly by TLR4, but not by TLR9, activation (Figure 4C). In the same cultures, pDCs expressed cytokines only upon TLR7 or TLR9 triggering, with a high induction of IFN-α and TNF and a milder expression of IFN-λ than cDC1s (Figures 4B and 4C). Hence, in-vitro-generated pDCs and cDC1s harbored the same responses to TLR triggering than previously described for their in vivo counterparts (Galan et al., 2014; Hémont et al., 2013; Ito et al., 2006; Jongbloed et al., 2010; Lauberbach et al., 2010; Meixlsperger et al., 2013).

**In-Vitro-Generated pDCs and cDC1s Share Key Phenotypic Characteristics with Their In Vivo Equivalents**

We analyzed our cultures for cell-surface expression of classical DC type markers. For a more unbiased flow cytometry data analysis, we used viSNE applied to all live Lineage (Lin)−HLA-DR+ cells. This algorithm takes into consideration all the parameters analyzed to regroup cells harboring similar expression patterns close together on a two-dimensional plot (Amir et al., 2013). This approach identified two clusters matching the phenotypes of blood cDC1s and pDCs, respectively, namely CD34−CX3CR1−SIRPa−BDCA2low−BDCA2low−ILT7+BDCA2+CX3CR1+BDCA2+CD123+ cells (Figures 5A and S3A). In-vitro-derived cDC1s expressed CD1c, contrary to blood cDC1s but consistent with cDC1s isolated from FLT3L-injected human volunteers or differentiated in vitro from CD34+ CB cells (Breton et al., 2015; Lee et al., 2015). In our cultures, some CD34−CADM1− cells co-expressed CD1c, SIRPa and CX3CR1, putatively encompassing pre-cDC2(BTLA+) or cDC2 (BTLA+) (Figure 5A) (See et al., 2017). Overlapping the viSNE plot with manual gates for pDCs, cDC1s, CD1c+ BTLA+ and CD1c+ CX3CR1+ BTLA+ cells, confirmed

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**Figure 3. Notch Signaling Impacts cDC1 Differentiation from Human CD34+ CB Cells Early During Development**

(A) Experimental setup. Medium (untreated), DAPT, or DMSO was added on one or several days during differentiation to define when DAPT inhibits cDC1 development.

(B) Frequency of cDC1s (left) and pDCs (right) among total live cells after DMSO or DAPT treatment. Data from triplicate wells of one donor representative of 5 tested are depicted.

(C) Numbers of live cDC1s (left) and pDCs (right) after DMSO or DAPT treatment. Numbers are normalized to DMSO and represent mean values of triplicate wells for 3 donors from one experiment representative of two. The mean ± SD across the three donors is shown for each experimental condition. Statistics were performed using the Student’s paired t test.
Figure 4. pDCs and cDC1s Generated from Human CD34+ CB Cells Share Key Functional Characteristics with Their Blood Counterparts

OP9+OP9_DLL1 FT7 cultures were stimulated with the indicated agonists. cDC1s were gated as live CD14^- CD206^- CD123neg-to-low BDCA2+ CADM1+ cells and pDCs as CD14^- CD206^- CD123+ BDCA2+ cells. Plots are representative of 4 donors from 2 experiments.

(A) Activation marker expression.
(B) Intracellular expression of IFN-α/β.
(C) Intracellular expression of IL-12p40 and TNF.

Cell Reports 24, 1902–1915, August 14, 2018
the localization of these cell populations in the expected areas of the viSNE plot (Figures 5B and 5S). In summary, in our culture system, we identified pDCs and cDC1s that share key phenotypic characteristics with their blood equivalents.

**Single-Cell RNA Sequencing Demonstrates a Strong Homology between In-Vitro-Derived cDC1s and pDCs and Their Blood Counterparts and Unravels an Overlooked Heterogeneity within cDC1s**

We next performed SmartSeq2 single-cell RNA sequencing (scRNAseq) of selected, index-aged cell populations from our in vitro cultures in order to further evaluate the extent of their homology to blood DC types and assess the degree of homogeneity within each population. Unlike bulk transcriptomics, scRNAseq allows rigorously assessing the lack of contamination of any flow cytometry gated cell population by other cell types and also enables distinguishing in an unbiased way different cell states for a given cell type (Trapnell, 2015). Single live Lin– HLA-DR+ cells were sorted from OP9+OP9_DLL1 FT7 cultures. pDCs were sorted as CD141 low−/− CADM1−/− BDC2+ CD123+ cells and cDC1s as CD141−/− CADM1+ cells (Figure S3). We also included the putative pre-cDC2s (CD1c+ BTLA+) and cDC2s (CD1c+ CX3CR1+) that we had identified (Figures S3B and S3C). Unsupervised dimensional reduction and graph-based clustering of the data identified 7 cell clusters based only on gene expression profiling (Figure 5C).

Cluster 4 contained only (and the majority of) sorted putative pDCs (13 out of 15). Many genes known to be selectively expressed in pDCs (Robbins et al., 2008) were specifically expressed to high levels in cluster 4. These genes encompassed GZMB, PTCRA, SPIB, LILRA4, CLEC4C, TCF4, IL3RA, IRF7, TLR7, and PLAC8 (Figures 5D and S4). A connectivity map (cMAP) analysis was performed on each single cell, using human mononuclear phagocyte (MNP)-type-specific transcriptomic fingerprints generated from previous, independent microarray data (Carpentier et al., 2016; Robbins et al., 2008). For cluster 4, cMAP scores were enriched for pDC, but not cDC1 or cDC2, genes (Figure 5E). Lymphoid genes were also enriched in cluster 4, consistently with pDC ontogeny (Sathe et al., 2013) and with their known high expression of B cell-related transcripts (Cao et al., 2007; Villani et al., 2017).

Clusters 6 and 7 contained exclusively all the cells sorted as putative cDC1s (Figure 5C). The genes highly expressed selectively in these two clusters encompassed many genes known to be cDC1 specific (Robbins et al., 2008), including CADM1, CLEC9A, IDO1, C1orf54, BATF3, SLAMF8, SNX22, CPN3, GCSAM, THBD, and CLNK (Figures 5D and S4). cMAP analysis also identified exclusively for clusters 6 and 7 an enrichment for cDC1 transcriptomic fingerprints (Carpentier et al., 2016; Robbins et al., 2008) (Figure 5E). Hence, scRNAseq demonstrated a strong homology between in vitro-derived pDCs or cDC1s and their in vivo counterparts.

The identity of the cells sorted as putative pre-cDC2s or cDC2s remained elusive. Whereas most of the cells of these respective phenotypes constituted cluster 1 versus 3 and 5, respectively, some combined together in cluster 2. Hence, our phenotypic CD1c+BTLA+−/− key did not clearly segregate distinct MNP types. Moreover, putative cDC2s from clusters 3 and 5 selectively expressed high levels of not only CLEC10A and FCER1A, the hallmark blood cDC2 genes (Heidkamp et al., 2016; See et al., 2017; Villani et al., 2017), but also OSCAR and MRC1 (CD206), genes considered as more associated with...
monocytes or macrophages (Figure 5D). Consistent with the cMAP scores of cell clusters 3 and 5 were high for the cDC2 versus cDC1 signature but low for the cDC2 versus monocyte and macrophage signature (Figure 5E). Hence, whether our FT7 cultures contain cDC2 equivalents remained unclear based on these data.

Surprisingly, scRNAseq analysis unraveled an overlooked heterogeneity within in-vitro-derived cDC1s. Indeed, cDC1 clusters 6 and 7 showed reciprocal expression of XCR1 and CLEC7A versus CXCR4, CLEC12A, and genes associated with cell proliferation (Figures 5F and S4). Flow cytometry analysis of in-vitro-derived CLEC9A/CADM1+ cDC1s confirmed a reciprocal expression of XCR1 and CXCR4 on these cells (Figure 5G). Re-analysis of an independent, public scRNAseq dataset encompassing in-vitro-derived cDC1s differentiated from CD34+/CD100+ progenitors on MS5 stromal cells (Villani et al., 2017) confirmed the existence of a fraction of cDC1s expressing higher levels of CXCR4, CLEC12A, and mitosis genes than the remaining cDC1s (Figure S5). Hence, independently and in two systems of in vitro differentiation of CD34+ progenitors, two distinct cDC1 states were identified: terminally differentiated XCR1+ cells and their likely immediate precursors harboring a transcriptomic signature associated with mitosis.

Notch signaling did not promote cDC1 expression of XCR1, since it was not required for induction of this receptor on XCR1- cDC1s sorted from CD34+ cell cultures and replated on an OP9_DLL1 feeder layer (Figure S5B), and the frequencies of XCR1+ cells in cDC1s in the CD34+ cell cultures were not higher on OP9_DLL1 than on OP9 feeder cells (Figure S6C).

Blood CADM1+CLEC9A+ cDC1s also encompassed XCR1+ and XCR1- cells (Figures 5H and S6A). We sorted both fractions (Figure S6B) and cultured them for 14 days on OP9_DLL1 feeder layers with FT7 in the presence of GM-CSF that was crucial to preserve cellular viability (FT7+G). Upon in vitro culture, the XCR1- subset of peripheral blood cDC1s acquired XCR1 expression (Figure 5I) and expanded (Figure 5J). Hence, peripheral blood cDC1s encompass an XCR1- fraction that can proliferate and acquire XCR1 expression, suggesting that these cells are in vivo equivalents of the immediate precursors of the XCR1+ cDC1s identified in our in vitro differentiation system.

**GM-CSF Boosts cDC1 Differentiation, Allowing Their Efficient Generation from Non-mobilized Adult Blood CD34+ Precursors**

Since GM-CSF is known to promote cDC differentiation and survival, we tested whether adding it to FT7 cultures further improved cDC1 yields. Adding GM-CSF at the beginning of differentiation (day 0 or 7) increased cDC1 frequencies and numbers (Figures 6A and 6B) without significantly affecting pDC differentiation (Figures 6A and 6C). Specifically, GM-CSF enhanced cell proliferation during the initial phase of differentiation (Figures 6D and 6E) rather than protecting DCs from cell death (Figure 6F). Moreover, the addition of GM-CSF to OP9_DLL1 FT7 cultures significantly increased the frequency of XCR1+ cells within cDC1s (Figure 6G). These results are consistent with the expansion and acquisition of XCR1 expression by ex-vivo-isolated peripheral blood XCR1- cDC1s upon in vitro culture with FT7+G on OP9_DLL1 feeder layers (Figures 5I and 5J). Importantly, our optimized protocol combining the OP9+OP9_DLL1 feeder with the FT7+G cytokine differentiation cocktail allowed efficient generation of both pDCs and cDC1s from non-mobilized adult blood CD34+ precursors (Figure 6H). This is particularly relevant for therapeutic applications, since the use of autologous cells is preferable (Sabado et al., 2017).

**Unbiased, High-Throughput, scRNAseq Analysis of the OP9+OP9_DLL1 FT7 Culture Broadens Identification of DC Types and Differentiation States**

To further characterize the cellular composition of our OP9+OP9_DLL1 FT7 cultures, we performed scRNAseq experiments using the 10X Genomics droplet system, enabling a higher throughput and bypassing the need for enrichment of rare cell types or states through the use of biased phenotypic keys (Figure 7). At day 19 of differentiation, bulk hematopoietic (CD45+) live cells were sorted and used for scRNAseq, leading to a detection of 2,563 single cells in 12 clusters (Figure 7A). Cell cluster identity was inferred by systematic high-throughput gene set enrichment analyses (GSEA) using transcriptomic signatures established independently from microarray analyses (Vu Manh et al., 2015b) or from scRNAseq profiling of Lin- HLA-DR+ peripheral blood mononuclear cells (PBMCs; Figure S7; Data S1). This approach identified two clusters of pDCs (C1 and C11), one cluster of cDC1s (C7), one cluster of cDC2s (C4), and one cluster of monocyte-derived cells (C10). The smallest pDC cluster (C11) expressed cell-cycle genes (Figures 7B and 7E), likely representing pre-terminally differentiated pDCs. The most prominent cell cluster (C0) and a smaller one (C8) were not strongly enriched in lymphoid or myeloid transcriptomic signatures (Figures S7, 7B, and 7E) or cell-cycle genes (Figures 7B and 7E), likely representing multipotent hematopoietic progenitors in consistency with high CD34 expression by C0 cells (Figure 7E). Three cell clusters (C2, C3, and C5) were enriched in lymphoid gene signatures (Figures S7, 7B, and 7E), with C5 expressing cell-cycle genes (Figures 7B and 7E). These cells are likely hematopoietic progenitors committed to lymphoid differentiation, since no differentiated lymphocytes were detected in our cultures (<0.1% of cells were positive for lymphoid lineage markers). Cluster C9 harbored high levels of genes selectively co-expressed in pro-myelocytes, myeloid progenitors, or neutrophils (Mabott et al., 2013) (http://biogps.org/dataset/BDS_00013/primary-cell-atlas/). Cluster C6 belonged to the myeloid lineage (Figure S7), but its precise identity remained ambiguous. Whereas this analysis uncovered a cluster of mitosis-gene-expressing pre-pDCs, it did not identify any clear heterogeneity in the cDC1 population, contrary to our SmartSeq2 experiment. Hence, to better characterize the heterogeneity of MNP s in our OP9+OP9_DLL1 FT7 cultures, we performed another experiment focused on sorted Lin-HLA-DR+ cells (Figures 7C, 7D, and 7F). Strikingly, two clusters were identified for each of the DC populations: C1 and C7 for cDC2s, C2 and C9 for pDCs, and C6 and C11 for cDC1s (Figures 7C, 7D, and 7F). The smallest of each of these cluster pairs encompassed cells expressing mitosis genes (Figures 7D and 7F), thus corresponding to
pre-terminally differentiated cells equivalent to those identified in our SmartSeq2 experiment for cDC1s and in the bulk 10X Genomics experiment for pDCs but newly revealed for cDC2s. In conclusion, our OP9+OP9_DLL1 FT7 cultures encompassed prominent populations of hematopoietic progenitor cells engaged in lymphoid, myeloid, or erythroid differentiation, monocyte-derived cells, and the three major types of human DCs, including pre-terminal differentiation states of these cells expressing cell-cycle genes.

**DISCUSSION**

In our culture system, DLL1-dependent Notch inhibited human pDC development, consistent with a previous report (Dontje et al., 2006) but in contrast to another study claiming the opposite (Olivier et al., 2006). These discrepant results may be explained by different effects of Notch signaling depending on the DC developmental pathways. A recent study demonstrated a critical role of Notch signaling among the microenvironmental cues instructing the development of human HLA-DR+ CD13+ CD11c+ BDCA1+ DCs in the thymus, without specific investigation regarding the presence of cDC1s (Martín-Gayo et al., 2017). As compared to our protocol, the cell yields in this study were 50 times lower for pDCs and at least 10 times lower for cDCs. Hence, we demonstrated unequivocally that DLL1-dependent Notch signaling and GM-CSF cooperate to promote human cDC1 generation. Moreover, we developed a cell culture system yielding much higher numbers of human pDCs, cDC1s, and...
cDC2s than previously reported. Our results are consistent with those reported in an independent study that was conducted in parallel to ours and published very recently (Kirkling et al., 2018).

cDC1s and pDCs share developmental dependence on the transcription factor IRF8. Constitutive loss of TCF4 expression in murine pDCs induces their upregulation of ID2 and their differentiation into cDC1-like cells (Ghosh et al., 2010). Hence, bifurcation from the common DC progenitor between the pDC and cDC1 cell lineages is likely enforced by reciprocal and antagonistic expression of ID2 and TCF4. Notch2 receptor knockdown in murine CD11c<sup>+</sup> splenocytes interferes with cDC2 and, to a lesser extent, cDC1 development, whereas pre-DC numbers are unaffected (Lewis et al., 2011). Mouse splenic cDC1s do not express Notch target genes (Lewis et al., 2011). Hence, Notch signaling likely promotes murine splenic cDC1 development at a precursor stage (Lewis et al., 2011). Combined with these observations in mice, our data on in vitro differentiation of human DC types strongly suggest an evolutionary conserved Notch-dependent lineage bifurcation between pDCs and cDC1s from their common precursor stage. Hence, we propose that Notch-dependent interactions between precursors and stromal cells contribute to instruct commitment into the human cDC1 lineage.

Phenotypic and functional characterization of in-vitro-derived cDC1s and pDCs confirmed their strong homology with their in vivo counterparts based on their shared expression of key cell-surface markers and the similarity of their phenotypic maturation and cytokine production upon TLR stimulations. CD11c expression was low on in-vitro-derived cDC1s, consistent with the lower levels of CD11c expression observed on human cDC1s as compared to cDC2s in blood, secondary lymphoid organs, and the parenchyma from non-lymphoid tissues (Haniffa et al., 2012). Hence, to identify cDC1s ex vivo in tissues and in vitro in culture models, caution should be taken not to use too high a threshold for CD11c expression.

The identity of the DC types generated in our cultures and their strong homology to their blood counterparts were unambiguously demonstrated by scRNAseq analyses. Strikingly, heterogeneity was revealed in each DC type, with a minor cell cluster characterized by mitosis gene expression and lack of XCR1 but increased expression of CXCR4 in the case of cDC1s. These cells likely represent previously overlooked immediate precursors of terminally differentiated DCs. We confirmed at the protein level that CLEC9A<sup>+</sup>/CADM1<sup>+</sup> cDC1s split into XCR1<sup>-</sup> and XCR1<sup>+</sup> cells, with the first expressing higher levels of CXCR4. We also confirmed that XCR1<sup>-</sup> cDC1s represent an immediate precursor of terminally differentiated XCR1<sup>+</sup> cDC1s and can be found circulating in the blood. Thus, we demonstrated the power of scRNAseq to reveal overlooked differentiation states of human DC types. Further studies aiming at comparing the functions of XCR1<sup>-</sup> versus XCR1<sup>+</sup> cDC1s should help better understand the differentiation of this DC lineage and its coupling to the acquisition of functional specialization. In mice, there are two forms of splenic cDC1s (as reviewed in Dresch et al., 2012): a pre-cross-presentation stage and a later stage that has acquired the capacity to cross-present antigens (the latter ability being induced by GM-CSF and correlating with XCR1 expression; Bachem et al., 2012). It remains to be tested whether the human XCR1<sup>-</sup>cDC1s identified in our study represent the counterparts of the mouse pre-cross-presentation cDC1 state.

In conclusion, we provide a valuable in vitro protocol to generate large numbers of pDCs, cDC1s, and cDC2s within the same culture of CD34<sup>+</sup> cells. When combined with the inhibition of candidate regulators, through pharmacological or genetic manipulations, this culture system will allow experimental testing of the mechanisms controlling the differentiation or functions of human MNPs. Since our culture system encompasses hematopoietic progenitors engaged in differentiation toward multiple immune lineages, its further adaptation might enable terminal differentiation of additional cell types to facilitate studying their interactions with DCs. Different strategies are being pursued to harness DC types for innovative vaccines or immunotherapies against viral infections or cancer (Saxena et al., 2018). Therefore, our system will also be useful for translational applications, including in vitro drug and vaccine testing on DC types. Further deep characterization of cell cultures modeling the differentiation of human MNP, as achieved here, should also yield novel strategies to generate in vitro the best suited combinations of MNP types and states for adoptive cell transfer to boost antitumoral responses in cancer patients (Bakdash et al., 2016) or to prevent graft rejection upon organ transplantation (Moreau et al., 2017).

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING

Figure 7. Unbiased Analysis of the Composition of OP9+OP9 DLL1 FT7 Cultures through Droplet-Based High-Throughput scRNAseq
(A) scRNAseq-based identification of clusters of cell types or states from viable CD45<sup>+</sup> cells from one culture using t-SNE with graphical clustering. 12 cell clusters were identified. The numbers of cells in each cluster are indicated in the graphical legend box, together with the percentages they represent out of the total cells analyzed.
(B) Violin plots showing expression profiles of previously known cell-type-specific genes across all individual cells and in comparison between cell clusters.
(C) Identification of clusters of cell types or states from sorted Lineage<sup>+</sup>/HLA-DR<sup>+</sup> cells. 13 cell clusters were identified.
(D) Violin plots showing expression profiles of the same genes as in (B).
(E and F) Expression patterns of 156 genes representative of those that were the most differentially expressed across cell clusters, as a heatmap with hierarchical clustering of cell clusters (columns) and genes (rows), for total live CD45<sup>+</sup> cells (E) and enriched Lineage<sup>+</sup>/HLA-DR<sup>+</sup> cells (F). Genes clustered largely according to previously known co-expression in specific cell types, as highlighted by the vertical bars and their annotations on the right of each heatmap. See also Figure S7 and Data S1.
Experimental Model and Subject Details
- Human studies
- Cell lines

Method Details
- Expansion and differentiation of CB precursors on OP9 stromal cell lines
- Cell sorting
- Flow cytometry
- Inhibitor experiments
- Single-cell RNA sequencing
- Single cell RNA-Seq data processing
- Single cell RNA-Seq data analysis

Quantification and Statistical Analysis

Data and Software Availability

Supplemental Information
Supplemental Information includes seven figures, one table, and one data file and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.07.033.

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Author Contributions
Conceptualization, Methodology, Validation, and Formal Analysis, S.B., C.A.-S., N.B., and M.D.; Methodology for scRNAseq, A.A., T.-P.V.M., and A.-C.V.; Investigation, C.A.-S., S.B., F.I., and J.S.; Bioinformatics, T.-P.V.M. with N.C. and M.D.; Visualization, C.A.-S., S.B., N.C., T.-P.V.M., and M.D.; Writing, C.A.-S., M.D., S.B., and N.B.; Editing, all authors; Project Supervision and Administration, M.D. and N.B.

Declaration of Interests
S.B., C.A.-S., and M.D. are inventors on a patent application filed on the described method. The remaining authors declare no competing interests.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse Anti human CD303, PerCP/Cy5.5 conjugate, Clone 201A, dilution 1:100 | Biolegend | Cat # 354210 |
| Mouse Anti human CD303, APC conjugate, Clone 201A, dilution 1:100 | Biolegend | Cat #354206 |
| Mouse Anti-Human CD272 (BTLA), PE-CF594 conjugate, Clone J168-540, dilution 1:100 | BD Bioscience | Cat # 564801 |
| Chicken Anti human CADM1, Purified, Clone 3E1, dilution 1:1000 | MBL | Cat # CM004-3 |
| Chicken Anti human CADM1, Biotin conjugate, Clone 3E1, dilution 1:1000 | MBL | Cat # CM004-6 |
| Mouse anti-human CD1c, APC/Cy7 conjugate, Clone L161, dilution 1:100 | Biolegend | Cat # 331520 |
| Mouse anti-human CD11c, V450 conjugate, Clone B-ly6), dilution 1:100 | BD Bioscience | Cat # 655112 |
| Mouse Anti-Human CD123, PE-Cy7 conjugate, Clone 7G3, dilution 1:50 | BD Bioscience | Cat # 560826 |
| Mouse Anti-Human CD123, BV605 conjugate, Clone 6H6, dilution 1:200 | Biolegend | Cat # 306026 |
| Mouse Anti-Human CD14, BV421 conjugate, Clone 15-2, dilution 1:200 | Biolegend | Cat # 321110 |
| Mouse Anti-Human CD14, AF700 conjugate, Clone 15-2, dilution 1:200 | Biolegend | Cat # 321132 |
| Mouse Anti-Human CX3CR1, PE conjugate, Clone K0124E1, dilution 1:100 | Biolegend | Cat # 355704 |
| Mouse Anti-Human Thrombomodulin/BDCA-3, APC conjugate, Clone 501733, dilution 1:30 | R&D system | Cat # FAB3947A |
| Mouse Anti-Human Thrombomodulin/BDCA-3, BV785 conjugate, Clone M80, dilution 1:100 | Biolegend | Cat # 344116 |
| Mouse Anti-Human Thrombomodulin/BDCA-3, APC-Vio770 conjugate, Clone ADS-14H12, dilution 1:50 | Miltenyi Biotec | Cat # 130-098-217 |
| Mouse Anti-Human CD45, PE conjugate, Clone HI30, dilution 1:100 | BD Bioscience | Cat # 555483 |
| Mouse Anti-human CD45RA, BV 655 conjugate, Clone H100, dilution 1:200 | Biolegend | Cat # 304136 |
| Mouse Anti-Human CD80, PE conjugate, Clone L307.4, dilution 1:100 | BD Bioscience | Cat # 557227 |
| Mouse Anti-Human CD83, PE conjugate, Clone HB15e, dilution 1:100 | BD Bioscience | Cat # 556855 |
| Mouse Anti-Human CD86, Alexa Fluor® 700 conjugate, Clone 2331 (FUN-1), dilution 1:100 | BD Bioscience | Cat # 561124 |
| Mouse Anti-Human CLEC9A, APC conjugate, Clone 8F9, dilution 1:50 | Miltenyi Biotec | Cat # 130-097-371 |

(Continued on next page)
Continued

| REAGENT or RESOURCE SOURCE | IDENTIFIER |
|----------------------------|------------|
| Mouse Anti-Human CLEC9A, Vio blue conjugate, Clone 8F9, dilution 1:50 | Miltenyi Biotec Cat # 130-097-406 |
| Mouse Anti-Human CLEC9A, PE conjugate, Clone 8F9, dilution 1:200 | Biolegend Cat # 353804 |
| Mouse Anti-Human CXCR4, APC conjugate, Clone 1G2G5, dilution 1:50 | Miltenyi Biotec Cat # 130-098-357 |
| Hamster anti-mouse DLL1, PE conjugate, Clone HMD1-3, dilution 1:100 | Biolegend Cat # 128307 |
| Mouse Anti-Human HLA-DR, Alexa Fluor 700 conjugate, Clone LN3, dilution 1:100 | eBioscience Cat # 56-9956-42 |
| Mouse Anti-Human HLA-DR, BV786 conjugate, Clone G46-6, dilution 1:100 | BD Bioscience Cat # 564041 |
| Mouse anti-Human IFN-α[2b], PE conjugate, Clone 7N4-1, dilution 1:20 | BD Bioscience Cat # 560097 |
| Mouse Anti-Human IL-12 (p40/p70), PE conjugate, Clone C11.5, dilution 1:20 | BD Bioscience Cat # 554575 |
| Mouse Anti-Human IL-29/IFN-lambda 1, unconjugated, Clone 247801, dilution 1:1000 | R&D Systems Cat # MAB15981-100 |
| Mouse Anti-Human Lineage Cocktail 1 (CD3, CD14, CD16, CD20, CD56), FITC conjugates (Clones SK7, Mkp9 3G8, SJ25C1, L27, NCAM16.2), dilution 1:100 | BD Bioscience Cat # 340546 |
| Mouse anti-human CD172a/b, APC conjugate, Clone SE5A5, dilution 1:100 | Biolegend Cat # 323810 |
| Mouse Anti-Human TNF, Alexa Fluor® 700 conjugate, Clone MAb11, dilution 1:100 | BD Bioscience Cat # 561023 |
| Mouse anti-human XCR1, PE conjugate, Clone S15046E, dilution 1:200 | Biolegend Cat # 372604 |
| Mouse anti-human CD3, PE conjugate, Clone SK7, dilution 1:100 | Biolegend Cat # 344806 |

Biological Samples

| Biological Samples | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| CB CD34+ cells     | ABCell Bio | Cat # CD34-CT-CG |
| CB                 | Hospital de la Conception, Marseille (Balan et al., 2014) | N/A |
| CB                 | NYC blood center | N/A |
| Peripheral blood   | NYC blood center | N/A |

Chemicals, Peptides, and Recombinant Proteins

| y-secretase inhibitor XXI, compound E (DAPT~ 5 μM) | Calbiochem | Cat # 565790-1MG |
| Dimethyl sulfoxide | Sigma Aldrich | Cat # D2650-100ML |
| Recombinant human Flt3L | Peprotech | Cat # 300-19 |
| Recombinant human Flt3L | R&D Systems | Cat # 308-FKN-025 |
| Recombinant human SCF | Peprotech | Cat # 300-07 |
| Recombinant human SCF | R&D Systems | Cat # 255-SC-050 |
| Recombinant human IL-7 | Peprotech | Cat # 200-07 |
| Recombinant human IL-7 | R&D Systems | Cat # 207-IL-025 |
| Recombinant human TPO | Peprotech | Cat # 300-18 |
| Recombinant human TPO | R&D Systems | Cat # 288-TP-025 |
| Recombinant human GM-CSF | Peprotech | Cat # 300-03 |
| Annexin-V (Pacific blue conjugate) | Biolegend | Cat # 640918 |
| Propidium Iodide Solution | Biolegend | Cat # 421301 |
| Annexin V Binding Buffer | Biolegend | Cat # 422201 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse Anti-Chicken IgA-FITC | SouthernBiotech | Cat# 8330-02 |
| APC/Fire 750 Streptavidin | Biolegend | Cat# 405250 |
| PE/Dazzle 594 Streptavidin | Biolegend | Cat# 405247 |
| Lipopolysacharide (Ultrapure LPS, Salmonella Minnesota) | Invivogen | Cat# tlrl-smlps |
| Poly(I:C) HMW | Invivogen | Cat# tlrl-pic |
| R848 (Resiquimod) | Invivogen | Cat# tlrl-r848 |
| Class A CpG oligonucleotide: ODN 2216 | Invivogen | Cat# tlrl-2216 |
| Brefeldin A | Sigma Aldrich | Cat# B7651-5MG |
| FBS | Biowest | Cat# S1820-500 |
| FBS | Biowest | Cat# S1620-500 |
| α-MEM glutamax | Thermofisher scientific | Cat # 32561-029 |
| α-MEM glutamax | Thermofisher scientific | Cat # 32561102 |
| Sodium Pyruvate (100 mM) | Thermofisher scientific | Cat # 11360070 |
| Penicillin-Streptomycin(10,000 U/mL) | Thermofisher scientific | Cat # 15140163 |
| 2-Mercaptoethanol (50 mM) | Thermofisher scientific | Cat # 31350010 |
| Trypsin-EDTA (0.05%), phenol red | Thermofisher scientific | Cat # 25300054 |
| LIVE/DEAD Fixable Aqua Dead Cell Stain Kit | Thermofisher scientific | Cat# L34957 |
| Ficoll-Paque PLUS density gradient media | GE Healthcare | Cat# 17144003 |

Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| EasySep Human Pan-DC Pre-Enrichment Kit | STEMCELL technologies | Cat # 19251 |
| Pan-DC Enrichment Kit, human | Miltenyi | Cat # 130-100-777 |
| Fixation/Permeabilization Solution Kit | BD Bioscience | Cat # 554714 |
| CD34 MicroBead Kit UltraPure | Miltenyi Biotec | Cat # 130-100-453 |
| APEX Alexa Fluor 647 Antibody Labeling Kit | Thermofisher scientific | A10475 |
| TCL | QIAGEN | Cat # 1031576 |
| beta-mercaptoethanol | ThermoFisher: Life technologies | Cat # 21985-023 |
| Agencourt RNA Clean XP | Beckman-Coulter | Cat # A63987 |
| Recombinant RNase Inhibitor | clontech | Cat # 2313B |
| dNTP (10 mM each) | ThermoFisher: Life technologies | Cat # R0192 |
| Betaine (5 M) | Sigma-Aldrich | Cat # B0300-5VL |
| Reverse Transcription DNA oligonucleotide primer | Integrated DNA Technologies (IDT) | N/A |
| AAG CAG TGG TAT CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTVN | Idtdna.com | N/A |
| PCR oligonucleotide primer | IDT | N/A |
| AAG CAG TGG TAT CAA CGC AGA GT | Exiqon | N/A |
| SMARTER TSO (with LNA) | Exiqon | N/A |
| AAGGCACTGTTATCAAGCGAGATCAATG+G | Sigma-Aldrich | Cat # M1028-10X1ML |
| Magnesium chloride solution | Sigma-Aldrich | Cat # M1028-10X1ML |
| SuperScript II Reverse Transcriptase | ThermoFisher: Life technologies | Cat # 18064-071 |
| KAPA HiFi HotStart PCR ReadyMix | KAPA Biosystems | Cat # KK2602 |
| Agencourt AMPure XP | Beckman-Coulter | Cat # A63881 |
| High Sensitivity DNA BioAnalyzer kit | Agilent | Cat # 5067-4626 |
| High Sensitivity DNA Qubit kit | ThermoFisher: Life technologies | Cat # Q32854 |
| Qubit assay tubes | ThermoFisher: Life technologies | Cat # Q32856 |
| TE buffer (500mL) | TEKNOVA | Cat# T0228 |
| Ethanol (100%) | VWR | Cat# 89125-170 |
| NexteraXT Library preparation kit | Illumina | Cat# FC-131-1096 |
| Nextera Indexing | ILLUMINA | Cat# FC-131-1002 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marc Dalod (dalod@ciml.univ-mrs.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human studies
Human nontherapeutic-grade CB samples were collected from informed and consenting mothers at the Marseille University Hospital de la Conception birth clinic, obtained through the Marseille CB bank and used for CD34+ cell enrichment (> 95%), as described (Balan et al., 2014), or from the UCB (USA national CB program). Alternatively, already purified CB CD34+ cell were purchased from a properly licensed commercial company (ABCell Bio). PBMCs were prepared by Ficoll (GE Healthcare) gradient centrifugation from buffy coats received from EFS (Marseille) or New York blood center (Long island city, NY, USA). Non-mobilized adult blood CD34+ cells were isolated using the CD34 MicroBead Kit UltraPure (Miltenyi). Donor cells were negative for HBV, HCV, HIV1/2, bacteria, fungi, and mycoplasma.

Cell lines
The OP9 (Kodama et al., 1994) and OP9-DLL1 (Schmitt et al., 2004) cell lines were initially imported at CIML by Bernard and Marie Malissen, from the laboratory of Pr. Juan Carlos Zuniga-Pflücker, and provided to us through the laboratory of Pierre Ferrier (CIML).
METHOD DETAILS

Expansion and differentiation of CB precursors on OP9 stromal cell lines

Purified CD34\(^+\) human CB cells were thawed and expanded in 96 well round bottom plates (Falcon) at 25,000 cells/ml in \(\alpha\)-MEM glutaMAX (Life Technologies), 10% FCS (Biowest), Penicillin / Streptomycin, 1mM sodium pyruvate, 50 \(\mu\)M \(\beta\)-mercaptoethanol, 25ng/ml hFLT3L, 5ng/ml hIL7, 5ng/ml hTPO, 2.5ng/ml hSCF, and for some experiments hGM-CSF (cytokines from Peprotech or R&D). Expansion was performed in 200 \(\mu\)l per well for 7 days at 5% CO\(_2\), +37 \(^\circ\)C. Expanded cells were either frozen for later use or directly differentiated. 24h before differentiation start, OP9 or OP9_DLL1 cells were harvested using 0.05% trypsin. 12,500 cells were seeded in 500 \(\mu\)L \(\alpha\)-MEM, 20% FCS, Penicillin / Streptomycin, 1mM sodium pyruvate, 50 \(\mu\)M \(\beta\)-mercaptoethanol in a 24 well flat bottom plate (Falcon). In case of the mixed feeder layer, a 3:1 ratio of OP9 and OP9_DLL1 was used. The stromal cell layer was 70%–80% confluent on the day of the experiment. Before differentiation, the medium was gently replaced and the concentration of the expanded precursor cells was adjusted to 20,000 cells/ml. Cytokines were added v/v at a 2x concentration, to achieve final concentrations of 15ng/ml hFLT3L, 5ng/ml hIL7, and 2.5ng/ml hTPO, with eventual addition of hGM-CSF as described in the relevant Figures. In each well prepared with a stromal cell layer, 500ul of expanded precursor suspension was gently added. Cells were incubated for 14-21 days at 5% CO\(_2\), +37 \(^\circ\)C. On day7 and 14 of the differentiation, half of the medium was replaced by fresh one supplemented with cytokines. Cells were harvested by pipetting and filtered through a 30 \(\mu\)m nylon mesh, between days 14 and 21.

Cell stimulations

Cell cultures were stimulated with medium, 5 \(\mu\)g/ml R848, 5 \(\mu\)g/ml CpG2216, 1 \(\mu\)g/ml LPS, 5 \(\mu\)g/ml poly(I:C), or 5 \(\mu\)g/ml R848 + 5 \(\mu\)g/ml HMW poly(I:C) (InvivoGen) directly in the 24 well plates used for differentiation. Half of the culture medium was replaced by fresh one supplemented with the TLR agonists and 200ng/ml hFLT3L, 50ng/ml hIL3 (Peprotech), Brefeldin A (Sigma) was added at a final concentration of 10 \(\mu\)g/ml after 2h (6h time point) or 14h (16h time point) of stimulation. For the analysis of activation markers, cells were stimulated for 16h without Brefeldin A.

Flow cytometry

Cells were stained with the live dead fixable aqua dead kit (Invitrogen) and with the antibodies listed in the supplemental information. Intracellular staining were performed using the Fix/Perm kit (BD). Cells stained for cell surface markers were assessed for apoptosis by incubation with Annexin V for 30 min followed by addition of PI immediately before acquisition, without further washing. Samples were acquired on LSR II or Fortessa (BD) and analyzed with FlowJo (Tree Star) or FCS express 6.04, (De Novo Software) software. ViSNE analysis from compensated FCS files was performed using Cytobank.

Inhibitor experiments

The \(\gamma\)-secretase inhibitor XXI, compound E (DAPT; Calbiochem), was used to block Notch signaling at a concentration of 5 \(\mu\)M. DMSO was used as control.

Cell sorting

Cells were sorted on a FACSAria (BD biosciences). For SmartSeq2 scRNAseq, single cells were index sorted using the indicated gating strategy, from FT7 cultures differentiated on OP9+OP_DLL1 for 18 days. For the 10X Genomics scRNAseq experiments from cultures, cells were harvested by pipetting, filtered through a 30 \(\mu\)m nylon mesh, and sorted by flow cytometry as live (LIVE/DEAD Fixable Aqua Dead) total hematopoietic (CD45\(^+\)) or Lin\(^-\) HLA-DR\(^+\), using PE-CD45, or FITC-lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56) with BV786-HLA-DR, APC-BDCA2/CD303 and V450-CD11c, before loading into the Chromium Single Cell Controller apparatus. For the 10X Genomics scRNAseq experiment with cells enriched from adult PBMCs, cells from a healthy blood donor were separated by density gradient centrifugation, enriched by magnetic bead-based sorting using the pan-DC enrichment kit from Miltenyi, stained and sorted for Lin\(^-\) HLA-DR\(^+\) cells as done for the cells from cultures, before loading into the Chromium Single Cell Controller apparatus. Peripheral blood XCR1\(^+\) and XCR1\(^-\) cDC1 subsets were sorted from PBMCs enriched with the EasySep Human Pan-DC Pre-Enrichment Kit (stem cell technologies), by gating on Lin\(^-\) HLA-DR\(^+\) CD123\(^-\) CD45RA\(^-\) CD1c\(^+\) SIRPa\(^-\) CD141\(^+\) CLEC9A\(^-\) CADM1\(^+\) cells. Sorted cells were cultured for 14 days on OP9_DLL1 by plating 10,000 to 30,000 cells/ml with the FT7+G cytokine cocktail.

Single-cell RNA sequencing

RNA purification and library generation for SmartSeq2 was performed as described (Villani et al., 2017). For the droplet-based scRNAseq, sorted cell samples were processed by HaloDx testing laboratory, using the Chromium Single Cell Controller apparatus with the single cell 3’ Library, Gel beads & multiplex kit (10X Genomics, Pleasanton) as per the manufacturer protocol. Libraries were sequenced by 50-bp versus 75-bp paired-end reading on a High Seq 4000 versus NextSeq500 sequencer (Illumina) for SmartSeq2 versus 10X Genomics data respectively. Reads were aligned on the GRC38 human genome assembly. Data analysis was performed using the R software package Seurat (https://github.com/satijalab/seurat) (Satija et al., 2015).
**Single cell RNA-Seq data processing**

For both SmartSeq2 and 10X Genomics data, sequencing reads were mapped to the human reference genome (GRCh38.87) using the STAR aligner (v2.5.3a) (Dobin et al., 2013) and read counts were generated using HTSeq (v0.9.1) (Anders et al., 2015).

Count matrices were normalized and filtered using the Setup function of the Seurat (v1.4.0.16) package (Satija et al., 2015) in the R environment (v3.4.1).

For SmartSeq2 data, cells with less than 5000 genes expressed at detectable levels and genes detected in less than 3 cells were filtered out; 86 cells and 18017 genes were retained and the corresponding data was log-transformed \([\log(data+1)]\) prior to further analysis.

For 10X Genomics data, cells with less than 200 genes expressed at detectable levels and genes detected in less than 3 cells were filtered out. The high-quality of the retained cells was confirmed by additional QC filters, based on the mitochondrial gene expression rate (cells in which more than 10% of total genes were mitochondrial genes were removed) and the read alignment rate. Overall, 2605 cells and 16495 genes from the total in vitro culture, 4462 cells and 17295 genes from the Lin-HLA-DR+ enriched culture and 5050 cells and 15107 genes from the PBMCs were kept for further analyses.

**Single cell RNA-Seq data analysis**

Following the Seurat pipeline, highly-variable genes (HVGs) were first selected based on thresholds on average expression and dispersion z-score; this method allowed us to take into account the intrinsic relationship between the mean and the variance of the expression values of the genes across the cells. In order to reduce the dimensionality of the dataset, a principal component analysis (PCA) was performed, using as input the expression values of the HVGs across the cells. The highly contributing principal components (PCs) were identified through the jackstraw permutation test (1000 iterations for SmartSeq2 and 500 permutations for 10X Genomics data). Then, a proportion test is performed to assess, for each PC, the robustness of the association of each gene to this PC and the statistical significance of the difference between the distribution of the obtained p values and the uniform distribution. Cell loadings for the highly contributing PCs subsequently served as input to perform both t-distributed stochastic neighbor embedding (t-SNE; perplexity \(p = 10\) for SmartSeq2 data, \(p = 80\) for 10X PBMCs and Lin-HLA-DR+ enriched in vitro culture, \(p = 40\) for 10X total culture) and graph-based clustering of the cells (\(k = 4\); resolution = 0.6 for Smartseq2 data; \(k = 10\), resol = 0.4 for 10X Lin-HLA-DR+ enriched culture, \(k = 10\), \(r = 0.3\) for 10X PBMCs, \(k = 15\), \(r = 0.4\) for 10X total culture); the obtained clusters were finally projected on top of the t-SNE plot, thus allowing to visualize the structure and the heterogeneity of the dataset in two dimensions. In brief, clustering relied on the superposition of two graphical clustering algorithms (K-Nearest Neighbors, performed using an Euclidean distance matrix as input, and Shared Nearest Neighbors) and of the Smart Local Moving (SLM) algorithm, aiming at optimizing the modularity of the clustering, which quantifies the ratio between intra-cluster connectivity and inter-cluster connectivity.

In order to identify putative markers for the clusters while keeping full benefit from the single-cell resolution of the data, we used the Receiver-Operating characteristic (ROC) statistical test, which quantifies the classification power of each individual gene between two clusters (or groups of clusters) through an AUC (Area Under the Curve) value.

C-MAP analysis was performed in R (v3.4.1) using an adaptation for single cells of the original R script, with the following parameters: 1000 permutations, ref = NULL, scaling = “none.”

Hierarchical clustering and heatmaps were made with the Morpheus software (https://software.broadinstitute.org/morpheus/).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

High-throughput GSEA was performed using BubbleMap from the BubbleGUM (v1.3.19) suite (Spinelli et al., 2015), with the default parameters except: permutation type = phenotype, Max geneset size = 5000, Min geneset size = 5.

The Prism software (GraphPad) was used for calculating statistics. Unless stated otherwise, statistical analyses were performed using Mann Whitney non parametric two tailed paired t test. Only statistically significant differences are shown. \(p < 0.05; **, p < 0.01\).

**DATA AND SOFTWARE AVAILABILITY**

The accession numbers for the raw single cell RNA-sequencing data reported in this paper are GEO: GSE108546 and GSE115405.
Supplemental Information

Large-Scale Human Dendritic Cell

Differentiation Revealing Notch-Dependent

Lineage Bifurcation and Heterogeneity

Sreekumar Balan, Catharina Arnold-Schrauf, Abdenour Abbas, Norbert Couespel, Juliette Savoret, Francesco Imperatore, Alexandra-Chloé Villani, Thien-Phong Vu Manh, Nina Bhardwaj, and Marc Dalod
Figure S1. DLL1 expression by OP9_DLL1 and role of different cytokines on DC generation. Related to Figure 1.
(A) Mouse DLL1 expression on OP9_DLL1 cells was confirmed by flow cytometry.
(B) Cell expansion is shown as a function of different cytokine combinations.
(C) The dot plots show the frequency of pDCs and cDC1s as a function of different cytokine combinations, from one donor representative of the four studied.
(D-E) pDC (D) and cDC1 (E) frequency and absolute numbers per well as a function of different cytokine combinations.
(B, D, E) The symbols correspond to the individual donors used for the experiment (n=4), with overlay of mean±SD. Statistical analysis was performed with Mann-Whitney two tailed t test.
Figure S2. FT7 cultures contain a minor fraction of AXL−Siglec-6+ cells. Related to Figure 1.
FT7 cultures on OP9+OP9_DLL1 were harvested on day 18 of differentiation.
(A) Representative plots illustrate the gating strategy to identify AXL−Siglec-6+ cells in the Live−Lin−HLA-DR+ and Live−Lin−HLA-DR−CD123−BDCA2− gates, respectively.
(B) Fluorescence minus one (FMO) controls for the stainings shown in (A) from the Live−Lin−HLA-DR+ gate.
(C) Frequencies of AXL−Siglec-6+ cells in the Live−Lin−HLA-DR+ and Live−Lin−HLA-DR−CD123−BDCA2− gates. Data shown (mean±SD) are representative of 3 independent experiments each with a different donor (3 data points for each gating strategy).
Figure S3. Phenotypic characterization and flow cytometry sorting of putative DC types from the cultures for scRNAseq. Related to Figure 5.

(A) viSNE plots from live LinHLA-DR⁺ cells harvested from FT7 cultures differentiated on OP9+OP9_DLL1 for 18 days. Color codes indicate the relative surface expression for the listed surface markers.

(B) Overlay of the viSNE plot shown in (A) with the indicated manually gated populations listed in the legend. All cells were live LinHLA-DR⁺; pDCs were gated as BDCA2⁺CD123⁺, and cDC1s as BDCA2⁺CD123⁻ to low CADM1⁺CD1c⁺. In the remaining population of CADM1⁻ cells, CD1c⁺ cells were subdivided into BTLA⁺ and BTLA⁻ cells. Plots are representative of 4 donors from 2 independent experiments. The position of population
clusters varies between this figure and Figure 5 as clustering by the viSNE algorithm was performed independently from separate stainings.

(C) Lineage HLA-DR− cells were index sorted from FT7 cultures differentiated on OP9+OP9_DLL1 for 18 days, for phenotypes of cDC1s (CD141−CADM1+), pDCs (CADM1−CD123−BDCA2+) or CADM1−BDCA2− cells containing putative pre-cDC2 (CD1c−BTLA+) versus putative differentiated cDC2 (CD1c−BTLA−) as shown in the gating strategy.

(D) Graphs display the positions of the individual index-sorted single cells within the sorting gates before RNA extraction.
Figure S4. Heat map and hierarchical clustering of individual index sorted cells based on 100 representative differentially expressed genes. Related to Figure 5.

Heatmap displays the relative mRNA expression levels of 100 genes across individual single cells sorted from FT7 cultures differentiated on OP9+OP9_DLL1 and sequenced as described in Figure S3. The color codes on the top line refer to the cell identity based on index sorting and the color codes below to the cell clusters identified by t-SNE analysis in Figure 6. Cells and genes are grouped by hierarchical clustering using Euclidian distance and Pearson correlation respectively. The numbers on the very left identify gene clusters of special interest. Gene cluster (A) is mainly composed of genes expressed in mitotic cells and found here to be selectively expressed in XCR1+ cDC1s and a subset of HLA-DR CLEC9A CD1c+ cells, (B): genes known to be selectively expressed in cDC1s, (C): gene found to be selectively expressed in HLA-DR CLEC9A CD1c+ BTLA+ cells, (D): genes found to be selectively expressed in HLA-DR CLEC9A CD1c+ BTLA+ cells and encompassing both genes known to be...
selectively expressed in cDC2s or in macrophages, (E): genes known to be selectively expressed in pDCs, and (F): CXCR4 and CLEC12A genes found to be selectively expressed in XCR1+ cDC1s.
Figure S5. Re-analysis of public single cell RNAseq data of cDC1s differentiated from CD100⁺CD34⁺ cells on MS5 stromal cells confirms the existence of two cellular states of cDC1s. Related to Figure 5.

(A) A part of the GEO GSE94820 dataset of single cell RNAseq performed on cells differentiated from CD100⁺CD34⁺ cells on MS5 stromal cells (Villani et al., 2017) was retrieved and re-analyzed. Cell types were identified based on key genes described in the original study that reported these data, and analyzed for their expression patterns of key genes identified in Figure S5 as differentially expressed between in vitro derived XCR1⁺ versus XCR1⁻ cDC1s. Cells and genes are grouped by hierarchical clustering using Euclidian distance and Pearson correlation respectively. The letters on the very left identify gene clusters of special interest. A: gene module expressed in mitotic cells already illustrated on Figure S6. B: genes selectively expressed in cDC1s. G: genes reported in the initial publication as selectively expressed in blood cDC2. H: genes reported in the initial publication as selectively expressed in blood CD100⁺CD34⁺ DC progenitors.

(B) Role of Notch signaling on XCR1 expression by cDC1s. Histograms show XCR1 staining on pre-sort cDC1s or post-sort XCR1⁺ cDC1s after 14 days culture on OP9_DLL1 feeder cells with FT7+G in the presence of DMSO or DAPT. cDC1s were generated from expanded CD34⁺ cells cultured on OP9_DLL1 feeder cells with the FT7 cytokine combination for 18 days. cDC1s were identified as CD206⁻CD14⁻CD45RA⁻CD123⁻CD141⁻CLEC9A⁺ cells, examined/sorted for differential expression of XCR1, and cultured for 14 days with FT7 + GM-CSF (0.25ng/mL) on OP9_DLL1 feeder cells, in the presence of DAPT or DMSO. Cells were replenished on day 7 by removing 500ul of medium and replacing with fresh medium, cytokines and DMSO or DAPT. On day 14, the cells were analyzed for XCR1 expression. Dark lines, common fluorescent minus one control prepared by pooling DMSO- and DAPT- treated cultures of sorted XCR1⁺ and XCR1⁻ cells from two donors; red lines, XCR1 staining. The data shown is from one experiment representative of two independent ones.
Figure S6. Frequencies of the XCR1- versus XCR1+ fractions of human peripheral blood cDC1s and purity control after ex vivo sorting of these cells. Related to Figure 5.

(A) Peripheral blood cDC1s were gated as Lin- HLA-DR+ CD11c+ CD1c- CD141+ CADM1+ CLEC9A+ and analyzed for XCR1 expression. The frequency of XCR1- vs XCR1+ cells within cDC1s is shown for 3 independent experiments encompassing altogether 6 different donors (symbols), with overlay of mean±SD.

(B) Phenotypic analysis of peripheral blood cDC1s before and after their sorting into XCR1- vs XCR1+ populations. Data shown are from one experiments representative of 3 independent ones, each with a different donor.

(C) XCR1 expression on cDC1 generated with the FT7 cytokine combination on OP9 or OP9-DLL1 feeder cells. Red lines, XCR1 staining; dark lines, fluorescent minus one control, on gated cDC1s. The data shown is from one donor representative of the three tested. The graph on the right shows the frequency of XCR1+ cells within cDC1s from cultures on OP9 versus OP9-DLL1 feeders; each symbol represents one cord blood donor.
Figure S7. scRNAseq characterization of Lineage-HLA-DR+ cells enriched from PBMCs and use of their transcriptomic fingerprints to analyze scRNAseq data from the cord blood CD34+ cell cultures. Related to Figure 7.

(A) Identification of clusters of cell types/states from adult peripheral blood Lineage-HLA-DR+ cells. PBMCs from a healthy blood donor were separated by density gradient centrifugation, enriched by magnetic bead-based
negative sorting using the pan-DC enrichment kit from Miltenyi, stained with aqua dead fixable kit, FITC-lineage cocktail, BV785-HLA-DR, APC-BDCA2 and V450-CD11c. Live Lineage-HLA-DR+ cells were then sorted by flow cytometry before loading into the Chromium Single Cell Controller apparatus. Data were analyzed through unsupervised dimensional reduction with the Seurat package using t-SNE with graphical clustering. 12 cell clusters were identified. The absolute numbers of cells in each cluster are indicated in the graphical legend box, together with the percentages they represent out of the total cells analyzed.

(B) Violin plots showing expression profiles of previously known cell type-specific genes, across all individual cells and in comparison between cell clusters. These genes were amongst the most differentially expressed across cell clusters.

(C) Heatmap of the expression patterns of the genes illustrated in figures 7E and 7F. Genes found to be invariant across the clusters of the Lineage-HLA-DR+ cells from PBMCs were removed. Hierarchical clustering was performed on cell clusters (columns) and genes (rows). For each cluster, mean value of gene expression across all individual cells was used. Genes clustered largely according to previously known co-expression in specific cell types as highlighted by the vertical bars and their annotations on the right of the heatmap.

(D and E) Analysis of the homologies between human blood cell subsets and cells from the in vitro cultures by high throughput GSEA using BubbleGUM (Spinelli et al., 2015). Gene signatures specific to each subset of human blood cells were generated from the scRNAseq dataset of the Lineage-HLA-DR+ enriched PBMCs (panels A-C), or from public microarray data in which human cell subsets were clearly identified (Vu Manh et al., 2015b). These signatures were assessed for enrichment in all possible pairwise comparisons between scRNAseq clusters of the in vitro cultures identified in figures 7A and 7C, using the BubbleMap module of BubbleGUM. Data are represented as Bubbles, bigger and darker for stronger and more significant enrichment, in a color matching that of the cluster in which the signature was enriched (blue for the cluster indicated in blue characters on the annotation on the left of each figure, red for the cluster to which the comparison is performed). The strength of the enrichment is quantified by the NES which represents the number and differential expression intensity of the genes enriched. The significance of the enrichment is measured by the false discovery rate (FDR) value (q) representing the likelihood that the enrichment of the GeneSet was a false-positive finding (e.g., if q = 0.25, a similar enrichment is found in 25% of the random GeneSets used as controls). This q-value was further corrected for multiple testing, leading to a higher stringency of the significance threshold used. The absolute NES values generally vary between 1 (no enrichment) and 5 (extremely high enrichment). The enrichment is considered significant for absolute NES values > 1 with an associated q value < 0.25. The boxes correspond to coherent enrichments allowing the identification of the cluster in blue based on the associated signature. (D) Human signatures assessed for enrichment across clusters identified from the total in vitro culture. (E) Human signatures assessed for enrichment across clusters identified from the Lineage-HLA-DR+ cells enriched from the in vitro culture.
| Feeder layer | Cord blood sample identity | CB3  | CB20 | CB7  | CB8  | CB3  | CB51 | mean  | SD   |
|--------------|---------------------------|------|------|------|------|------|------|-------|------|
| Total fold increase of live cells | | | | | | | | | |
| Expansiona | | 2.67 | 3.2  | 2.13 | 2.46 | 1.5  | 5.6  | 2.93  | 1.43 |
| Expansion & differentiationb | OP9 | 641  | 870  | 682  | 541  | 216  | 829  | 630   | 236  |
| & OP9 DLL1 | 160  | 518  | 192  | 192  | 198  | 470  | 288  | 161   |
| & OP9+OP9 DLL1 | 363  | 960  | 328  | 472  | 360  | 504  | 498  | 237   |
| Total numbers of cDC1s (x10^5) generated from 10^4 human CD34+ cord blood cells.c | OP9 | 0.10 | 0.00 | 0.26 | 0.27 | 0.14 | 0.04 | 0.14  | 0.11 |
| & OP9 DLL1 | 1.79 | 4.21 | 0.53 | 1.40 | 3.16 | 0.75 | 1.97 | 1.44  |
| & OP9+OP9 DLL1 | 2.37 | 6.25 | 2.81 | 1.26 | 2.38 | 2.16 | 2.87  | 1.73 |
| Total numbers of pDCs (x10^5) generated from 10^4 human CD34+ cord blood cells.c | OP9 | 15.5 | 7.24 | 7.89 | 8.35 | 2.75 | 9.45 | 11.50 | 7.77 |
| & OP9 DLL1 | 3.87 | 7.31 | 0.08 | 0.16 | 1.56 | 1.51 | 2.42  | 2.76  |
| & OP9+OP9 DLL1 | 8.12 | 6.46 | 6.00 | 6.81 | 7.17 | 19.17 | 11.14 | 6.81  |

aCalculations are based on the expansion of 5,000 CD34+ CB cells / well under FST7 conditions.
bCalculations are based on the expansion of 5,000 CD34+ CB cells / well under FST7 conditions with subsequent differentiation of 10,000 expanded cells / well under FT7 conditions on the indicated feeder layers for 18-19 days.
cCalculations are based on the expansion of 5,000 CD34+ CB cells / well under FST7 conditions with subsequent differentiation of 10,000 expanded cells / well under FT7 conditions on the indicated feeder layers for 18-19 days. cDC1s and pDCs were gated as described in Figure 1B.

dFor comparison, equivalent yields were 1.2 for CD141+CLEC9A+ cells and thus less than that for bona fide CD141+CLEC9A+ cells in (Thordardottir et al., 2014) and 0.25 in (Lee et al., 2015), thus about 3 to 10 times less than with our protocol.
eFor comparison, equivalent yields were 3.8 in (Thordardottir et al., 2014) and 0.5 in (Lee et al., 2015), thus about 3 to 20 times less than with our protocol.