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3.DCSupplemental

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Human XCR1+ Dendritic Cells Derived In Vitro from CD34+ Progenitors Closely Resemble Blood Dendritic Cells, Including Their Adjuvant Responsiveness, Contrary to Monocyte-Derived Dendritic Cells

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Human monocyte-derived dendritic cell (MoDC) have been used in the clinic with moderately encouraging results. Mouse XCR1+ DC excel at cross-presentation, can be targeted in vivo to induce protective immunity, and share characteristics with XCR1+ human DC. Assessment of the immunoactivation potential of XCR1+ human DC is hindered by their paucity in vivo and by their lack of a well-defined in vitro counterpart. We report in this study a protocol generating both XCR1+ and XCR1− human DC in CD34+ progenitor cultures (CD34-DC). XCR1+ DC were strongly activated by polyinosinic-polycytidylic acid but not LPS, and conversely for MoDC. XCR1+ DC and MoDC expressed strikingly different patterns of molecules involved in inflammation and in cross-talk with NK or T cells. XCR1+ DC and XCR1− DC but not MoDC efficiently cross-presented a cell-associated Ag upon stimulation by polyinosinic-polycytidylic acid or R848, likewise for what was reported for XCR1+ bDC. Hence, it is feasible to generate high numbers of bona fide XCR1+ human DC in vitro as a model to decipher the functions of XCR1+ bDC and as a potential source of XCR1+ DC for clinical use. The Journal of Immunology, 2014, 193: 1622–1635.

Dendritic cells (DC) are a heterogeneous family of rare leukocytes that sense danger signals and convey them to lymphocytes for the orchestration of adaptive immune defenses. Clinical trials used monocyte-derived DC (MoDC) to attempt to promote protective immunity in patients suffering from infections or cancer. These immunotherapies showed limited efficacy, owing to the poor recirculation of MoDC to lymph nodes (1, 2) and likely to other yet uncharacterized functional differences.
between MoDC and lymphoid residing DC (LT-DC). Hence, major efforts are being made to better characterize human LT-DC and to evaluate their immunoregulatory potential.

Steady-state human blood and secondary lymphoid organs contain three major DC subsets, CD141(BDCA3)+CLEC9A+ DC, CD1c(BDCA1)+ DC, and CLEC4C(BDCA2)+ plasmacytoid DC (pDC) (3). Homologies exist between mouse and human LT-DC subsets (4, 5). Comparative transcriptomics (4–7) and functional studies (7–12) showed that human CD141+CLEC9A+ DC are homologous to mouse spleen CD8α+ DC, which are specialized in cross-presentation. Mouse CD8α+ DC and human CD141+CLEC9A+ DC specifically express the XCR1 chemokine receptor (4, 8, 9, 13, 14) and can therefore be coined XCR1+ DC. The ligands of XCR1 are selectively expressed in NK and CD8 T cells, promoting their interactions with XCR1+ DC (13). Human XCR1+ DC have been described in many tissues (6, 7, 15). Human and mouse XCR1+ DC specifically express high levels of TLR-3 (16) and respond to its triggering with hepatitis C virus or with the synthetic ligand polyinosinic-polycytidylic acid (PolyI:C) by IFN-α production (12, 15, 17) and by enhanced cross-presentation (7–11). The extent to which human XCR1+ DC are more efficient for cross-presentation than other human DC subsets is debated. It depends on the tissue origin of the DC subsets, on their activation status, and on the mode of Ag delivery (7–10, 18–23). However, several independent studies showed that human XCR1+ DC (bDC) excel at cross-presentation of cell-associated Ags (8–10) and respond to its triggering with hepatitis C virus or with the synthetic ligand polyinosinic-polycytidylic acid (PolyI:C) by IFN-α production (12, 15, 17) and by enhanced cross-presentation (7–11). The extent to which human XCR1+ DC are more efficient for cross-presentation than other human DC subsets is debated. It depends on the tissue origin of the DC subsets, on their activation status, and on the mode of Ag delivery (7–10, 18–23). However, several independent studies showed that human XCR1+ DC (bDC) excel at cross-presentation of cell-associated Ags (8–10) and respond to its triggering with hepatitis C virus or with the synthetic ligand polyinosinic-polycytidylic acid (PolyI:C) by IFN-α production (12, 15, 17) and by enhanced cross-presentation (7–11). The extent to which human XCR1+ DC are more efficient for cross-presentation than other human DC subsets is debated. It depends on the tissue origin of the DC subsets, on their activation status, and on the mode of Ag delivery (7–10, 18–23). However, several independent studies showed that human XCR1+ DC (bDC) excel at cross-presentation of cell-associated Ags (8–10) and respond to its triggering with hepatitis C virus or with the synthetic ligand polyinosinic-polycytidylic acid (PolyI:C) by IFN-α production (12, 15, 17) and by enhanced cross-presentation (7–11).

**Materials and Methods**

**Isolation of cord blood CD34+ cells**

Human nontherapeutic-grade cord blood (CB) samples were collected from informed and consenting mothers at the Etablissement Français du Sang (EFS) Lyon in the frame of an EFS–INSERM agreement or at the Marseille University Hospital de la Conception birth clinic and obtained through the Marseille CB bank. Marseille CB bank is a not-for-profit allogeneic CB bank affiliated with the French consortium of CB bank (RFSP, Réseau Français du Sang Placentaire) and the “Agence de Biologie.” CB units that were disqualified for therapeutic use upon reception at the Institut Paoli-Calmettes cell therapy facility, because of insufficient volume or total cell number, were used for research, as indicated to pregnant mothers in the information document. The protocol was reviewed by the Institut Paoli-Calmettes Comité d’Orientation Stratégique (COS or IRB).

**In vitro generation of MoDC from enriched monocytes derived from CB CD34+ cells**

MoDC were differentiated from enriched monocytes derived from CB CD34+ cells, as adapted from a published protocol (35). CD34+ cells were expanded for 21 d in expansion#1 medium (stemspan from StemCell Technologies; 10% FBS, 25 ng/ml Flt-3L, 20 ng/ml stem cell factor [SCF], and 10 ng/ml TPO). The cells were replated in weekly intervals at 5 × 10^5 cells/ml, 2 ml/well, in 6-well plates, in fresh expansion#1 medium. On day 14, the cells were plated in 100-mm dishes at 2 × 10^5 cells/ml, 10 ml/dish, in fresh expansion#1 medium. On day 21, CD14+ monocytes were enriched by positive selection through MACS (Miltenyi Biotec) and differentiated for 7 d in differentiation#1 medium (RPMI 1640 medium (Sigma-Aldrich), 10% FBS, 50 ng/ml GM-CSF, and 25 ng/ml IL-4) (GM4 protocol). The growth factors and cytokines used were from PeproTech unless specified otherwise.

**In vitro generation of XCR1+ and XCR1− DC from CB CD34+ cells**

XCR1+ CD34+DC cultures were performed accordingly to the FS36 protocol (11) and modifications thereof as described below. For the FS36 protocol, CD34+ cells were cultured for 7 d at 5 × 10^5 cells/ml, 2 ml/well, in 6-well plates, with expansion#2 medium (stemspan; 100 ng/ml Flt-3L, 100 ng/ml SCF, 20 ng/ml IL-3, and 20 ng/ml IL-6) in the absence of serum supplementation. Expanded cells were harvested on day 7. They were used for DC generation, either directly or after cryopreservation, through culture in U-bottom 96-well plates in differentiation#2 medium (RPMI 1640 medium 10% FCS, 100 ng/ml Flt-3L, 20 ng/ml SCF, 20 ng/ml GM-CSF, and 20 ng/ml IL-4). On day 6, half of the medium was replaced, and fresh cytokines were added. Cultures were harvested at days 8–11. In the adapted version FS36A, 5% heat-inactivated autologous plasma was added to the expansion medium. In the FS3TA protocol, for the expansion phase, 50 ng/ml TPO was substituted to IL-6 (expansion#3 medium), and 5% heat-inactivated autologous plasma was added. The FS3T protocol was based on the FS3TA with substitution of autologous plasma by 10% FBS. The optimized FS3T* protocol was based on the FS3T protocol, with reduced cell density and culture in 96-well plates during the expansion phase and decreased concentration of GM-CSF and IL-4 during the differentiation phase. In detail, for the FS3T* protocol, CD34+ cells were cultured for 7 d at 2.5 × 10^5 cells/ml, 200 μl/well, in round-bottom 96-well plates, with expansion#3 medium (stemspan; 100 ng/ml Flt-3L, 100 ng/ml SCF, 20 ng/ml IL-3, and 50 ng/ml TPO) with 10% FCS. Expanded cells were differentiated in RPMI 1640 medium plus 10% FCS, with 100 ng/ml Flt-3L, 20 ng/ml SCF, 2.5 ng/ml GM-CSF, and 2.5 ng/ml IL-4 in U-bottom 96-well plates (6.25 × 10^5 cells/ml and 200 μl/well). On day 6, half of the medium was replaced, and fresh cytokines were added. Cultures were harvested on days 8–13, depending on the experiments.

**Surface staining and sorting of in vitro-generated DC**

Cells were stained with Abs (Table I) for 30 min at 4°C, washed with 1× PBS, and acquired on a LSR II (BD Biosciences) flow cytometer (BD Biosciences). Human XCR1 was stained with a fluorescently coupled recombinant human chemokine ligand vaccibody (rhXCL1-mCherry) (36). FS36 and FS3T cultures were sorted into >97% pure DC subsets with a FACSAria cytometer (BD Biosciences), based on viability (negativity for Sytox blue; Invitrogen) and expression of CD141 and CD11c.

**Purification of peripheral blood DC**

PBMC were prepared by Ficoll-Hypaque (GE Healthcare) gradient centrifugation from buffy coats received from EFS (Marseille) and depleted of monocytes. B cells, and T cells by negative selection using LD columns and magnetic beads coupled to Abs against CD3, CD14, and CD19 (Miltenyi Biotec). Enriched cells were stained for 30 min at 4°C in 1× PBS and 0.5% BSA. Viable (Sytox blue-negative) DC subsets were sorted with a FACSAria cytometer (BD Biosciences) to a purity >96%, as Lin− HLA−
DR CD1c^+CD1c^+CLEC4C^+CD141^high cells for XCR1^+ DC, Lin^− HLA-DR-CD1c^+CD14-CLEC4C^+CD141^low cells for CD1^+ DC, and Lin^− HLA-DR-CD1c^+CD1c^−CLEC4C^+CD141^low cells for pDC.

**Cytospin and Wright–Geimsa stainings**

Cells were diluted at 10–15 × 10^6 cells/50 μl and used for the cytospin at 400 rpm for 4 min. The slides were air-dried for 15 min, fixed in methanol for 7 min, and stained with Wright–Geimsa solution (Sigma-Aldrich). Images were taken with a Nikon eclipse (camera "DS-FI2") microscope.

**TLR stimulation of DC subsets**

Optimal doses of adjuvants were defined by titration curves in assays assessing the phenotypic maturation of CD34-DC (data not shown). Sorted DC subsets were stimulated for 16 h with PolyIC (5 μg/ml), R848 (10 μg/ml), or LPS (1 μg/ml) (all from InvivoGen). Cells were then examined for phenotypic maturation or used for RNA extraction. Sorted bDC were maintained in Flt-3L (100 ng/ml) plus GM-CSF (20 ng/ml). MoDC were stimulated on day 6.

**Cytokine production assays**

Cytokines were titrated in culture supernatants with a custom procarta immune assay kit following the instructions from the manufacturer. Data were acquired with the Magpix reader using the xPONENT 4.2 software and analyzed with the Milliplex Analyst 5.1 Software.

**RNA extraction and microarray experiments**

Total RNA was prepared with the RNeasy Micro kit (Qiagen). The quantity and integrity of mRNA were measured using the 2100 Bioanalyzer system with picochips (Agilent Technologies). The RNA integrity numbers were generally above 8 and never below 7. Microarray experiments were performed at the Plate-forme Biopuces (Strasbourg, France) (http://www.microarrays.u-strasbg.fr). In brief, cDNA was generated and labeled using the Ribo-SPATM Whole Transcript Amplification Process (NuGen) using as a starting material 20 ng total RNA for most of the in vitro–derived DC and 1 ng for ex vivo–isolated blood DC. GeneChip Human Gene 1.0 ST DAT files were processed using Affymetrix GeneChip Operating System to generate .CEL files. Quality control of the array hybridization and normalization of the raw Affymetrix expression data with Robust Multi-chip Analysis 2 (37) were performed through Bioconductor in the R statistical environment (version 2.14.1) using the oligo package. Density plots of the normalized expression data were generated to estimate the background noise. For TLR stimulations of CD34-DC subsets, differentially expressed genes between conditions were identified by General Linear Model with paired analysis comparing PolyIC, R848, or LPS-treated samples to unstimulated controls for each DC subset of each individual CB culture tested. For hierarchical clustering and heat mapping, each gene was represented by only one ProbeSet selected as the one giving the highest signal. Principal component analysis (PCA) was computed with the MeV software and represented with the R software (scatterplot3D package). Hierarchical clustering was made with the pvclust package in R to evaluate the robustness of the branches of the tree through multiscale bootstrap resampling of the genes (http://www.is.i.teich.ac.jp/~shimo/prog/pvclust/). Heat-map representations were made with the GeneG software (http://www.broadinstitute.org/cancer/software/GENE-E/). Genes were clustered based on coregulation analysis using BioLayout Express3D for computing Pearson’s correlation coefficients and for graphical representation (38).

**Allogeneic CD4^+ T cell stimulation**

CD4^+ T cells were isolated from buffy coat with the RosetteSep human CD4^+ T cell enrichment mixture (StemCell Technologies). CD4^+ T cells were labeled with CFSE (2 μM) and cocultured with DC at 20:1 ratio for 6 d. T cell proliferation and CFSE dilution was assessed by FACS after addition of 10% FBS during the expansion phase. During the differentiation phase, lower concentrations of GM-CSF and IL-4 were sufficient to yield maximal numbers of XCR1^+ CD34-DC (data not shown). XCR1^+ CD34-DC were not detected at the end of the differentiation phase.

**Generation of K562-N9V or GFP expressing cell lines**

We used lentiviral–based vectors to generate the K562 cell line expressing the N9V epitope of the CMV pp65 fused in frame at the 5' end to cytoplasmic OVA. The HIV lentiviral backbone is based on self-inactivating 3'-long terminal repeat vectors. This vector was engineered to carry first the human CMV early enhancer/chicken β-actin hybrid promoter driving the N9V-OVA protein, followed by the spleen focus-forming virus pro-moter driving the enhanced GFP reporter gene. To increase the level of transcription the woodchuck hepatitis virus posttranscriptional regulatory element was inserted downstream of N9V-OVA. Amplification of the fusion protein was done using a vector encoding a full OVA as template and the following primers: forward N9V-CytOVA, 5'-GGCTCGAGTGGACCATATGATCCGACCATGGCGGCCACCATGCCTGTCGTCGCTGCTATCCGGGTGGTCACAGACAAATAGTGGTGTTCG-3' and reverse OVA, 5'-CTGCTAAGGCTGAGACTCTC-3'. Viruses were pseudotyped with vesicular stomatitis virus glycoprotein, infections at MOI 5 were performed, and 5 d posttransduction, GFP-positive K562 cells were sorted, expanded in culture, and used as a stable bulk population. GFP expression was checked before each experiment. Negative controls for cross-presentation experiments always included K562 cells infected with a lentivirus encoding GFP alone, which never induced IFN-γ production by the CD8^+ T cell clone (data not shown).

**Cross-presentation assay**

A total of 10^5 K562 cells expressing the N9V (pp65^103-105) epitope from the major CMV structural protein and 0.5 × 10^5 DC were cocultured in round-bottom 96-well plates in complete medium for 24–36 h with or without PolyIC (5 μg/ml) or R848 (10 μg/ml) (InvivoGen). As positive control, 10 nM soluble N9V peptide was added to DC for 1 h before extensive washes. An N9V-specific T cell clone (0.5 × 10^5 cells/well) (39) was then added in the presence of GolgiStop (BD Biosciences) for an additional 12 h (ratio DCT = 1:2). After fixation and permeabilization, T cell activation was determined as the percentage of IFN-γ–expressing cells within CD3^+GFP^+ cells.

**Statistics**

Statistics for gene chips data analyses are detailed in Materials and Methods and the figure legends. Significance for other experimental data was evaluated with Graph pad Prism 6, using the tests indicated in the figure legends.

**Accession numbers**

The microarray data have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE57671.

**Results**

**Combined use of FLT3-L and TPO in CD34^+ progenitor cultures yields high numbers of XCR1^+ DC**

We reproduced what to our knowledge is the first published protocol for in vitro generation of XCR1^+ DC from CB CD34^+ progenitors (11). It consisted in an initial 7-d expansion phase with FLT3-L, SCF, IL-3, and IL-6 (“FS36” cultures), followed by a 13-d differentiation phase with FLT3-L, SCF, GM-CSF, and IL-4 (Supplemental Fig. 1A). We tested modifications of this protocol by varying the cytokines used for the expansion phase (data not shown), in particular IL-6 replacement by TPO (“FS3T” cultures). FS36 and FS3T cultures were both composed in majority of CD1c^highCD14^−HLA-DR^− cells (Fig. 1A, Table I, Supplemental Fig. 1B), which lacked expression of XCR1, CLEC9A, and CADM1 (Fig. 1B, Supplemental Fig. 1C) and were therefore named XCR1^− CD34-DC. The cultures also consistently encompassed CD1c^lowCD14^highHLA-DR^− cells (Fig. 1A, Supplemental Fig. 1B), which shared with XCR1^+ DC34-DC (36) specific expression of XCR1, CLEC9A, and CADM1 (Fig. 1B, Supplemental Fig. 1C) and were thus named XCR1^+ CD34-DC. MoDC were derived from the same CB CD34^+ cells (Supplemental Fig. 1A) (35). The morphology of XCR1^+ CD34-DC was similar to that of XCR1^− DC34-DC and XCR1^− CD34-DC (Supplemental Fig. 1D, 1E). The yields of XCR1^+ CD34-DC were improved by lowering cell density and adding 10% FBS during the expansion phase. During the differentiation phase, lower concentrations of GM-CSF and IL-4 were sufficient to yield maximal numbers of XCR1^+ CD34-DC (data not shown). XCR1^+ CD34-DC were not detected at the end of the
FIGURE 1. Characterization of in vitro–generated CD34-DC subsets. (A) Identification of cell subsets in cultures of CD34⁺ CB progenitors based on the expression of CD11c and CD141 for FS3T cultures (left contour plot, double-negative cells in violet, XCR1⁺ CD34-DC in green and XCR1⁺ CD34-DC in red) and G4 cultures (right contour plot, MoDC in black). (B) Expression of XCR1, CLEC9A, and CADM1 on each of the cell subsets identified in (A), using the same color code. Isotype control stainings are shown as gray curve on each histogram. For (A) and (B), one representative result of at least three independent cultures is shown. (C) Microscopy analysis of the morphology of Giemsa/May–Grünwald-stained DC subsets sorted from the cultures of CD34⁺ CB progenitors or from the blood of adult healthy donors. (D–G) Gene expression profiling of XCR1⁺ CD34-DC, XCR1⁻ CD34-DC, and MoDC. Microarrays were performed on total RNA extracted from DC subsets FACS purified from FS3T culture (XCR1⁺ CD34-DC and XCR1⁻ CD34-DC), from G4 cultures (MoDC), or from the blood of adult healthy donors (bpDC, CD1c⁺ bDC, and XCR1⁺ bDC). (D) PCA performed on all ProbeSets. (E) Hierarchical clustering performed on the 3934 genes showing a fold change ≥ 2 between at least two of the cell types studied, using Pearson and average as distance metric/linkage parameters. The numbers above edges indicate the robustness of the corresponding node, calculated as (Figure legend continues).
expansion phase; their development during the differentiation phase required both GM-CSF and IL-4; they reached maximal frequencies ∼11 d after the initiation of differentiation (data not shown). Hence, unless specified otherwise, XCR1+ CD34-DC were generated with an optimized protocol coined FS3T*, by culturing cells during expansion at 2.5×10^4 cells/ml, in U-bottom 96-well plates, with 10% FBS and, during differentiation, by decreasing GM-CSF and IL-4 concentrations to 2.5 ng/ml and harvesting cultures at day 11. FS3T* cultures yielded 1.8×10^6 XCR1+ CD34-DC from 10^5 CD34+ CB progenitors (Supplemental Fig. 1E).

XCR1+ and XCR1- CD34-DC respectively expressed XCR1+ bDC and MoDC transcriptomic fingerprints

To determine in the broadest and most unbiased way possible to which extent XCR1+ CD34-DC resembled XCR1+ bDC, we profiled their gene expression. PCA (Fig. 1D) demonstrated a clear proximity between XCR1+ CD34-DC and XCR1+ bDC, and between MoDC and XCR1- CD34-DC, along the PC1 axis representing the majority of gene expression variability in the dataset. Cell type distribution along PC1 was explained by their differential expression of many genes previously identified as being selectively expressed either in XCR1+ bDC or in MoDC (4, 40) (Supplemental Table I). On the PC2 axis, XCR1+ CD34-DC clearly segregated away from XCR1+ bDC, which resulted in part from their higher expression of mitotic genes and lower expression of lysosomal genes (Supplemental Table I). Hence, XCR1+ CD34-DC are a good model of human XCR1+ bDC, contrary to MoDC, but their differentiation might be stopped at a preterminal stage as it has been reported for in vitro generated mouse XCR1+ DC (41). Hierarchical clustering confirmed the strong similarity between XCR1+ CD34-DC and XCR1+ bDC and between MoDC and XCR1- CD34-DC (Fig. 1E). Coregulation analysis using BioLayoutExpress (Fig. 1F) clustered together genes with a high selective expression in MoDC and XCR1- CD34-DC (C1, n=1228) or in XCR1+ CD34-DC and XCR1+ bDC (C2, n=747 and C4, n=188), in consistency with the transcriptomic fingerprints previously reported for in vitro-derived (4) or ex vivo-isolated (40) human MoDC versus for human blood, skin, and intestine XCR1+ DC (Fig. 1G) (4, 6, 7). CD34-DC subsets from FS36 and FS3T cultures harbored similar gene expression programs (Supplemental Fig. 2). The expression patterns of several genes were confirmed at the protein level (Fig. 2). Bona fide XCR1+ DC also could be efficiently generated from CD34+ progenitors iso-

### Table I. Abs and other reagents used for flow cytometry stainings

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To determine in the broadest and most unbiased way possible to which extent XCR1+ CD34-DC resembled XCR1+ bDC, we profiled their gene expression. PCA (Fig. 1D) demonstrated a clear proximity between XCR1+ CD34-DC and XCR1+ bDC, and between MoDC and XCR1- CD34-DC, along the PC1 axis representing the majority of gene expression variability in the dataset. Cell type distribution along PC1 was explained by their differential expression of many genes previously identified as being selectively expressed either in XCR1+ bDC or in MoDC (4, 40) (Supplemental Table I). On the PC2 axis, XCR1+ CD34-DC clearly segregated away from XCR1+ bDC, which resulted in part from their higher expression of mitotic genes and lower expression of lysosomal genes (Supplemental Table I). Hence, XCR1+ CD34-DC are a good model of human XCR1+ bDC, contrary to MoDC, but their differentiation might be stopped at a preterminal stage as it has been reported for in vitro generated mouse XCR1+ DC (41). Hierarchical clustering confirmed the strong similarity between XCR1+ CD34-DC and XCR1+ bDC and between MoDC and XCR1- CD34-DC (Fig. 1E). Coregulation analysis using BioLayoutExpress (Fig. 1F) clustered together genes with a high selective expression in MoDC and XCR1- CD34-DC (C1, n=1228) or in XCR1+ CD34-DC and XCR1+ bDC (C2, n=747 and C4, n=188), in consistency with the transcriptomic fingerprints previously reported for in vitro-derived (4) or ex vivo-isolated (40) human MoDC versus for human blood, skin, and intestine XCR1+ DC (Fig. 1G) (4, 6, 7). CD34-DC subsets from FS36 and FS3T cultures harbored similar gene expression programs (Supplemental Fig. 2). The expression patterns of several genes were confirmed at the protein level (Fig. 2). Bona fide XCR1+ DC also could be efficiently generated from CD34+ progenitors iso-
lated from bone marrow (Fig. 3) or blood (data not shown) of adult donors. In conclusion, to our knowledge, we demonstrated for the first time that a culture protocol of CB CD34+ cells generated simultaneously different DC subsets along two independent ontogenic pathways, XCR1+ CD34-DC related to the monocyte lineage and similar to MoDC as well as XCR1+ CD34-DC resembling XCR1+ bDC.

**XCR1+ versus XCR1− CD34-DC show reciprocal responsiveness to TLR3 versus TLR4 triggering**

Because we generated XCR1+ CD34-DC and MoDC-like XCR1+ CD34-DC from the same CD34+ cells in the same cultures, it allowed a rigorous side-by-side comparison of the cell-intrinsic immunomodulatory potential of these two DC subsets, without potential confounding effects linked to differentiation from distinct cell progenitors and with different cytokines. XCR1+ CD34-DC matured phenotypically in response to PolyI:C but not to LPS, and conversely for XCR1− DC and MoDC (Fig. 4A), in consistency with the opposite expression pattern in CD34-DC subsets of the respective receptors of these adjuvants, TLR3 and TLR4 (Fig. 2). TLR7/8 activation by R848 induced an intermediate response in all DC subsets (Fig. 4A). These response patterns were confirmed by gene expression profiling. In particular, both PCA (Fig. 4B) and hierarchical clustering (Fig. 4C) showed that PolyI:C stimulation of XCR1+ DC and LPS stimulation of XCR1− CD34-DC and MoDC strongly affected their gene expression programs, setting them apart from their unstimulated counterparts. LPS stimulation of XCR1+ CD34-DC and PolyI:C stimulation of XCR1− CD34-DC and MoDC did not strongly affect their gene expression programs. The highest number of differentially expressed genes (n = 2010) was observed for PolyI:C stimulation of XCR1+ DC (Fig. 4D, Supplemental Table I), followed by LPS stimulation of MoDC (n = 1447) and XCR1− CD34-DC (n = 1047).

**Hundreds of genes are similarly modulated in PolyI:C-stimulated XCR1+ CD34-DC and LPS-stimulated XCR1− CD34-DC or MoDC**

PCA showed a parallel shift in the gene expression programs of each DC subset from unstimulated to optimally activated states (Fig. 4B). Indeed, 370 genes were similarly modulated in response to PolyI:C in XCR1+ CD34-DC and in response to LPS in XCR1− CD34-DC or MoDC (Fig. 5, Supplemental Table I). Ingenuity pathway analyses found many annotations to be significantly enriched for the genes commonly upregulated in mature DCs (Fig. 5), highlighting the contribution of this core maturation gene set to the canonical process of DC maturation, the activation of NK or T cells or more generally the immune defenses against bacteria or viruses, in a large part under the instruction of NFkB or type I IFN (IFN-I) (Fig. 5C).

**Many genes are modulated in specific combinations of DC subset/stimulus**

Many genes were significantly modulated in specific combinations of DC subset/stimulus (Fig. 5A, 5B Supplemental Table I), likely determining the nature of the specific functions exerted by mature DC subsets. Fewer annotations were enriched for these gene sets and with lower statistical significance. Under steady-state conditions, XCR1+ CD34-DC or MoDC harbored a higher expression of genes coding for inflammatory molecules than XCR1− CD34-DC (Fig. 6). TLR4 or TLR8 triggering of XCR1− CD34-DC and MoDC further enhanced the expression of these inflammatory molecules and induced additional ones, including *IL18* and *IL12B* (Fig. 6). IL-10 was only detected in XCR1− CD34-DC and MoDC in response to R848 or LPS (Fig. 6A, 6B), consistent with production of this cytokine only by CD14+ DC but not XCR1+ DC in skin (7, 42). As described for XCR1+ bDC in vitro (12), XCR1+ CD34-DC uniquely expressed IFN-α upon TLR3 triggering (Fig. 6A, 6B). Many other genes were newly found to be selectively induced to high levels in XCR1+ CD34-DC upon PolyI:C stimulation (Fig. 6, Supplemental Table I), including genes encoding antiapoptotic, antiviral, or costimulation molecules as well as cytokines/chemokines acting on NK or T cells.

The response pattern of XCR1+ CD34-DC and XCR1+ bDC to candidate adjuvants are strikingly similar

To examine to which extent the responses of XCR1+ CD34-DC to adjuvants were similar to those of XCR1+ bDC, we compared the gene expression profiling of CD1c+ bDC, XCR1+ bDC, XCR1+ CD34-DC, and XCR1− CD34-DC upon stimulation with R848, PolyI:C, or LPS. As observed for XCR1+ CD34-DC, XCR1+ bDC were not activated by LPS, moderately by R848, and strongly by PolyI:C (Fig. 7A, 7B). PolyI:C-stimulated XCR1+ CD34-DC, XCR1+ bDC, and CD1c+ bDC regrouped together, away from other conditions of stimulation of these cell types and from XCR1− CD34-DC (Fig. 7A, 7B) or MoDC (data not shown). Cell type distribution along PCA axes resulted in a large part from their differential expression of many genes already at steady state (Fig. 7C, clusters 1 and 5–8) as well as from selective induction of genes in 1) CD1c+ bDC and XCR1− CD34-DC by R848 and LPS (subcluster A), 2) bDC and XCR1+ CD34-DC by PolyI:C as well as XCR1− CD34-DC by both PolyI:C and LPS (cluster 2), and 3) bDC and XCR1+ CD34-DC by PolyI:C (cluster 4). *IL12A* was strongly and exclusively induced in bDC subsets by PolyI:C. Despite being based on a very stringent and constrained selection of genes bearing a heavy weight in the first three components of the PCA, this analysis highlighted striking similarities and differences between DC subset responses to PolyI:C. To extend this observation, PolyI:C-induced gene clusters harboring contrasting expression pattern across all conditions were identified through iterative self-organizing maps (Fig. 7D). This confirmed that DC subset responses to PolyI:C were characterized both by 1) the induction of many genes specifically in bDC subsets and XCR1+ CD34-DC (cluster 3, n = 388), with a higher induction of a number of these genes in XCR1+ bDC and/or XCR1− CD34-DC as compared with CD1c+ bDC (Supplemental Fig. 5), and 2) the induction of many genes, in particular interferon stimulated genes, by PolyI:C in all the DC subsets studied as well as by LPS in XCR1− CD34-DC (cluster 5, n = 203). In addition to *IL12A*, several genes were induced exclusively by PolyI:C in bDC (cluster 4, n = 63), including *IL23A* and *IL13*. Thus, in addition to inducing interferon stimulated genes similar to MoDC and XCR1− CD34-DC, bDC mounted a strikingly specific and strong response to PolyI:C, which was very well modeled by XCR1+ CD34-DC.

PolyI:C-activated XCR1+ CD34-DC increased their ability to stimulate allogeneic CD4+ T cells and have the highest capacity for cell-associated Ag cross-presentation

PolyI:C and R848 stimulations significantly increased the ability of XCR1+ CD34-DC to induce allogeneic CD4+ T cell proliferation, consistent with previous observations on human blood or skin XCR1+ DC (7, 10), although this was not the case with LPS (Fig. 8A). Under steady-state conditions, no significant differences were observed between DC subsets for their ability to cross-present a cell-associated Ag (Fig. 8B). PolyI:C stimulation increased this function in XCR1+ CD34-DC significantly above the levels observed at steady state and in XCR1− CD34-DC and MoDC (Fig. 8B, 8C). Although R848 activated all DC subsets to
In this paper, we report an optimized protocol for in vitro generation of high numbers of human XCR1+ DC from CD34+ hematopoietic progenitor cultures. We characterized these XCR1+ CD34-DC, as well as the XCR1- CD34-DC representing the major cell population in the same cultures, through an unbiased, high-throughput comparison with XCR1+ bDC and with MoDC. These results unambiguously demonstrated that XCR1- CD34-DC resemble MoDC, whereas XCR1+ CD34-DC are a good model of bDC and LT-DC, both under steady-state as well as activation conditions.

XCR1+ CD34-DC expressed the molecular signature characteristic of XCR1+ bDC, including the chemokine receptor XCR1, which likely promotes physical interactions with CD8+ T cells (8, 13), the endocytic receptor CLEC9A, which promotes cross-presentation of Ag derived from dying cells (43), the adhesion receptor CADM1, which may promote the induction of CTL responses (44), and the pattern recognition receptor TLR3, which allows sensing of dsRNA including responsiveness to the synthetic adjuvant PolyIC. CD1c and CD141 are not reliable markers of human DC subsets, because they were expressed on all the three in vitro-derived DC subsets studied in this work, consistent with similar observations reported for ex vivo–isolated human DC subsets (7, 11, 42). Hence, flow cytometry assessment of cell surface expression of the discriminative markers XCR1 or CLEC9A and CADM1, or gene expression profiling, are critical to ensure the identity of HLA-DR+CD141+ cells in humans (7, 8, 11, 36).

To examine the immunostimulation potential of XCR1+ CD34-DC, XCR1- CD34-DC, and MoDC, we selected for their stimulation three adjuvants widely used for basic research on DC maturation and/or for the design of novel immunotherapeutic treatments, LPS, PolyIC, and R848. Ag targeting to XCR1+ DC combined with PolyIC adjuvant is one of the best vaccination strategies to induce protective immune responses in mouse models of infectious diseases and cancer (45, 46). It is also very efficient for the induction of antiviral human CD4+ T cell responses in humanized mice in experimental settings where XCR1+ DC are engaged.

FIGURE 2. Phenotypic analyses confirm microarray data regarding the identity of CD34-DC subsets. DC cultures were stained with Abs against TLR3 or a variety of cell surface markers selected based on available Abs and differential expression of the corresponding genes between XCR1+ versus XCR1- CD34-DC as shown in Fig. 1G. Data were analyzed as in Fig. 1A and 1B. One representative result of at least three independent cultures is shown.

FIGURE 3. Efficient differentiation of XCR1+ DC in FS3T cultures of CD34+ hematopoietic progenitors from the bone marrow of adult donors. (A) Identification of cell subsets in cultures of adult bone marrow CD34+ progenitors based on the expression of CD11c and CD141, for FS3T cultures, similarly to what is shown for CB cultures on Fig. 1A. (B) Expression of selected markers on each of the cell subsets identified in (A), similarly to what is shown for CB cultures cultures on Figs. 1B and 2. One representative result of at least two independent cultures is shown.
a major source of IFN-I (47). The unique potency of PolyI:C in eliciting strong adaptive cellular immune responses also extends to nonhuman primate vaccination against SIV, papillomavirus, or *Plasmodium falciparum* (48–50). As compared with many other stimuli tested including LPS and R848, PolyI:C induces a unique combination of cytokines and chemokines in human whole blood assays (51), highlighting the particular immunoactivation potential of this adjuvant. However, the in vivo primary targets and specific
modes of action of PolyI:C are still largely unknown, which hampers efficient translation to human of preclinical animal vaccination trials using this adjuvant. XCR1+ and CD1c+ bDC were strongly and uniquely activated by PolyI:C, but not by LPS, and conversely for MoDC. The differential responses of bDC versus MoDC to candidate adjuvants were nicely reproduced by the contrasting responses of in vitro–derived XCR1+ versus XCR1− MoDC in response to LPS. Hence, XCR1+ CD34-DC are a good in vitro model for XCR1+ bDC not only under steady-state conditions but also in terms of responses to adjuvants. The differences in the responses to adjuvants of bDC versus MoDC are largely cell intrinsic rather than being imprinted by their development in distinct environments through exposure to different growth factors and cytokines. Of note, however, regulation of a core set of hundreds of genes

**FIGURE 5.** Comparison of the genetic reprogramming of XCR1+ DC in response to PolyI:C and XCR1− DC or MoDC in response to LPS. Venn diagrams were performed on the data from Fig. 4E to compare the sets of genes induced (A) or repressed (B) in XCR1+ DC in response to PolyI:C and XCR1− DC or MoDC in response to LPS. Ingenuity pathway analyses were performed for each set of genes as defined by the different areas of the Venn diagrams to determine whether these lists were enriched for genes annotated for specific pathways. The results are shown as heat maps where the color intensity increases with statistical significance of the annotation enrichment. (C) A heat map showing the expression patterns of the genes responsible for the enrichment of the pathways NF-KB signaling and IFN signaling shown in (A). The red font identifies the genes previously reported to be part of the core transcriptomic signature commonly upregulated in mouse DC subsets and in human pDC or MoDC upon activation with a variety of microbial stimuli.
FIGURE 6. Cytokine production by CD34-DC subsets and MoDC. DC subsets were sorted and stimulated as described in Fig. 4. (A) Expression pattern of selected genes. Genes highlighted in bold green are 1) already expressed to higher levels in steady state XCR1+ CD34-DC or MoDC, as compared with XCR1+ CD34-DC (except for IL12B and IL18, which are not detected at steady state), 2) further induced specifically in XCR1+ CD34-DC or MoDC upon R848 or LPS stimulation, and 3) encoded for molecules with proinflammatory or matrix remodeling functions, except for IL-10, which is anti-inflammatory. Genes highlighted in bold red are induced to higher levels in PolyI:C-activated XCR1+ CD34-DC as compared with all the other conditions examined and encode for antiviral, antiapoptotic, or NK/T cell–stimulating molecules. (B) Secretion of selected cytokines. Culture supernatants were used for Luminex-based detection of 18 different analytes. Results from three to eight independent cultures for each DC subset are shown as individual points, with bar overlays indicating mean ± SD. Dotted lines in the graph show detection (bottom) or saturation (top) thresholds. Color codes for molecule names are the same as in (A).
FIGURE 7. Comparison of the responses to adjuvants of CD34-DC and bDC subsets. XCR1⁺ bDC and CD1c⁺ bDC were FACS sorted from the blood of adult healthy donors, stimulated in vitro under the same conditions described in Fig. 4 for CD34-DC subsets, and processed for microarrays. Data were analyzed together with those of CD34-DC subsets shown in Fig. 4. (A) PCA performed on the 2978 genes showing a fold change $\geq 2$ between at least two of the conditions studied. (B) Hierarchical clustering performed on the same set of genes as in (A), using Pearson/Ward as distance metric/linkage parameters. (C) A heat map showing the expression patterns of the genes bearing the most weight on cell type distribution along the PC1, PC2, and PC3 axes of the PCA illustrated in (A). (D) A heat map showing the expression patterns of PolyI:C-induced genes harboring contrasting expression patterns across conditions. Individual expression patterns of a few selected PolyI:C-induced genes are shown on Supplemental Fig. 3 as bar graphs of mean ± SD of relative linear expression values for each combination of DC subset/stimulus to better show the differential expression between conditions.
was observed in all optimally activated human DC subsets irrespective of stimuli, including NFKB or IFN-I target genes. These results extend to human XCR1+ DC, our previous observations on mouse DC subsets and human MoDC and pDC (52), and emphasizes the relevance of MoDC as a practical model to study the cell biology and molecular regulation underlying this conserved maturation subprogram.

XCR1+ DC and MoDC expressed strikingly different patterns of molecules involved in the orchestration of inflammatory responses and in cross-talk with NK or T cells. Resting XCR1- CD34-DC and MoDC expressed a much broader array of inflammatory molecules than XCR1+ CD34-DC and XCR1+ bDC, a difference that was further increased upon stimulation by LPS or R848. Resting XCR1+ CD34-DC and XCR1+ bDC expressed higher levels of genes critical for Ag processing and presentation, homing to lymph nodes or cross-talk with NK and T cells. In PolyI:C-activated XCR1+ CD34-DC, XCR1+ bDC, and eventually CD1c+ bDC, a specific secretion of IFN-λ was measured as well as the selective induction of genes involved in cross-talk with NK or T cells. Likewise to what was reported for XCR1+ bDC (8, 10), XCR1+ CD34-DC efficiently cross-presented a cell-associated Ag upon PolyI:C or R848 stimulation. This was not the case for XCR1+ CD34-DC and MoDC. Hence, MoDC may be poised for inducing inflammation, whereas XCR1+ CD34-DC and XCR1+ bDC might be endowed with a stronger potential for the activation of NK cells and T cells. However, some differences were observed between XCR1+ bDC and XCR1+ CD34-DC. XCR1+ bDC expressed higher levels of lysosomal genes at steady state (Supplemental Table I) and, in response to PolyI:C, induced more strongly many cytokine genes including IFNLs, IFNB1, IL12A, and IL23A (Supplemental Fig. 5). In contrast, XCR1+ CD34-DC expressed higher levels of mitotic genes (Supplemental Table I). This suggests that XCR1+ CD34-DC might not be completely differentiated.

In summary, our results demonstrated that XCR1+ CD34-DC and XCR1+ bDC are similar in their gene expression programs and functions not only under steady-state conditions but also upon stimulation. In particular, they mount strikingly broad but specific responses to TLR3 stimulation and are specialized in cross-presentation of cell-associated Ags. In contrast, XCR1+ CD34-DC resemble MoDC and are specialized in the modulation of inflammation. Our study demonstrated the feasibility of generating high numbers of bona fide XCR1+ DC in vitro from clinically relevant sources of CD34+ progenitors and demonstrated their unique responsiveness to the PolyI:C adjuvant, which represents a significant advance toward the potential use of human XCR1+ DC in the clinic.

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Activation of T lymphocytes by resting and stimulated CD34-DC subsets and MoDC. (A) Allogeneic CD4+ T cell activation. FS3T* CD34-DC subsets and MoDC were cocultured for 6 d with CFSE-labeled allogeneic CD4+ T cells. The percentages of CD4+ T cells showing CFSE dilution are shown on the left graphs and the fold changes in CD4+ T cell numbers on the right graphs as a function of stimuli. Results are shown as mean ± SD for three to four independent MLR cultures for each DC subset with three replicate wells per culture. To determine significance, a Wilcoxon matched-pairs signed rank test was performed. (B and C) Cross-presentation of a cell-associated Ag by FS36 CD34-DC subsets and by MoDC derived from adult peripheral blood monocytes. (B) Each DC subset was cocultured 24 h with N9V/OVA-expressing K562 cells with or without R848 or PolyI:C (ratio DC:tumor = 1:1) and tested for induction of IFN-γ production by an N9V-specific T cell clone as assessed by intra-cellular staining. (C) N9V peptide–pulsed DC subsets were used as a positive control for the activation of the N9V-specific T cell clone and to ensure that all DC subsets were viable and had a similar efficacy for direct presentation of an optimal MHC-I–restricted epitope. No cross-presentation was observed in absence of cognate Ag when using K562 cells transfected with a vector expressing GFP only (data not shown). Results are shown as individual percentages of IFN-γ–expressing T cells for four to six independent experiments for each DC subset, with mean values indicated by black horizontal bars. To determine significance, a Wilcoxon matched-pairs signed rank test was performed. *p < 0.05, **p < 0.01.


FIGURE S1. Comparison between the previously reported FS36 protocol and the new FS3T protocols for efficiency of XCR1⁺ CD34-DC differentiation.
(A) Three different culture protocols were designed to generate DC from human CD34⁺ cord blood cell cultures: FS36 cultures (Poulin et al. 2010), FS3T cultures (reported here for the first time to the best of our knowledge) and G4 cultures (Balan et al., 2010). Each culture consisted in a first step of progenitor expansion followed by a second step of DC differentiation. For expansion, the cytokine cocktails used were FLT3-L, SCF and IL-3 with either IL-6 or TPO for the FS36 and FS3T protocols respectively, and FLT3-L, SCF and TPO for the G4 protocol. For differentiation, FS36 and FS3T cultures used FLT3-L, SCF, GM-CSF and IL-4 (FSG4), while G4 cultures used GM-CSF and IL-4 on enriched CD14⁺ cells. (B) Identification of cell subsets in cultures of CD34⁺ cord blood progenitors based on the expression of CD11c and CD141, for FS36 cultures, similarly to what is shown for FS3T cultures on Fig. 1A. (C) Expression of selected markers on each of the cell subsets identified in FS36 cultures, similarly to what is shown for FS3T cultures on Fig. 1B and Fig. 2. For B-C, one representative result of at least 3 independent cultures is shown. (D-E) Percentages (D) and yields (E) of XCR1⁺ CD34-DC in different culture protocols, FS36, FS36 with addition of 5% autologous plasma during expansion (FS36A), FS3T with addition of 5% autologous plasma during expansion (FS3TA), FS3T with addition of 5% fetal bovine serum during expansion (FS3T), and FS3T with decreased cell density during expansion and reduced concentration of GM-CSF and IL-4 during differentiation (FS3T*). Four to twelve different CB samples were used in each condition.
FIGURE S2. XCR1⁺ versus XCR1⁻ CD34-DC subsets harbor similar gene expression profiles irrespective of their differentiation with the FS36 or FS3T protocols.
XCR1⁺ CD34-DC and the CD141⁺ versus CD141⁻ subsets of XCR1⁻ CD34-DC from FS36 cultures were FACS-sorted based on their expression patterns of CD11c and CD141 and processed for microarrays. Data were analyzed together with those of FS3T CD34-DC subsets shown in Fig. 1. (A) Hierarchical clustering performed on all ProbeSets, using Pearson/Ward as distance metric/linkage parameters. (B) Principal component analysis (PCA) performed on all ProbeSets. The right curve displays the distribution of the Eigenvectors coefficients of all ProbeSets for PC1 and PC2 from the PCA analysis illustrated in (B). The genes bearing the most weight on cell type distribution along the PCA axes were selected as associated with Eigenvector coefficients above or below the thresholds indicated on the graphs. (C) Heatmap showing the expression patterns of the “PC1” genes with the highest Eigenvector coefficients. (D) Heatmap showing the expression patterns of the “PC1” genes with the lowest Eigenvector coefficients. (E) Heatmap showing the expression patterns of the “PC2” genes with the highest Eigenvector coefficients. (F) Heatmap showing the expression patterns of the “PC2” genes with the lowest Eigenvector coefficients.
FIGURE S3. Individual expression patterns of a few selected PolyI:C-induced genes.
Bar graphs show the expression pattern of selected PolyI:C-induced genes, as relative linear expression values across conditions. For each gene, linear expression values for each sample were normalized to the maximal linear expression value across all samples and the mean±SD of 3 to 6 replicates is shown for each combination of DC subset/stimulus. Bar graphs are regrouped into panels according to the belonging of the corresponding genes to the clusters shown on Fig. 6D.
Supplemental Table Legend

TABLE S1 is an Excel spreadsheet giving the Eigenvector coefficients for the PC1 and PC2 axis for Figure 1D, and the results of DAVID gene enrichment annotations analyses on the lists of genes with extreme PC2 Eigenvector coefficients, the details of the samples used for microarray analysis of CD34-DC subset responses to adjuvants, the lists of genes up or down in stimulated CD34-DC subsets and MoDC with their expression values, as well as the lists the potential up-stream regulators of the core gene expression program induced commonly in PolyI:C-stimulated XCR1+ CD34-DC and in LPS-stimulated XCR1- CD34-DC and MoDC, with their gene targets.