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*J Immunol* 2014; 193:1622-1635; Prepublished online 9 July 2014;
doi: 10.4049/jimmunol.1401243
http://www.jimmunol.org/content/193/4/1622

Supplementary Material

[http://www.jimmunol.org/content/suppl/2014/07/09/jimmunol.1401243.DCSupplemental](http://www.jimmunol.org/content/suppl/2014/07/09/jimmunol.1401243.DCSupplemental)

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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 CD34- progenitor cultures (CD34-DC). Gene expression profiling, phenotypic characterization, and functional studies demonstrated that XCR1+ CD34-DC are similar to canonical MoDC, whereas XCR1+ CD34-DC resemble XCR1+ blood DC (bDC). XCR1+ DC were strongly activated by polyinosinic-polycytidylic acid but not LPS, and conversely for MoDC. XCR1+ DC and XCR1+ bDC expressed strikingly different patterns of molecules involved in inflammation and in cross-talk with NK or T cells. XCR1+ CD34-DC but not MoDC efficiently cross-presented a cell-associated Ag upon stimulation by polyinosinic-polycytidylic acid or R848, likewise to 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.

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Received for publication May 15, 2014. Accepted for publication June 11, 2014.

This work was supported by the I2HD CIML-SANOFI project, INSERM, Agence Nationale de la Recherche (EMICIF, ANR-08-MIEN-008-02; to M.D.), Agence Nationale de Recherches sur le SIDA et les Hépatites Virales (to M.D.), Institut National du Cancer Grant 2011-153 (to C. Caux, J.V.-G., and M.D.), Ligue contre la Cancer (Comité de la Savoie), Lyon Biopole (France), Association for the Recherche sur le Cancer (France), Fondation pour la Recherche Médicale (Equipe Labellisée à M.D.), and the European Research Council under the European Community’s Seventh Framework Programme (FP7/2007-2013 Grant Agreement 281225; to M.D.). V.O. is a laureate from a LabEx DEVSweCAN PhD fellowship. S.B. was supported through the Agence Nationale de la Recherche (EMICIF, Grant ANR-08-MIEN-008-02). S.B., J.V.-G., and M.D. conceived the project and designed the experiments; S.B., V.O., J.V.-G., and M.D. gathered funding; and J.V.-G. and M.D. supervised the project.

The online version of this article contains supplemental material. Abbreviations used in this article: bDC, blood DC; CB, cord blood; DC, dendritic cell; EFS, Etablissement Français du Sang; FLT-L3-I, FLT-L3-igand; GSEA, Genet Set Enrichment Analysis; LT-DC, lymphoid tissue–resident DC; MoDC, monocyte-derived DC; PCA, principal component analysis; pDC, plasmacytoid DC; PolycE, polyinosinic-polycytidylic acid; SCF, stem cell factor; TPO, thrombopoietin.

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between MoDC and lymphoid tissue–resident DC (LT-DC). Hence, major efforts are being made to better characterize human LT-DC and to evaluate their immunostimulatory 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 polyinosin-polycytidylic acid (PolyI:LC) by IFN-λ 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+ blood DC (bDC) excel at cross-presentation of cell-associated Ags (8–10, 18) and of particulate Ags delivered through FcγRs, through lysosomes (19, 20) or upon polyI:LC stimulation (8, 10, 23). Because they share unique characteristics with mouse XCR1+ DC, human XCR1+ bDC constitute a distinct human DC subset that may have potential clinical applications (24–26). To determine whether and how human XCR1+ DC could be harnessed in the clinic, it is necessary to better characterize them. This includes determining their global responses to adjuvants. A major bottleneck for such studies is the paucity of XCR1+ DC in human tissues. Generating high numbers of XCR1+ DC in vitro from CD34+ hematopoietic progenitors will help overcome this problem.

Human DC derived in vitro from monocytes or CD34+ progenitors have been widely used to investigate DC biology. Only MoDC (27) and Langerhans cells (LC) (28) have been generated reproducibly in vitro by many teams. Gene expression profiling and/or ontogenic studies showed that MoDC, Langerhans cell, and DC lines strikingly differ from LT-DC subsets and are more similar to monocytes or macrophages (4, 29–32). Cultures of CD34+ progenitors with FLT3-ligand (FLT3-L) or thrombopoietin (TPO) have been reported to yield pDC, CD141+CLEC9A+ DC, or CD1c+ DC (11, 33, 34). The extent to which the CD141+ CLEC9A+, the CD1c+, or the other HLA-DR+ cells in these cultures resembled human bDC subsets was, however, not thoroughly examined. In this study, we report a protocol generating high numbers of both XCR1+ DC and XCR1− DC in CD34+ progenitor cultures (CD34+DC), with a high-throughput characterization of these DC subsets by gene expression and secreted molecule profiling under steady-state and activation conditions and by comparison with XCR1+ bDC and MoDC.

### 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, Reseau Francais 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 in the information document. The protocol was reviewed by the Institut Paoli-Calmettes Comité d’Orientation Stratégique (COS or IRB). CD34+ cells were enriched magnetically by using the Dynal (Invitrogen) or Miltenyi Biotec CD34+ cell isolation kit. CD34+ cells from adult donor bone marrow or mobilized peripheral blood were purchased from properly licensed commercial companies (AllCells and StemCells).

**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 × 10^7 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 × 10^7 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 × 10^3 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 × 10^4 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 × 10^3 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 PBS, and acquired on a LSR II or Canto II flow cytometer (BD Biosciences). Human XCR1 was stained with a fluorescently coupled recombinant human chemokine ligand vacciodyce (rhXCL1-nCherry) (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 60 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+CD11c–CLEC4+CD141hi cells for XCR1+ DC, Lin–HLA-DR-CD1c+CD11c–CLEC4+CD141–cells for CD1c+ DC, and Lin–HLA-DR-CD1c+CD11c–CLEC4+CD141–low cells for pDC.

Cytoxin and Wright–Geimsa stainings

Cells were diluted at 10–15 × 10³ cells/50 µl and used for the cytoxin 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 cultures (data not shown). Sorted DC subsets were stimulated for 16 h with PolyLC (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-SPAT Process Transcript Amendment 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 Gene Chip 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 PolyI:C, R848, or LPS-stimulated samples to unstimulated controls for each DC subset of each individual CB culture tested. For hierarchical clustering and heat maps, 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 for TLR stimulations of CD34-DC subsets.

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, GM-CSF, and IL-4 (“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 CD11c+CD141hiHLA-DR+ cells (Fig. 1A, Table I, Supplemental Fig. 1B), which lacked expression of XCR1, CLEC9A, and CADM1 (Fig. 1B, Supplemental Fig. 1C) and were thus named XCR1–CD34-DC. The cultures also consistently encompassed CD11c–CD141hiHLA-DR+ cells (Fig. 1A, Supplemental Fig. 1B), which shared with XCR1+ bDC (36) specific expression of XCR1, CLEC9A, and CADM1 (Fig. 1B, Supplemental Fig. 1C). The XCR1+ CD34-DC were improved by lowering cell density and 10% FBS during the expansion phase. 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–Grünewald-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 |
|----------------|-----------------|-----------------|-----------------|
| Ag             | Fluorochrome    | Clone or Reagent| Suppliers       |
| CD1c           | PE              | AD5-8E7         | Miltenyi Biotec |
| CD1c           | PerCp           | L161            | BioLegend       |
| CD1a           | Allophycocyanin-| HI149           | BioLegend       |
| BDCA2          | PerCp           | 201A            | BioLegend       |
| BDCA2          | Biotin          | AC144           | Miltenyi Biotec |
| BDCA3          | FITC            | AD5-14H12       | Miltenyi Biotec |
| BDCA3          | Allophycocyanin | AD5-14H12       | Miltenyi Biotec |
| BDCA3          | PE              | V1 E013         | BD Bioscience   |
| lin mixture 3 (CD3,14, 19, 20) | FITC         | R&D Systems     |
| Clec9a         | Allophycocyanin | 683409          | MBL             |
| SystCAM/TSLC1/CADM1 | Purified | 3E1             | BD Biosciences  |
| CD11c          | V450            | B-ly6           | BD Biosciences  |
| CD11b          | FITC            | ICFR44          | eBioscience     |
| CD1b           | Allophycocyanin | eBioSN13(SN13 K5-1B8)| eBioscience |
| CD23           | PE              | EBVCS2          | eBioscience     |
| CD32           | Allophycocyanin | CD32            | eBioscience     |
| CD206          | PE-Cy7          | 19.2            | eBioscience     |
| CD209          | Allophycocyanin | eB-h209         | eBioscience     |
| CD86           | AF700           | 2331(FUN-1)     | BD Biosciences  |
| CD83           | Allophycocyanin | HB15e           | BD Biosciences  |
| HLADR          | AF700           | LN3             | eBioscience     |
| CD40           | PE              | MAB89           | Beckman Coulter |
| TLR3           | PE              | 34A3            | Innate Pharma   |
| TLR4           | PE              | HTA125          | eBioscience     |
| CD172          | Allophycocyanin | 15-414          | eBioscience     |
| CD103          | PE-Cy7          | B-lY7           | eBioscience     |
| CD14           | FITC            | M5E2            | BD Biosciences  |
| XCR1           | mCherry         | rhXCL1          | Homemade        |
| Secondary anti-chicken | FITC    | Polyclonal Ab   | Southern Biotechnology Associates |
| Biotin         | Allophycocyanin | Streptavidin    | BD Bioscience   |

the percentage of occurrence of this node among 1000 independent trees generated by multiscale bootstrap resampling. (F) BioLayoutExpress clustering of the same set of genes as used in (E). On the left, the size of the different clusters and their relationships are shown as tridimensional clouds with each gene represented as a node colored accordingly to the gene clusters to which it belongs. The calculation parameters used were a cutoff for Pearson correlation coefficient at 0.8 and an inflation value at 2. Only clusters encompassing ≥50 genes are represented. On the right, the average gene expression pattern across all cell types examined is shown for each cluster. (G) Heat maps showing the expression patterns for 20 individual genes selected from each cluster mostly based on prior knowledge of their selective expression in ex vivo–isolated bDC subsets (bold red for XCR1+ bDC-specific genes) or monocyte-derived inflammatory DC (bold green) and/or based on prior knowledge of their functions.
lateral 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 ontogenetic 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 immunomodulation 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 consistence 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 1). 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 1), 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-A 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 1), 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 crosspresent 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
the same extent (Fig. 4), it induced cross-presentation to significantly higher levels only in XCR1⁺ CD34-DC (Fig. 8B, 8C).

**Discussion**

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 PolyI:C. 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 immunoactivation 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, PolyI:C, and R848. Ag targeting to XCR1⁺ DC combined with PolyI:C 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
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⁻ CD34⁻DC. 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 where 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 NFκB 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 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.** 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 intracellular 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.
Acknowledgments

We thank Pierre Grenot, Marie-Laure Thibult, Marc Barad, Atika Zouine, and Isabelle Durand for technical support on cell sorting, Violaine Alunni and Christelle Thibaut-Carpenter from the Plate-forme Biopuces (Strasbourg, France) for performing the microarray experiments (www.microarrays.ustrasbg.fr), and Lise-Marie Daufresne and Julie Bernard for management of the CB bank.

Disclosures

E.P. and B.B. are inventors on a patent application on XCL1-targeted vaccinodies. I.P. and C.A.-P. are employees of Innate Pharma and are inventors on a patent application on anti-human TLR3 Abs. H.L., M.S., N.C., and T.S. are employees of SANOFI. The other authors have no financial conflicts of interest.

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