Production of large numbers of plasmacytoid dendritic cells with functional activities from CD34+ hematopoietic progenitor cells: Use of interleukin-3

Stéphanie Demoulin, Patrick Roncarati, Philippe Delvenne, and Pascale Hubert

Laboratory of Experimental Pathology, GIGA-Cancer (Center for Experimental Cancer Research), University of Liege, Liege, Belgium

Plasmacytoid dendritic cells (pDC), a subset of dendritic cells characterized by a rapid and massive type-I interferon secretion through the Toll-like receptor pathway in response to viral infection, play important roles in the pathogenesis of several diseases, such as chronic viral infections (e.g., hepatitis C virus, human immunodeficiency virus), autoimmunity (e.g., psoriasis, systemic lupus erythematosus), and cancer. As pDC represent a rare cell type in the peripheral blood, the goal of this study was to develop a new method to efficiently generate large numbers of cells from a limited number of CD34+ cord blood progenitors to provide a tool to resolve important questions about how pDC mediate tolerance, autoimmunity, and cancer. Human CD34+ hematopoietic progenitor cells isolated from cord blood were cultured with a combination of Flt3-ligand (Flt3L), thrombopoietin (TPO), and one of the following cytokine: interleukin (IL)-3, interferon-β, or prostaglandin E2. Cells obtained in the different culture conditions were analyzed for their phenotype and functional characteristics. The addition of IL-3 cooperates with Flt3L and TPO in the induction of pDC from CD34+ hematopoietic progenitor cells. Indeed, Flt3L/TPO alone or supplemented with prostaglandin E2 or interferon-β produced smaller amounts of pDC from hematopoietic progenitor cells. In addition, pDC generated in Flt3L/TPO/IL-3 cultures exhibited morphological, immunohistochemical, and functional features of peripheral blood pDC. We showed that IL-3, in association with Flt3L and TPO, provides an advantageous tool for large-scale generation of pDC. This culture condition generated, starting from 2×10^5 CD34+ cells, up to 2.6×10^6 pDC presenting features of blood pDC.   © 2012 ISEH - Society for Hematology and Stem Cells.   Published by Elsevier Inc.

Dendritic cells (DC) represent a major class of professional antigen-presenting cells characterized by their capacity to prime naïve T cells and to initiate primary immune responses [1]. In humans, two major lineages of DC can be distinguished based on differential developmental origins, phenotypes, and anatomical locations, myeloid DC (mDC) and plasmacytoid DC (pDC).

Human pDC represent a rare peripheral cell blood population (0.2−0.8%), which can be distinguished from other blood cells based on selective expression of surface antigens BDCA-2 [2,3] and ILT7 [4,5]. Immature pDC also express CD4, CD45RA, CD123, and BDCA-4, but lack expression of the lineage markers CD3, CD19, CD14, CD16, and DC marker CD11c. pDC represent key effectors in innate and adaptive immune responses. They selectively express two microbial pattern-recognition receptors (i.e., Toll-like receptor [TLR] 7 and TLR9) and play a major role in antiviral immunity by rapidly producing massive amounts of type-1 interferon (IFN-α/β) after viral stimulation through induction of the TLR pathway [6,7]. Other consequences of pDC activation include secretion of cytokines, such as tumor necrosis factor−α and interleukin (IL)-6, and acquisition of antigen presentation ability contributing to the recruitment/activation of other cell types, such as DC, natural killer cells, and T cells [8–10].

pDC fully develop in the bone marrow and migrate into T-cell−rich areas of lymphoid organs through high endothelial venules under steady-state conditions. However, under pathological conditions, pDC are recruited from lymphoid organs to peripheral tissues through the action of different chemokines and adhesion molecules [6,11–13].

pDC have attracted a growing interest in recent years and fundamental questions remain concerning their

Offprint requests to: Stéphanie Demoulin, X.X., Laboratory of Experimental Pathology, Bât.B23 Anatomie et Cytologie Pathologiques Tour 3 +4, Avenue de l'Hôpital 3, 4000 Liège 1, Belgium; E-mail: stephanie.demoulin@ulg.ac.be

regulation and activities, especially in tolerance, autoimmunity, and cancer. The obtention of pDC in large numbers has proven to be difficult, limiting progress in the understanding of their functions. Here, we describe a simple method for the large-scale generation of pDC exhibiting morphological, immunohistochemical, and functional features of pDC present in the peripheral blood from a limited number of CD34^{+} cord blood progenitors.

Several cytokines, such as IL-3, prostaglandin E2 (PGE$_2$), and IFN-β have been shown to be implicated in pDC development, proliferation, and/or survival. IL-3 induces the proliferation of pDC, inhibits their apoptosis [14,15], and has a proliferative effect on CD34^{+} progenitor cells [16–18]. CD34^{+} cells cultured with mesenchymal stem cells or their conditioned medium have been shown to increase the number of cells generated and the percentage of pDC in culture through PGE$_2$ production [19]. Buelens et al. showed that monocytes cultured in IL-3 and IFN-β give rise to a population of DC showing several characteristics of pDC. Those cells express high levels of CD123 and secreted high levels of IL-6 and tumor necrosis factor−α [20]. To determine the best method to generate high amounts of pDC in vitro, we added one of those cytokines (i.e., IL-3, IFN-β, or PGE$_2$) to Flt3 ligand (Flt3L)/thrombopoietin (TPO) CD34^{+} hematopoietic progenitor cells (HPC) culture as previous studies showed that TPO acts in synergy with Flt3L for the differentiation and the expansion of HPC [21].

We showed that the conditions Flt3L/TPO, Flt3L/TPO/PGE$_2$, and Flt3L/TPO/IFN-β induced generation of low numbers of pDC. In contrast, TPO, Flt3L, and IL-3 synergistically induce generation of $>2.6 \times 10^6$ pDC presenting characteristics of peripheral blood pDC from a small initial number of precursor cells (2×10^5 HPC) after 21 days in culture. These findings suggest that, unlike IFN-β and PGE$_2$, IL-3 might represent a key factor in controlling pDC development.

Materials and methods

Isolation of pDC from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte-enriched Buffy-coats by centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield, Oslo, Norway). After washing PBMC at low centrifugation speed to discard a maximum of platelets, pDC were sorted by using a negative cell sorting kit following manufacturer's instructions (Human Plasmacytoid DC Enrichment kit; StemCell Technologies, Vancouver, Canada).

Generation of pDC from CD34^{+} HPC

CD34^{+} HPC were isolated from human umbilical cord blood obtained during normal full-term deliveries. This study was approved by the Ethics Committee of the University Hospital of Liege and informed consent was obtained from donors. CD34^{+} HPC were recovered by Ficoll-Hypaque density-gradient centrifugation (Lymphoprep; Axis-Shield). CD34^{+} cells were isolated from other mononucleated cells using the MACS Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) and MiniMacs separation columns (Miltenyi Biotec), according to manufacturer's protocol. CD34^{+} cells were cultured in 24-well plates (Nunc, Roskilde, Denmark) at 2×10^5 cells/mL in RPMI medium supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol and 1% penicillin-streptomycin, sodium pyruvate, and nonessential amino acid and (all purchased from Invitrogen, Merelbeke, Belgium). To obtain pDC differentiation, TPO (50 ng/mL), Flt3L (100 ng/mL), and one of the following recombinant human cytokines: IL-3 (20 ng/mL), IFN-β (1000 U/mL), or PGE$_2$ (15 ng/mL), were added to the medium. All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA) except for PGE$_2$ (Cayman Chemicals, Ann Arbor, MI, USA). Cell cultures were refreshed every 3 days with RPMI medium containing designated cytokines. pDC were isolated by using the Human Plasmacytoid DC Enrichment kit (StemCell Technologies). In some experiments, pDC were stimulated during 24 hours with a CpG oligodeoxynucleotide (ODN) at 12 μg/ml (CpG ODN 2216: 5′-ggGGGACGATCGTCgggggg-3′; Eurogentec, Seraing, Belgium). **Q5**

Flow cytometry analysis

Flow cytometry studies were performed by using procedures published previously [22] with the following antibodies: CD123-FITC (clone AC145; Miltenyi Biotec), CD11c-allophycocyanin (clone B-ly6; BD Pharmingen, Franklin Lakes, NJ, USA), BDCA-4-phycoerythrin (PE) (clone AD5-17F6; Miltenyi Biotec), CD40-PE (clone 5C3; BD Pharmingen), CD83-PE (clone HB15e; BD Pharmingen), CD86-PE (clone c2331 (FUN-1); BD Pharmingen), CCR7-PE (clone 150503, R&D Systems, Minneapolis, MN, USA), and HLA-DR-PE (clone AB3; DAKO, Glostrup, Denmark). Fluorescence intensity and positive cell percentages were measured on a FACSCanto (Becton Dickinson, NJ, USA) and data were analyzed using FACSDiva software V 6.1.2 (Becton Dickinson) and FlowJo software (TreeStar, Ashland, OR, USA). **Q6 Q7**

Reverse transcription polymerase chain reaction analysis

One microgram total RNA extracted from cell cultures (RNeasy mini kit; Qiagen, Valencia, CA, USA) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) were performed using the following primer sequences: TLR9 F: ACTGGAGGTGGCCCCGGG; TLR9 R: CAGGGGTTGGGAGCGTGG; HPRT F: GTTGGATA TAGGCCAGACTTTG TTG; and HPRT R: CAGATGTTTCC AAACTCAACTTGAA (Eurogentec). The housekeeping gene HPRT was used as an internal control.

Cytokine production assays

Culture supernatants collected from pDC cultures were assessed for IFN-α levels by using an enzyme-linked immunosorbent assay kit according to manufacturer's instructions (PBL Interferon-Source, Piscataway, NJ, USA).

pDC chemotaxis assay

pDC migration was evaluated using a chemotaxis microchamber technique (48-well Boyden microchamber; Neuroprobe, Cabin John, MD, USA) [23]. Human recombinant chemerin (10 pM, 100 pM, 10 nM, or 100 nM; R&D Systems) was added to the lower wells of the chamber. A nonconditioned medium was used as

control for random migration. Conditioned medium of human fibroblasts was used as positive control. A polyvinylpyrrollidone-free polycarbonate membrane 5-μM gelatin-coated pore filter (Poretics Corp., Livermore, CA, USA) was placed in the micro-chamber. After cell sorting, 55 μL pDC suspension (2×10^6 cells/mL) was applied into the upper wells of the chamber. The chamber was incubated for 5 hours at 37°C. Cells having migrated to the underside of the filter were fixed and stained with Diff Quick Stain set (Baxter Diagnostics AG, Düdingen, Switzerland). The upper side of the filter was scraped to remove residual nonmigrating cells. One random field was counted per well using an eyepiece with a calibrated grid to evaluate the number of fully migrated cells.

Mixed lymphocyte reaction assay

The stimulator population consisted of pDC sorted from periph-eral blood or Flt3L/TPO/IL-3 culture. Those cells were irradiated at 5000 rads and placed in RPMI 5% human pooled AB serum. Varying numbers of stimulator cells (312−40,000 cells per well) were added to round-bottomed 96-well Nunclon plates containing 2×10^5 allogeneic PBMC per well. A proliferative response was measured after 5 days of culture by adding 1 μCi ^{3}H-thymidine to each well. Cells were harvested 18 hours later using an automated sample harvester (Packard, Canberra, Tilburg, The Netherlands) and counted using a liquid scintillation counter (Top Count, Pack-ard). DCs obtained by culturing adherent fraction of PBMC with IL-4 and granulocyte-macrophage colony-stimulating factor, as described previously [22,24], were used as positive control.

Morphology of pDC

Cytospins of pDC sorted from peripheral blood or Flt3L/TPO/IL-3 cultures were prepared by spinning (200 rpm, 3 minutes) 1×10^5 cells onto methanol-treated slides. May−Grünwald Giemsa stain-ing was performed using standard procedures and slides were examined using a FSX100 microscope (Olympus, Aartselaar, Belgium).

Statistical analysis

Statistical evaluation of the results was performed using unpaired Student's *t* test. Comparisons of means were studied by analysis of variance (ANOVA), followed by a Newman−Keuls multiple comparison test (one-way ANOVA) or a Bonferroni post-test (two-way ANOVA). ANOVA tests were performed on log-transformed data. Differences were considered as statistically significant when $p < 0.05$. Statistical tests were performed using the GraphPad Prism 5 software (Graph-Pad Software, La Jolla, CA, USA).

Results

IL-3 induces massive expansion of HPC and generation of large numbers of pDC from CD34$^+$ HPC cultivated in the presence of Flt3L and TPO

Previous works showed that TPO cooperates with Flt3L in the generation of pDC from CD34$^+$ HPC [21]. In this study, we tested the combinations of Flt3L (100 ng/mL) and TPO (50 ng/mL) with other growth factors that have been shown to be implicated in pDC development, proliferation, and/or
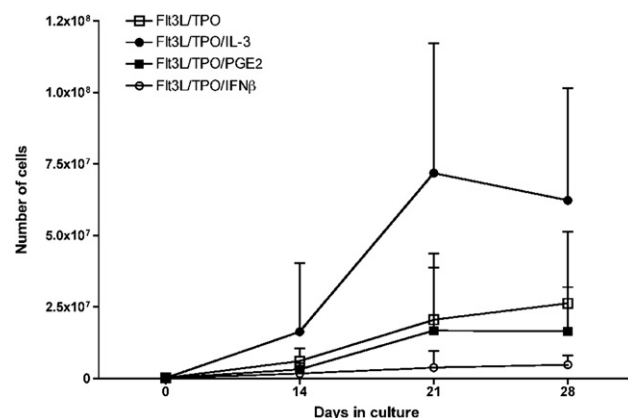

Figure 1. IL-3 in combination with Flt3L and TPO promotes the prolifer-ation of CD34$^+$ HPC. CD34$^+$ HPC were cultured with Flt3L and TPO alone or with IL-3, PGE$_2$, or IFN-β for 28 days. Total number of cells were counted and analyzed at days 14, 21, and 28. Data shown represent mean ± standard deviation of six independent experiments using different donors.

survival, such as IL-3 (20 ng/mL), IFN-β (1000 U/mL), and PGE$_2$ (15 ng/mL) [14,16,17,19,25].

In six different experiments, 2×10^5 CD34$^+$ HPC were incubated in media containing Flt3L/TPO alone or with IL-3, IFN-β, or PGE$_2$ for 28 days. Total numbers of generated cells were determined at periodic intervals between days 14 and 28. Interestingly, IL-3 together with Flt3L and TPO induced a massive expansion of cultured cell numbers from $1.6 \times 10^7 \pm 2.4 \times 10^7$ cells at day 14, to $7.2 \times 10^7 \pm 4.5 \times 10^7$ cells at day 21 (Fig. 1). ANOVA tests showed that the number of cells generated in Flt3L/TPO/IL-3 condition was significantly higher at day 21 ($p < 0.001$) and 28 ($p < 0.05$) compared with the number of cells generated at day 14 in that condition. In addition, statistical test also showed that, after 21 days of culture, the number of cells generated in Flt3L/TPO/IL-3 condition was significantly higher compared with all the other culture conditions tested (Flt3L/TPO, $p < 0.01$; Flt3L/TPO/PGE$_2$, $p < 0.05$; and Flt3L/TPO/IFN-β, $p < 0.001$). After 28 days of culture, numbers of cells generated in Flt3L/TPO/IL-3 condition was only significantly higher than the Flt3L/TPO/IFN-β ($p < 0.05$) condition.

In three independent experiments, we also analyzed the percentages of hematopoietic cell types present in our culture conditions. Cells were examined for their expres-sion of markers related to pDC (BDCA-4$^+$/CD123$^+$/CD11c$^-$), mDC (CD1a$^+$/CD11c$^+$), T cells (CD3$^+$), natural killer cells (CD3$^-$/CD16$^+$), B cells (CD19$^+$), granulocytes (CD3$^-$/CD11a$^+$/CD14$^+$ and CD3$^-$/CD11a$^+$/CD14$^-$) and monocytes (CD3$^-$/CD11a$^-$/CD14$^+$) by flow cytometry. We showed that mDC, T cells, and granulocytes were the main cell types produced in the different culture conditions (Fig. 2). By using an anti-CD34 antibody we also showed that only a small number of CD34$^+$ HPC remained in the culture medium after 21 days of culture, as in Flt3L/TPO,
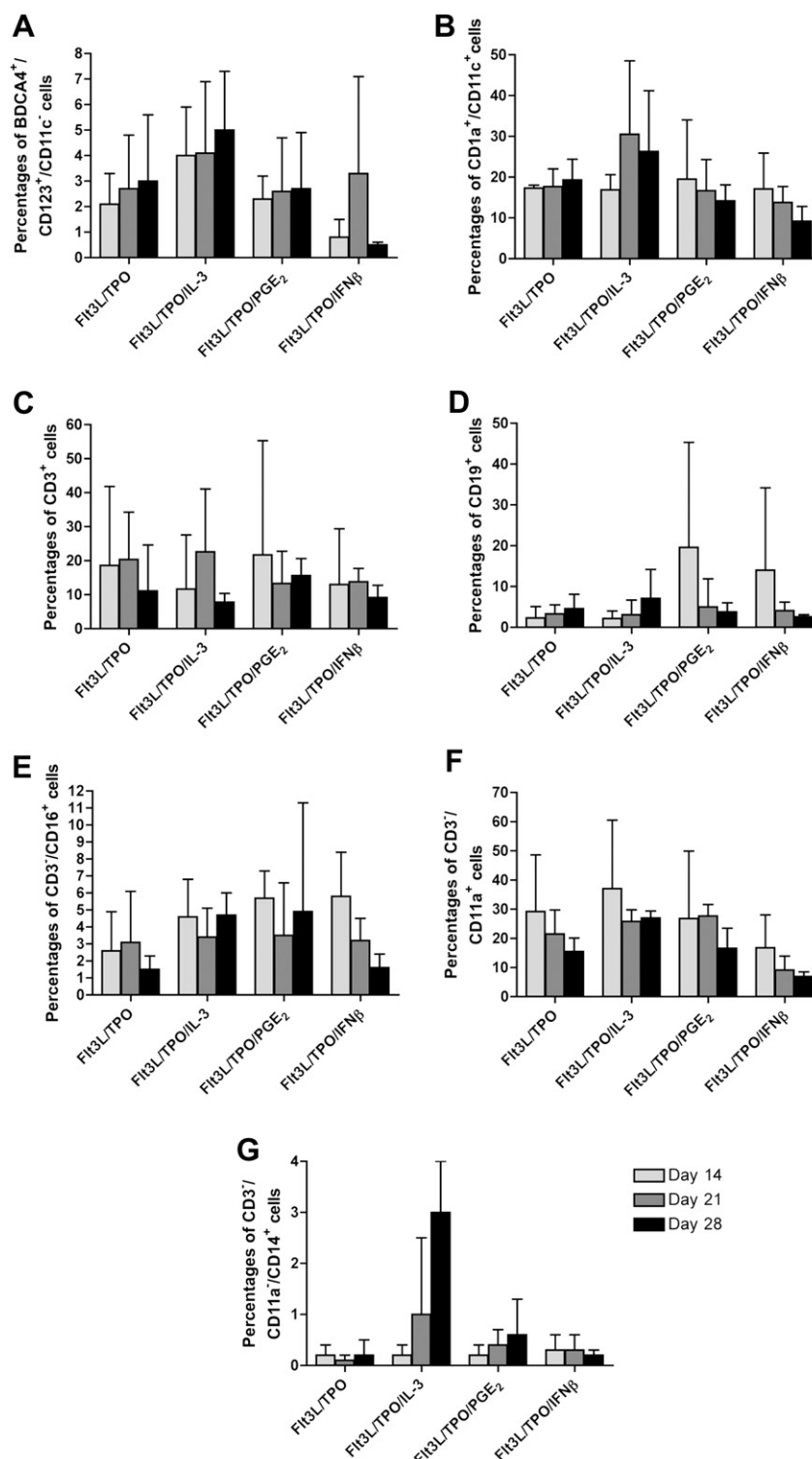
Figure 2. Various hematopoietic cell types are produced in Flt3L/TPO culture alone or supplemented with IL-3, PGE$_2$, or IFN-β. Percentages of pDC (BDCA-4$^+$/CD123$^+$/CD11c$^-$) (**A**), mDC (CD1a$^+$/CD11c$^+$) (**B**), T cells (CD3$^+$) (**C**), B cells (CD19$^+$) (**D**),natural killer cells (CD3$^-$/CD16$^+$) (**E**), granulocytes (CD3$^-$/CD11a$^+$/CD14$^+$ and CD3$^-$/CD11a$^+$/CD14$^-$) (**F**), and monocytes (CD3$^-$/CD11a$^-$/CD14$^+$) (**G**) in cultures, at days 14 (light gray), 21 (dark gray), and 28 (black), were determined by flow cytometry using their corresponding phenotypes. Data are from three different experiments using different donors and mean values are shown as percentages of positive cells ± standard deviation.

408 Flt3L/TPO/IL-3, Flt3L/TPO/PGE$_2$, and Flt3L/TPO/IFN-β
409 conditions, only up to 6.2%, 0.8%, 2%, and 1.3% of cells
410 were CD34$^+$ HPC, respectively (data not shown). At day
411 14, the maximum yield of pDC was observed using IL-3
412 in the culture medium. With this condition, 4% ± 1.9%
413 of cells showed a phenotype of fully differentiated pDC
414 (BDCA-4$^+$/CD123$^+$/CD11c$^-$). The range of increase
415 compared with pDC obtained at day 14 in other culture
416 conditions was from >170% to 500%. After long-term
417 cultures (28 days), the percentage of pDC increased and
418 remained the highest in Flt3L/TPO/IL-3 culture (Fig. 2A).
419   Next, we determined the number of immature pDC
420 based on their percentages in culture and showed that the
421 combination of Flt3L, TPO, and IL-3 induced an enhance-
422 ment in pDC generation. At 14, 21, and 28 days of culture,
423 the number of pDC was higher in Flt3L/TPO/IL-3 condi-
424 tion compared with the other culture conditions (Fig. 3).
425 The highest number of pDC was detected at day 21 in
426 Flt3L/TPO/IL-3 culture (2.6 × 10^6 ± 1.2 × 10^6) and the
427 number of pDC was significantly higher in Flt3L/TPO/IL-
428 3 compared with Flt3L/TPO (*p* < 0.05), Flt3L/TPO/
429 PGE$_2$ (*p* < 0.05), and Flt3L/TPO/IFN-β (*p* < 0.01)
430 cultures. These results are concordant with apoptotic tests
431 showing that the Flt3L/TPO/IL-3 condition presents the
432 highest percentages of living cells in culture. Proliferation
433 tests also showed that at day 14, 21, and 28 of culture,
434 cell proliferation was higher in Flt3L/TPO/IL-3 condition
435 than in the other conditions tested. Flt3L/TPO/PGE$_2$ condi-
436 tion was characterized by low percentages of proliferating
437 cells. In addition, IFN-β strongly blocked pDC generation
438 from HPC in Flt3/TPO culture, as this culture condition
439 was characterized by low percentages of proliferating cells
440 and high numbers of apoptotic cells (data not shown).
441   We showed that Flt3L/TPO/IL-3 condition was respon-
442 sible for the generation of the highest amount of pDC
443 in vitro. We therefore decided to study more extensively
444 cells obtained only with this condition. In addition, as
445 pDC numbers were the highest at day 21 in Flt3L/TPO/
446 IL-3 culture, subsequent studies were performed on pDC
447 obtained after 21 days of culture.

pDC derived from HPC in Flt3L/TPO/IL-3 culture
differentiate into mature pDC after activation by CpG
ODN
452 We performed a phenotypic analysis on pDC sorted from
453 Flt3L/TPO/IL-3 culture at day 21. After pDC isolation,
454 we showed that a majority of cells were CD11c$^-$. Only
455 2% of cells were CD11c$^+$. BDCA-4$^+$/CD123$^+$ pDC repre-
456 sented 25.7% of CD11c$^-$ cells. Interestingly, 11.2% of
457 CD11c$^+$ cells expressed the pDC markers BDCA-4$^+$ and
458 CD123$^+$ (Fig. 4A). Sorted-pDC were then cultured 24
459 hours with IL-3 to assure their survival and with CpG
460 ODN (a pDC stimulus recognized by their TLR9) to induce
461 their maturation. Phenotype analysis of sorted cells cultured
462 in medium complemented with IL-3 alone showed that the
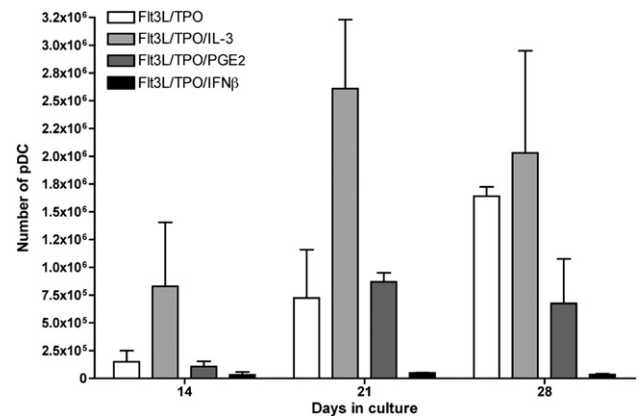
Figure 3. IL-3 induces the generation of large numbers of pDC from CD34$^+$ HPC cultivated with Flt3L and TPO. CD34$^+$ HPC were cultured with Flt3L and TPO alone (white) or with IL-3 (light gray), PGE$_2$ (dark gray), or IFN-β (black) for 28 days. Numbers of pDC generated at day 14, 21, and 28 in the different culture conditions were determined based on their percentages in culture. Data shown represent mean ± standard deviation of three independent experiments using different donors.

484 number of CD11c$^-$ and CD11c$^+$ cells expressing the
485 markers BDCA-4 and CD123 is increased in the presence
486 of IL-3 from 25.7% to 53.4% and from 11.2% to 33.4%,
487 respectively (Fig. 4B). Sorted cells cultured for 24 hours
488 with IL-3 and CpG ODN showed a higher increase of
489 BDCA-4$^+$/CD123$^+$/CD11c$^-$ (from 25.7% to 91.5%) and
490 BDCA-4$^+$/CD123$^+$/CD11c$^+$ (from 11.2% to 77.5%) cells
491 compared with sorted cells cultured with IL-3 alone
492 (Fig. 4C). These results demonstrated that BDCA-4$^+$
493 CD123$^+$ CD11c$^-$ cells are immature pDC that differentiate
494 into mature pDC expressing CD11c when cultured with
495 IL-3, with or without CpG ODN. However, the combination
496 of IL-3 and CpG ODN induced a higher number of mature
497 pDC than IL-3 alone.
498   We were also interested in determining whether non-
499 sorted pDC exposed to Flt3L, TPO, and IL-3 were capable
500 of maturation with CpG ODN. Nonsorted pDC generated in
501 Flt3L/TPO/IL-3 condition were assessed for their expres-
502 sion of maturation markers (CD40, CD83, CD86, HLA-
503 DR, CCR7, and CD11c) in the presence or absence of
504 CpG ODN. After 24 hours of activation by CpG ODN,
505 we showed that pDC stimulated with CpG ODN were
506 matured. The fluorescence intensity of all maturation
507 markers was increased in stimulated-pDC compared to non-
508 stimulated pDC (Fig. 5).

pDC generated in Flt3L/TPO/IL-3 cultures display
characteristics of peripheral blood pDC
512 In order to determine whether pDC generated in Flt3L/
513 TPO/IL-3 culture display characteristics of peripheral blood
514 pDC, we sorted pDC from Flt3L/TPO/IL-3 cultures and
515 from peripheral blood by magnetic bead isolation.
516   Sorted-pDC from Flt3L/TPO/IL-3 cultures (Fig. 6Aa)
517 exhibited typical peripheral blood pDC plasma cell
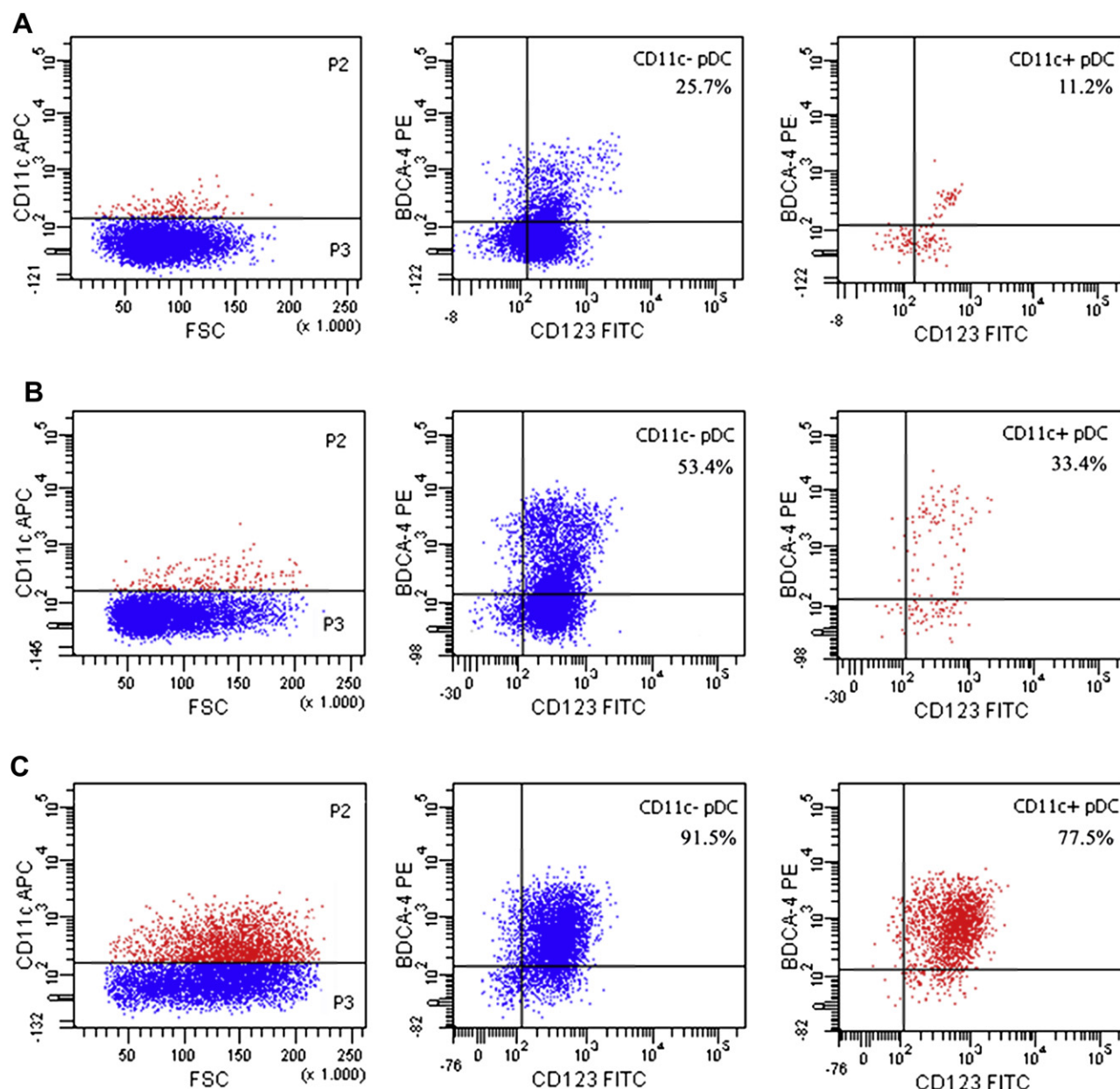
Figure 4. CD11c$^-$ pDC are immature cells and differentiate into mature pDC expressing CD11c when cultured with IL-3, with or without CpG ODN. Sorted-pDC from Flt3L/TPO/IL-3 cultures were analyzed for their expression of CD11c directly after their sorting (**A**) or 24 hours after maturation with IL-3, with (**C**) or without (**B**) CpG ODN. The values correspond to percentages of BDCA-4$^+$/CD123$^+$ pDC in the population of CD11c$^-$ (P3) and CD11c$^+$ cells (P2). Data are representative of three experiments showing similar results.

morphology (Fig. 6Ab) with a high nucleus−cytoplasm ratio on a May−Grünwald Giemsa−stained cytospin.

By using a mixed lymphocyte reaction, we showed that pDC generated in Flt3L/TPO/IL-3 culture as well as peripheral blood pDC have the capacity to provide accessory signals for an efficient proliferation of allogeneic T lymphocytes (Fig. 6B). pDC generated in culture or sorted from peripheral blood did not differ in their ability to stimulate an allogeneic response when the stimulator/responder ratio was the lowest (1:40 and 1:20), but the response was significantly more important for the population of pDC

generated in culture when the stimulator/responder ratio was higher (1:10 and 1:5) ($p < 0.05$). In addition, as it has already been shown [21,26], the stimulatory capacity of pDC was significantly less important ($p < 0.01$) than that of the mDC population used as control in the assay (Fig. 6B).

pDC function is associated with the expression of TLR different from that expressed by mDC [27]. We confirmed by quantitative real-time PCR that, unlike mDC, peripheral blood pDC and pDC produced in our culture system, express high levels of TLR9 messenger RNA (Fig. 6C).
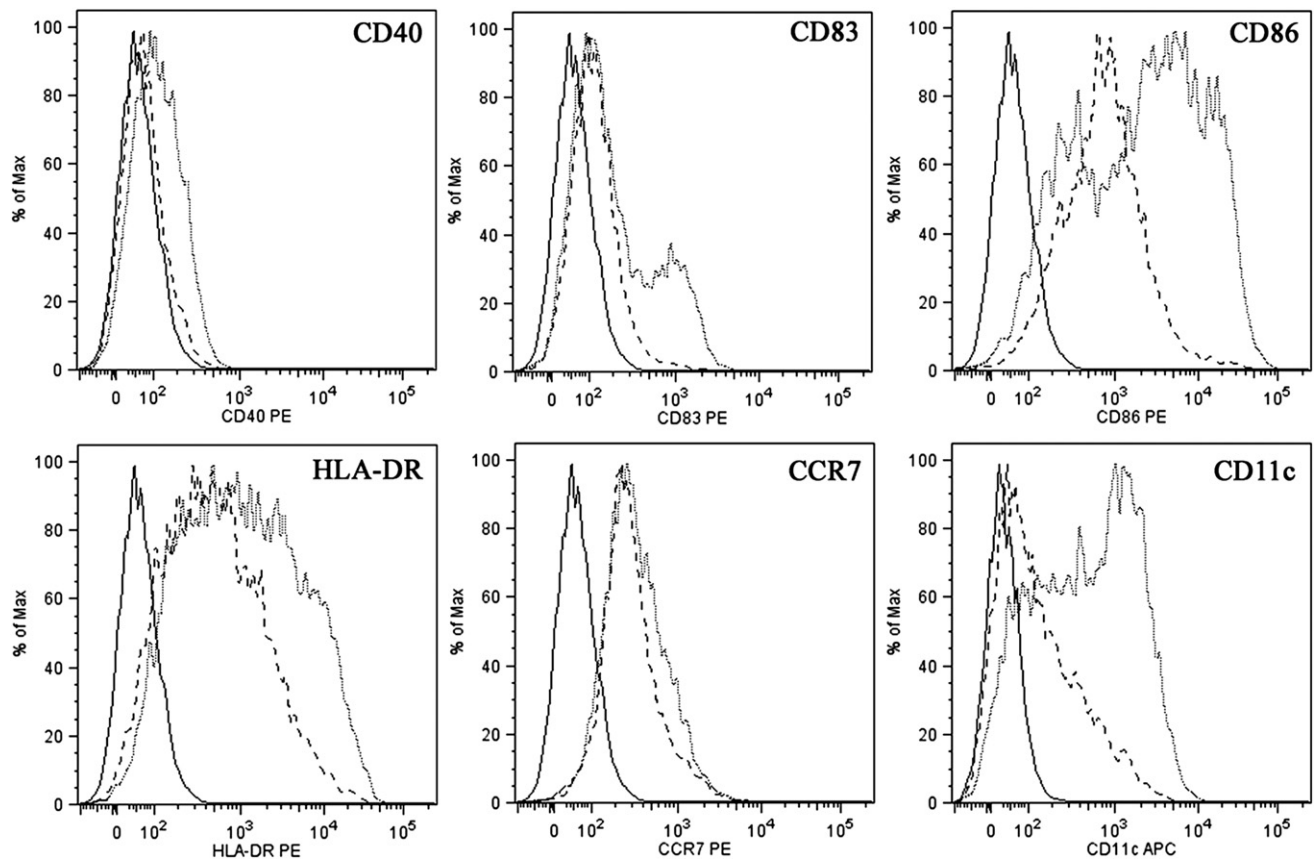
Figure 5. Immature pDC differentiate into mature pDC on activation by CpG ODN. Nonsorted pDC were analyzed for their expression of the maturation markers CD40, CD83, CD86, HLA-DR, CCR7, and CD11c after stimulation with CpG ODN for 24 hours. Fluorescence intensity (x-axis) is plotted against the percent of Max (y-axis), where the maximum y-axis value in absolute count becomes 100% of total. Patterns of isotype-matched control antibodies (full lines), unstimulated pDC (long dashed lines), and CpG ODN-stimulated pDC (dotted lines) are included in each histogram. Data are representative of six independent experiments showing similar results.

This messenger RNA expression was correlated to the TLR9 protein level detected in pDC by intracellular staining (Fig. 6D).

We also showed that in pDC sorted from the Flt3L/TPO/IL-3 culture, TLR9 stimulation by CpG ODN induces the secretion of high amounts of IFN-α (Fig. 6E) compared with unstimulated pDC.

As pDC migrate in response to chemerin due to their expression of CMKLR1 [28–30], we investigated the ability of pDC generated in Flt3L/TPO/IL-3 cultures to migrate in the presence of human recombinant chemerin by using a Boyden chamber assay. A significantly increased migration of pDC was observed in the presence of human fibroblasts media ($p < 0.001$) compared with a nonconditioned medium used as control. We also showed that different concentrations of chemerin (100 pM, 10 nM, and 100 nM) induced a significantly increased migration of pDC, with a peak observed at 10 nM, compared with the nonconditioned medium (Fig. 6F).

These results demonstrated that pDC generated in vitro have characteristics and functional properties similar to those of peripheral blood pDC.

Discussion

Because of their unique ability to secrete IFN-α and their central roles in innate and adaptive immunity, pDC have attracted growing interest for several years. pDC investigation is also of potential interest because of their implication in the pathogenesis of several diseases in which they often present a pathogenic/tolerogenic role mainly related to either the increase or the reduction of their function [13]. Sustained overproduction of type-I IFN by pDC in response to host-derived self-nucleic acid is associated with the development of autoimmunity in systemic lupus erythematosus [31] and **Q11** psoriasis [32]. On the other hand, pDC have also been shown to present a limited responsiveness to certain viruses (e.g., hepatitis C virus and human immunodeficiency virus) **Q12** [33,34]. In addition, in the microenvironment of certain tumors [35–37], pDC are often responsible for the development of a immunosuppressive/tolerogenic response [38].

Although the function and clinical manipulation of pDC have become topics of great interest, progress in the understanding of pDC is limited by their low percentage in peripheral blood and the difficulty producing large quantities of these cells in culture. Several groups have already
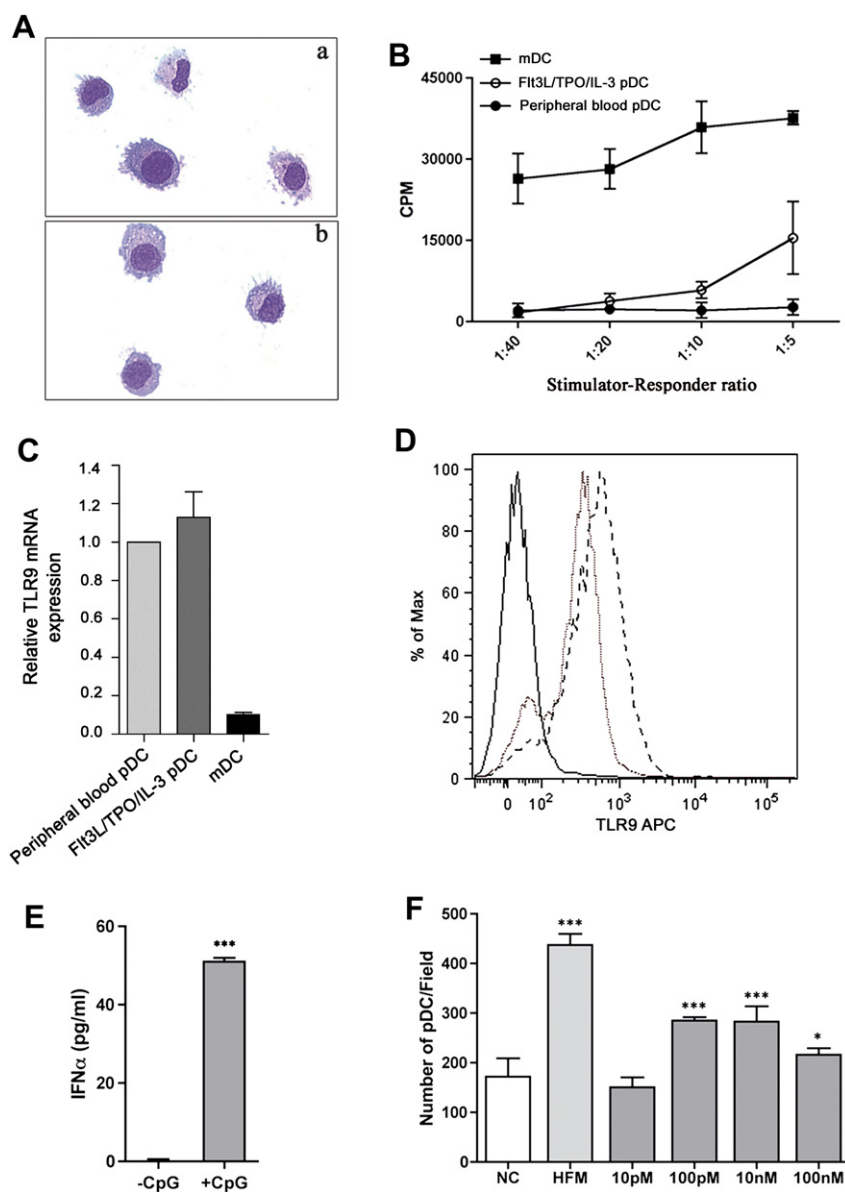
Figure 6. pDC generated with Flt3L/TPO/IL-3 cultures present several characteristics of peripheral blood pDC. (**A**) pDC morphology on a cytospin (May−Grünwald Giemsa coloration). (**a**) Sorted pDC from a Flt3L/TPO/IL-3 culture at day 21, (**b**) sorted pDC from peripheral blood. Magnification × 400. (**B**) Mixed lymphocyte reaction of sorted pDC from peripheral blood or generated in Flt3L/TPO/IL-3 cultures with allogeneic T lymphocytes. Responder cells: lymphocytes T; stimulator cells: pDC. mDC were used as positive control. Data represent mean ± standard deviation of three independent experiments using different donors. (**C**) Real-time quantitative analysis of TLR9 messenger RNA expression in CpG ODN-stimulated pDC generated in Flt3L/TPO/IL-3 culture (dark gray), peripheral blood pDC (light gray), and mDC (black). Data represent mean ± standard deviation of three independent experiments. (**D**) TLR9 protein expression analysis on CpG ODN-stimulated pDC generated in Flt3/TPO/IL-3 culture or sorted from peripheral blood. Fluorescence intensity (x-axis) is plotted against the percent of Max (y-axis). Patterns of isotype-matched control antibody (full line), stimulated-pDC generated in Flt3L/TPO/IL-3 culture (long dashed line) and stimulated-pDC sorted from peripheral blood (dotted line) are included in the histogram. (**E**) Secretion level of IFN-α with (+CpG) or without (−CpG) CpG ODN in culture supernatants of sorted pDC from Flt3L/TPO/IL-3 culture. Data represent mean ± standard deviation (***$p < 0.001$) of three independent experiments using different donors. (**F**) Influence of recombinant chemerin on pDC migration in a Boyden chamber assay. HFM = conditioned medium of human fibroblasts (positive control); NC = nonconditioned medium (negative control). NC was supplemented with four different concentrations (10 pM, 100 pM, 10 nM, and 100 nM) of human recombinant chemerin. Data represent mean ± standard deviation (*$p < 0.05$; ***$p < 0.001$) of three independent experiments using different donors.

induced the differentiation of pDC in vitro from CD34^{+} HPC [14,19,21,39,40]. However, these approaches generated relatively small amounts of pDC. Here, we describe an original procedure using a combination of Flt3L, TPO,

and IL-3 for large-scale generation of pDC exhibiting morphological, immunohistochemical, and functional features of pDC present in the peripheral blood from a limited number of CD34^{+} HPC.

Originally, pDC were thought to be in the lymphoid lineage. Interestingly, recent studies indicate that these cells have diverse origins and can develop from lymphoid or myeloid precursors [41,42]. Even if the origin and affiliation of pDC is controversial, partly because pDC show features of lymphocytes and DC, clear evidences indicate that Flt3L and TPO might represent major regulators of pDC development. First, previous reports showed that Flt3L is responsible for development of pDC in vitro [21,39,43,44] and in vivo [45]. Interestingly, mice and human subjects injected with Flt3L have an increase in pDC number [46–48]. Second, TPO has been shown to support the proliferation and long-term expansion of HPC and to induce their differentiation into pDC in synergy with Flt3L [21,49,50]. As IL-3 was reported to induce the proliferation of pDC, to inhibit their apoptosis [14,15] and to have a proliferative effect on $CD34^+$ HPC in vitro [16–18], we postulated that this cytokine in combination with Flt3L and TPO could have an impact on pDC generation from $CD34^+$ HPC in culture. Other cytokines (e.g., IFN-β and PGE_2) potentially implicated in pDC development and proliferation [19,20,25] were also tested in combination with Flt3L and TPO for their induction of pDC from $CD34^+$ HPC.

We showed that IL-3 together with Flt3L and TPO allowed the generation after 21 days of culture of $>2.6 \times 10^6$ pDC from 2×10^5 HPC. Flt3L/TPO alone or in combination with PGE_2 induced the generation of smaller amounts of pDC compared with Flt3L/TPO/IL-3 culture. At day 21, the combination of Flt3L, TPO, and IL-3 was associated with a mean increase of 152% and 158% in pDC production, compared with a culture without IL-3 or with a culture where IL-3 was replaced by PGE_2, respectively. In addition, the addition of IFN-β to Flt3L/TPO culture induced a very low proliferation of HPC, high number of apoptotic cells, and inhibited pDC differentiation. Our results indicate that, in contrast to PGE_2 and IFN-β, IL-3 is an efficient cofactor in triggering development and survival of human pDC from $CD34^+$ HPC. The effects of IFN-β on pDC production could be progenitor specific. Indeed, the poor pDC differentiation/proliferation effect of IFN-β on $CD34^+$ cells might be due to the fact that this cytokine only acts on monocytes to induce their differentiation in DC with pDC characteristics as shown by Buelens et al. [20] and Huang et al. [25].

Interestingly, we demonstrated that $CD11c^-$ pDC sorted from our culture system at day 21 and stimulated with IL-3 alone or with IL-3 and CpG ODN acquire the expression of the CD11c marker. This implies that IL-3 presents the ability to induce the maturation of a fraction of pDC, as already shown by Grouard et al. [15] and Defays et al. [51]. It must be emphasized that $CD11c^+$ cells maintain their expression of the pDC markers BDCA-4 and CD123 and do not differentiate into DC. Even if the manipulation of a pure population of immature pDC might require cell sorting after their generation, our culture system only requires a small quantity (2×10^5) of HPC, allowing functional studies on large quantities of pDC coming from the same donor and does not require pooling pDC obtained from different patients due to their very low frequency in the peripheral blood. We found that $BDCA-4^-/CD123^-$ and $BDCA-4^-/CD123^+$ cells sorted from cultures might be pDC precursors, as a fraction of those cells differentiated into $BDCA4^+/CD123^+$ pDC in the presence of IL-3 alone or in combination with CpG ODN. We also observed an increased expression of the BDCA-4 and CD123 markers in the pDC population after their maturation with CpG ODN and IL-3 when cells were not sorted (data not shown). This implies that although cells generated in culture have been exposed to IL-3 for 21 days, they maintain their capacity to be matured by CpG ODN.

Cytometry analysis showed that stimulated-pDC obtained in Flt3L/TPO/IL-3 cultures exhibit a matured phenotype as shown by the increased fluorescence intensity of their maturation markers HLA-DR, CD83, CD86, CD40, CCR7, and CD11c. This full pDC matured phenotype was obtained with an IL-3 concentration of 20 ng/mL in culture. We also tested two other concentrations of IL-3 (40 ng/mL and 60 ng/mL) in our Flt3L/TPO culture system in order to determine if we could obtain more benefit for pDC generation or shorten the time frame needed for successful pDC generation. Our results showed that even if we obtained more pDC at days 14 and 21 of culture with 60 ng/mL than with 20 ng/mL of IL-3, the use of 20 ng/mL IL-3 is more appropriate to generate pDC, which could be used to perform functional studies, as pDC generated with 60 ng/mL IL-3 could not be fully matured by CpG ODN in culture (data not shown).

pDC generated in culture displayed characteristics of peripheral blood pDC as demonstrated by their plasma cell morphology, their stimulation of allogeneic T lymphocytes proliferation in a mixed lymphocyte reaction assay, and their expression of TLR9 messenger RNA. We also showed that pDC generated in the Flt3L/TPO/IL-3 condition keep, after 21 days of culture, their main characteristic, including the secretion of IFN-β, after their stimulation with CpG ODN. Also, pDC generated in culture were chemoattracted in a Boyden chamber assay by chemerin, their chemoattractant.

In conclusion, we have described a simple and reproducible method for the generation, from small amounts of progenitor cells, of a large number of cells with phenotypical, structural, and functional features of pDC. The number of pDC was twice as important as the maximal number of pDC generated in a previous report using a combination of Flt3L and TPO [21]. This ratio is still underestimated, as Chen et al. used a double staining (HLA-DR/CD123) to identify pDC, which is less specific for pDC than the double staining BDCA-4/CD123 used in this study. Indeed, in the Flt3L/TPO/IL3 culture system, the number of

CD123$^+$ cells was 1.7 fold more important than the number of BDCA4$^+$/CD123$^+$ cells.

Achievement of generation of high amounts of pDC in vitro opens up an exciting possibilities for the initiation of functional studies allowing for better comprehension of pDC regulation and function in homeostatic and pathological conditions, and also for the development of therapeutic approaches targeting human pDC.

Funding disclosure

Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

1. Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18:767–811.
2. Dzionek A, Sohma Y, Nagafune J, et al. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med. 2001;194:1823–1834.
3. Dzionek A, Fuchs A, Schmidt P, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol. 2000;165:6037–6046.
4. Rissoan MC, Duhen T, Bridon JM, et al. Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. Blood. 2002;100:3295–3303.
5. Cho M, Ishida K, Chen J, et al. SAGE library screening reveals ILT7 as a specific plasmacytoid dendritic cell marker that regulates type I IFN production. Int Immunol. 2008;20:155–164.
6. Reizis B, Bunin A, Ghosh HS, Lewis KL, Sisirak V. Plasmacytoid dendritic cells: recent progress and open questions. Annu Rev Immunol. 2011;29:163–183.
7. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu Rev Immunol. 2005;23:275–306.
8. Swiecki M, Colonna M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. Immunol Rev. 2010;234:142–162.
9. Zhang Z, Wang FS. Plasmacytoid dendritic cells act as the most competent cell type in linking antiviral innate and adaptive immune responses. Cell Mol Immunol. 2005;2:411–417.
10. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol. 2008;8:594–606.
11. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. Nat Immunol. 2004;5:1219–1226.
12. McKenna K, Beignon AS, Bhardwaj N. Plasmacytoid dendritic cells: linking innate and adaptive immunity. J Virol. 2005;79:17–27.
13. Sozzani S, Vermi W, Del Prete A, Facchetti F. Trafficking properties of plasmacytoid dendritic cells in health and disease. Trends Immunol. 2010;31:270–277.
14. Encabo A, Solves P, Mateu E, Sepulveda P, Carbonell-Uberos F, Minana MD. Selective generation of different dendritic cell precursors from CD34+ cells by interleukin-6 and interleukin-3. Stem Cells. 2004;22:725–740.
15. Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J Exp Med. 1997;185:1101–1111.
16. Saeland S, Caux C, Favre C, et al. Combined and sequential effects of human IL-3 and GM-CSF on the proliferation of CD34+ hematopoietic cells from cord blood. Blood. 1989;73:1195–1201.
17. Kawano Y, Takaue Y, Hirao A, et al. Synergistic effect of recombinant interferon-gamma and interleukin-3 on the growth of immature human hematopoietic progenitors. Blood. 1991;77:2118–2121.
18. Astori G, Malangone W, Adami V, et al. A novel protocol that allows short-term stem cell expansion of both committed and pluripotent hematopoietic progenitor cells suitable for clinical use. Blood Cells Mol Dis. 2001;27:715–724. discussion 725–717.
19. Chen L, Zhang W, Yue H, et al. Effects of human mesenchymal stem cells on the differentiation of dendritic cells from CD34+ cells. Stem Cells Dev. 2007;16:719–731.
20. Buelens C, Bartholome EJ, Amraoui Z, et al. Interleukin-3 and interferon beta cooperate to induce differentiation of monocytes into dendritic cells with potent helper T-cell stimulatory properties. Blood. 2002;99:993–998.
21. Chen W, Antonenko S, Sederstrom JM, et al. Thrombopoietin cooperates with FLT3-ligand in the generation of plasmacytoid dendritic cell precursors from human hematopoietic progenitors. Blood. 2004;103:2547–2553.
22. Hubert P, Greimers R, Franzen-Detrooz E, et al. In vitro propagated dendritic cells from patients with human-papilloma virus-associated preneoplastic lesions of the uterine cervix: use of Flt3 ligand. Cancer Immunol Immunother. 1998;47:81–89.
23. Herfs M, Herman L, Hubert P, et al. High expression of PGE2 enzymatic pathways in cervical (pre)neoplastic lesions and functional consequences for antigen-presenting cells. Cancer Immunol Immunother. 2009;58:603–614.
24. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med. 1994;179:1109–1118.
25. Huang YM, Hussien Y, Yarilin D, Xiao BG, Liu YJ, Link H. Interferon-beta induces the development of type 2 dendritic cells. Cytokine. 2001;13:264–271.
26. Kohrgruber N, Halanek N, Groger M, et al. Survival, maturation, and function of CD11c- and CD11c+ peripheral blood dendritic cells are differentially regulated by cytokines. J Immunol. 1999;163:3250–3259.
27. Hornung V, Rothenfusser S, Britsch S, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 2002;168:4531–4537.
28. Zabel BA, Silverio AM, Butcher EC. Chemokine-like receptor 1 expression and chemerin-directed chemotaxis distinguish plasmacytoid from myeloid dendritic cells in human blood. J Immunol. 2005;174:244–251.
29. Vermi W, Riboldi E, Wittamer V, et al. Role of ChemR23 in directing the migration of myeloid and plasmacytoid dendritic cells to lymphoid organs and inflamed skin. J Exp Med. 2005;201:509–515.
30. Skrzeczynska-Moncznik J, Wawro K, Stefanska A, et al. Potential role of chemerin in recruitment of plasmacytoid dendritic cells to diseased skin. Biochem Biophys Res Commun. 2009;380:323–327.
31. Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. Am J Pathol. 2001;159:237–243.
32. Nestle FO, Conrad C, Tun-Kyi A, et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med. 2005;202:135–143.

33. O'Brien M, Manches O, Sabado RL, et al. Spatiotemporal trafficking of HIV in human plasmacytoid dendritic cells defines a persistently IFN-alpha-producing and partially matured phenotype. J Clin Invest. 2011;121:1088–1101.

34. Lai WK, Curbishley SM, Goddard S, et al. Hepatitis C is associated with perturbation of intrahepatic myeloid and plasmacytoid dendritic cell function. J Hepatol. 2007;47:338–347.

35. Zou W, Machelon V, Coulomb-L'Hermin A, et al. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. Nat Med. 2001;7:1339–1346.

36. Bekeredjian-Ding I, Schafer M, Hartmann E, et al. Tumour-derived prostaglandin E and transforming growth factor-beta synergize to inhibit plasmacytoid dendritic cell-derived interferon-alpha. Immunology. 2009;128:439–450.

37. Treilleux I, Blay JY, Bendriss-Vemare N, et al. Dendritic cell infiltration and prognosis of early stage breast cancer. Clin Cancer Res. 2004; 10:7466–7474.

38. Hirsch I, Caux C, Hasan U, Bendriss-Vermare N, Olive D. Impaired Toll-like receptor 7 and 9 signaling: from chronic viral infections to cancer. Trends Immunol. 2010;31:391–397.

39. Olivier A, Lauret E, Gonin P, Galy A. The Notch ligand delta-1 is a hematopoietic development cofactor for plasmacytoid dendritic cells. Blood. 2006;107:2694–2701.

40. Blom B, Ho S, Antonenko S, Liu YJ. Generation of interferon alpha-producing predendritic cell (Pre-DC)2 from human CD34(+) hematopoietic stem cells. J Exp Med. 2000;192:1785–1796.

41. Reizis B. Regulation of plasmacytoid dendritic cell development. Curr Opin Immunol. 2010;22:206–211.

42. Karsunky H, Merad M, Mende I, Manz MG, Engleman EG, Weissman IL. Developmental origin of interferon-alpha-producing dendritic cells from hematopoietic precursors. Exp Hematol. 2005;33:173–181.

43. Gilliet M, Boonstra A, Paturel C, et al. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. J Exp Med. 2002;195:953–958.

44. Onai N, Obata-Onai A, Tussiwand R, Lanzavecchia A, Manz MG. Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. J Exp Med. 2006;203:227–238.

45. Maraskovsky E, Daro E, Roux E, et al. In vivo generation of human dendritic cell subsets by Flt3 ligand. Blood. 2000;96:878–884.

46. Maraskovsky E, Brasel K, Teepe M, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med. 1996;184:1953–1962.

47. Bjorck P. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. Blood. 2001;98:3520–3526.

48. Pulendran B, Banchereau J, Burkeholder S, et al. Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets in vivo. J Immunol. 2000;165:566–572.

49. Piacibello W, Sanavio F, Garetto L, et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood. 1997;89:2644–2653.

50. Arrighi JF, Hauser C, Chapuis B, Zubler RH, Kindler V. Long-term culture of human CD34(+) progenitors with FLT3-ligand, thrombopoietin, and stem cell factor induces extensive amplification of a CD34(-)CD14(-) and a CD34(-)CD14(+) dendritic cell precursor. Blood. 1999;93:2244–2252.

51. Defays A, David A, de Gassart A, et al. BAD-LAMP is a novel biomarker of non-activated human plasmacytoid dendritic cells. Blood. 2011;118:609–617.

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