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Purification of circulating plasmacytoid dendritic cells using counterflow centrifugal elutriation and immunomagnetic beads

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Abstract

Background aims. Plasmacytoid dendritic cells (pDC) are a dendritic cell (DC) subset specialized in the production of high amounts of interferon (IFN) type I (IFN-α, -β) in response to viruses. They can be purified from peripheral blood mononuclear cells (PBMC), usually using magnetic bead sorting. *Methods.* In this study, we set up a counterflow centrifugal elutriation (CCE) procedure to enrich pDC from PBMC. We first analyzed each CCE fraction for the presence of pDC using CD123 and BDCA-2 as markers. We then purified pDC using CCE and magnetic beads and verified that their functions were not affected by this procedure. *Results.* pDC were sorted by CCE into intermediate fractions between those containing lymphocytes and monocytes. The pDC frequency in these intermediate fractions was 3-fold that in PBMC. Using negative-magnetic bead sorting, starting with the same number of cells and beads, we obtained more than twice as many pDC from intermediate fractions as from PBMC. The phenotypes and IFN-α production capacities of sorted pDC from PBMC and from intermediate fractions were similar, both immediately after sorting and after stimulation with CpG-A oligodeoxynucleotides. In addition, we showed that intermediate fractions could be cryopreserved and that magnetic bead sorting could be performed with the same efficiency after thawing. *Conclusions.* Altogether, our results show that CCE can be used to enrich lymphocytes, monocytes and pDC from the same donor, without magnetic beads on their surface. Our method should be useful for the purification of these cells for experimental research and may also be adaptable for clinical use in immunotherapy.

Key Words: *counterflow centrifugal elutriation, monocytes, negative-magnetic bead sorting, plasmacytoid dendritic cells*

Introduction

During the last decade, since their clear identification as professional, type I, interferon (IFN)-producing cells, human plasmacytoid dendritic cells (pDC) have been studied extensively (1). pDC are characterized by the expression of CD4, CD123 and the c-type lectin BDCA-2, and, contrary to myeloid DC, by a lack of CD11c expression (2). They produce huge amounts of type I IFN (IFN-α, -β and -ω) in response to viruses such as Herpes Simplex Virus (HSV) (1), influenza (3) and Human Immunodeficiency Virus (HIV) (4). After contact with viruses and infected cells, they are able to present and cross-present viral antigens to amplify antiviral T-cell responses (5,6). For these types of studies, pDC are usually purified from blood; however, their frequency is relatively low among human peripheral blood mononuclear cells (PBMC) (*c.* 0.4%). pDC were initially purified by negative-magnetic bead sorting combined with fluorescence-activated cell sorting (1). A commercial, positive-magnetic bead-enrichment kit, based on the expression of BDCA-2, and negative-magnetic bead-enrichment kits are currently available for pDC purification, but these methods have the disadvantage that the magnetic beads attach to some of the cells (pDC with the positive cell sorting, other cell subsets with the negative cell sorting).

Counterflow centrifugal elutriation (CCE) is a technology based on differences in sedimentation velocity, which is used to separate cells according to their size and density (7). In research laboratories, monocyte purification (>90% $CD14^+$) from human blood mononuclear cells can be easily and routinely performed using a semi-closed system

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(8). This method has been shown to result in high purity, good recovery and excellent viability and function of 'untouched' monocytes (not stained with magnetic beads). A clinical-grade closed system has been developed for the isolation of large numbers of monocytes by leukapheresis for monocyte-derived DC vaccines (9). The enrichment of lymphocytes for immunotherapy using CCE has also been described (10). CCE can not only be used to purify monocytes and lymphocytes, but also to enrich rare blood cell populations such as blood dendritic cells (DC) (11) or circulating tumor cells (12). For these rare blood cell populations, an additional immunologic-based selection is required for further purification. The advantage of CCE is that it allows purification of different subsets of cells without the attachment of beads.

In this study, we investigated whether CCE could be used to enrich pDC from PBMC obtained after platelet apheresis. Such a procedure would allow the mechanical removal of a large proportion of unwanted PBMC and the use of a smaller quantity of immunologic reagents to purify this rare subset of DC. Thus we studied the presence of pDC in the different fractions obtained after CCE. We measured a 3-fold enrichment of these cells in the intermediate fractions obtained between the fractions containing lymphocytes and those containing monocytes. We then compared negative-magnetic bead sorting of pDC from PBMC and from cells of the intermediate fractions. We found that more than twice as many pDC were purified from the pool of intermediate fractions than from PBMC, using the same amount of magnetic beads, without significant differences in phenotype or function, and with the same purity, >97%. Finally, we showed that intermediate fractions could be cryopreserved and that pDC sorting by magnetic beads could be performed efficiently after thawing. Altogether, our results show that CCE can be used to enrich highly 'untouched' monocytes and lymphocytes and to pre-enrich pDC, which can then be further purified by immunologic procedures, without affecting their phenotype and function.

Methods

Blood collection and PBMC separation

Platelet apheresis residues were collected from healthy donors, after informed consent was given, at the Etablissement Français du Sang (Nantes, France). Blood was diluted 4-fold with RPMI-1640 (Sigma, Saint-Quentin Fallavier, France) before PBMC were isolated by Ficoll–Paque density-gradient centrifugation (Eurobio, Courtaboeuf, France) at 400 ***g*** for 30 min at room temperature without braking. Collected PBMC were washed in 50 mL RPMI-1640, once at 330 ***g*** for 10 min and once at 230 ***g*** for 10 min, to eliminate remaining platelets. Cell viability and cell counting were performed by eosin Y exclusion. Cell viability was >98%.

CCE

Different cell populations were enriched from PBMC by CCE using a Beckman Avanti J20 centrifuge equipped with a JE-5.0 rotor and a 40-mL elutriation chamber (Beckman Instrument Inc., Fullerton, CA, USA). The flow was maintained with a peristaltic pump (Masterflex) for which the speed had been previously calibrated and adjusted. By this method, cells are separated according to their sedimentation velocity, which depends on their density and size.

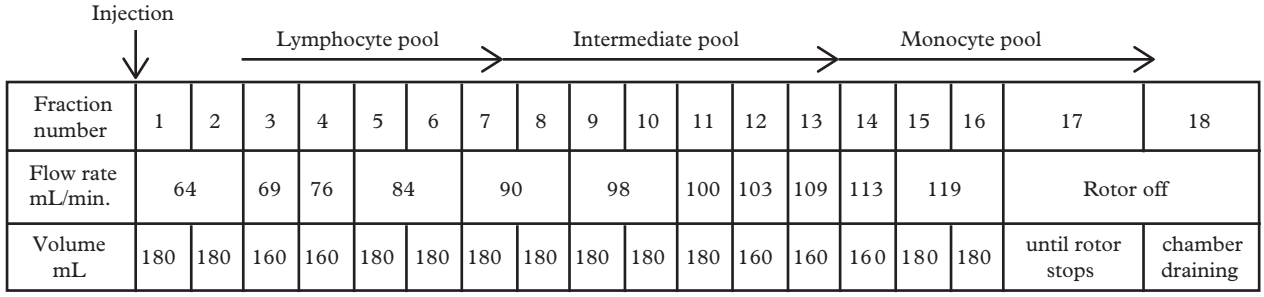
Before use, the CCE system was filled with an aqueous solution containing 0.5% sodium hypochlorite, so that elutriation was performed under sterile conditions. Air bubbles were completely removed from the CCE system using manual techniques. A 0.9% NaCl solution containing 4% autologous plasma-RPMI collected after density-gradient centrifugation was used as the effluent. Cells were centrifuged at 4°C with a constant rotor speed (602 ***g***) and stepwise increments in flow rate (Table I). The elutriation was initiated at a flow rate of 64 mL/min and 50-mL cell suspensions were injected into the CCE system. As suspended cells are introduced into the chamber, they migrate according to their sedimentation velocity to positions where the effects of the two opposing forces on them are balanced (i.e. centrifugal force field versus effluent velocity).

After introduction of the PBMC suspension, remaining platelets (despite previous centrifugation) and red cells were elutriated first. The flow rate was modified after each collection of 160 mL or 360 mL effluent. The cells were then collected in 18 fractions. 'Rotor off' consisted of the maintenance of a terminal constant flow rate of 119 mL/min as the elutriation rotor was stopped. The elutriation processing time from PBMC loading to rotor stop was approximately 30 min and an additional 1 h was needed to wash and prepare the CCE device for the next purification. After use, the CCE system was refilled with an aqueous solution containing 0.5% sodium hypochlorite.

Flow cytometry

Flow cytometry acquisition and analysis were performed using a FACSCalibur cytometer (Becton Dickinson, Pleasanton, CA, USA) with MacIntosh-CellQuest software. Fluorescein isothiocyanate (FITC)-conjugated anti-CD14, CD3,

Table I. CCE Procedure. CCE was performed at a constant rotor speed (602 ***g***). It was initiated at a flow rate of 64 mL/min before cell suspensions were injected into the system. The flow rate was increased after each collection of 160–360 mL effluent.



Fraction number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Flow rate mL/min.	64		69	76	84		90		98		100	103	109	113	119		Rotor off	
Volume mL	180	180	160	160	180	180	180	180	180	180	180	160	160	160	180	180	until rotor stops	chamber draining

CD123, HLA-DR, CD80 and isotype-matched control monoclonal antibody (MAb), phycoerythrin (PE)-conjugated anti-CD16, CD19, CD4, CD40, CD83 and isotype-matched control MAb, and Allophycocyanin (APC)-conjugated anti-CD56, CD8, CD86 and isotype-matched MAb, were purchased from BD Biosciences (Le Pont-De-Claix, France). PE-conjugated anti-BDCA-2 and BDCA-4 MAb, and APC-conjugated anti-BDCA-4 MAb, were purchased from Miltenyi Biotec (Paris, France). Isotype-matched antibodies were used as controls. Labeling was carried out at 4°C for 30 min in RPMI-1640 supplemented with 10% fetal calf serum (FCS; PAA Laboratory, Les Mureaux, France). Cells were washed twice in phosphate-buffered saline (PBS) and analyzed on a cytometer. The results are expressed as the percentage of positive cells or the relative median fluorescence intensity (RMFI) calculated by dividing the median fluorescence intensity of cells stained with specific antibody by the median fluorescence intensity of cells stained with isotypic control antibody.

pDC purification and culture

pDC were negatively selected from PBMC or intermediate pool fractions (IP) using an EasySep® human plasmacytoid DC enrichment kit according to the manufacturer's instructions (Stemcell, Grenoble, France). In some experiments, pDC were purified from PBMC and IP using a plasmacytoid dendritic cells isolation kit (Miltenyi Biotech).

pDC were cultured in AIM-V culture medium (Invitrogen, Saint Aubin, France) supplemented with 5 μg/mL CpG-A oligodeoxynucleotides (ODN2216; Invitrogen) at a concentration of 0.5×10^6 cells/mL for 16 h at 37°C, in a humidified atmosphere containing 5% CO_2. Supernatants were collected and stored at −80°C for IFN-α measurement by enzyme-linked immunosorbent assay (ELISA) (MabTech, Sophia Antipolis, France). Cell phenotype was analyzed by immunofluorescence and flow cytometry.

Cryopreservation

Cells were cryopreserved in FCS containing 10% dimethylsulfoxide (DMSO) at a concentration of 35×10^6 cells/mL, stored at −80°C for at least 24 h and then at −194°C in liquid nitrogen until further use.

Statistical analysis

For statistical analyses, experiments were repeated with at least three donors, and statistical significance was assessed with the non-parametric Friedman test using GraphPad PRISM.

Results

Enrichment of monocytes, lymphocytes and pDC from platelet apheresis residues by CCE

At the Plateforme de Développement et de Transfert Clinique (Development and Clinical Transfer Facility, Centre Hospitalier Régional Universitaire de Nantes, Nantes, France), we set up a CCE procedure to enrich monocytes and lymphocytes from platelet apheresis residues, for use in experimental research (Table I). Our procedure was based on a protocol published previously by Berger *et al.* (9). All of the CCE fractions were first displayed by use of forward- and side-angle light scatter properties (Figure 1). Fractions containing >85% lymphocytes (lower gate) and <2% monocytes (upper gate) were called the lymphocyte pool (LP), usually corresponding to fractions 3–7 (F3–F7). Fractions containing >85% monocytes and <10% lymphocytes were called the monocyte pool (MP) and usually corresponded to fractions 14–17 (F14–F17). Fractions between the LP and MP were called IP and in most cases corresponded to fractions 8–13 (F8–F13). Each group of fractions was then routinely pooled and counted, and the phenotype was checked by flow cytometry. The cell viability was performed by eosin Y exclusion and was >98%.

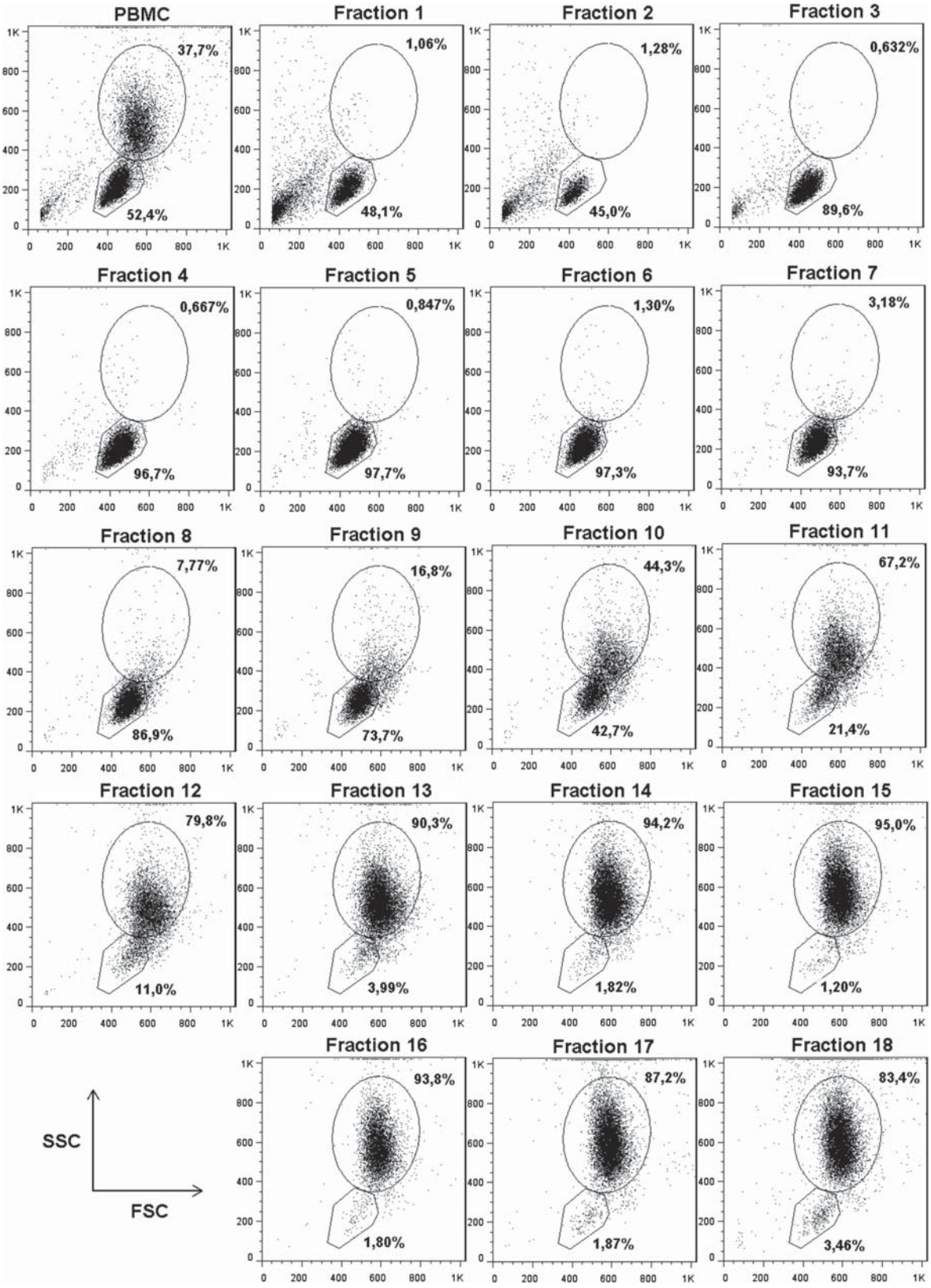


Figure 1. Size/structure analysis by flow cytometry of fractions obtained by CCE. At the end of the CCE procedure, samples of the 18 collected fractions were analyzed using a flow cytometer. Cell populations were identified by the use of forward- and side-angle light scatter properties (FSC and SSC). The upper gate contains the monocyte population and the lower gate the lymphocyte population.

Table II. Enrichment of monocytes by CCE. The quantity, purity and recovery of monocytes were measured by CD14 immunofluorescence staining and flow cytometry. Results are expressed as mean, standard deviation (SD) and coefficient of variation (CV).

N = 39 elutriations	Mean	SD	CV
Number of PBMC	2241×10^6	626×10^6	0.28
% $CD14^+$ in PBMC	34.4	7.1	0.21
Number of cells in monocyte pool	598×10^6	197×10^6	0.33
Elutriated monocyte purity (% $CD14^+$)	91.7	1.9	0.02
Elutriated monocyte recovery (%)	73.4	15.9	0.22

Platelet apheresis residues from 39 healthy donors were processed using this approach. The statistical results of these 39 elutriations are reported in Table II. A mean value of 2241×10^6 PBMC, with a mean of 34.4% $CD14^+$ cells corresponding to monocytes, was collected after Ficoll separation of platelet apheresis residues. We did not process samples with less than 1500×10^6 PBMC, or with PBMC containing less than 15% $CD14^+$ cells, after Ficoll separation, as this considerably reduces monocyte purity and recovery (data not shown). Monocytes were isolated in the MP, with a mean purity of 91.7%, based on CD14 expression, and a mean recovery of 73.4%. The frequency of $CD123^+$ BDCA-2^+ pDC was also measured in the IP and MP. We found a 2.84- and a 1.86-fold increase in pDC in the IP and MP, respectively, compared with the frequency in PBMC (Table III). Altogether, these results showed that pDC could be enriched from PBMC by CCE, ending up in the IP and MP. They also confirmed that monocytes could be enriched in the MP, with a purity >90%.

Enrichment of pDC in the IP and MP of CCE

In subsequent experiments, we determined precisely the cellular composition of each fraction obtained by CCE. Cell populations were quantified in each fraction by immunofluorescence using antibodies against phenotypic markers (CD3, CD4, CD8, CD14, CD16, CD19, CD56, CD123 and BDCA-2), analyzed by flow cytometry (Figure 2). As fractions 1 and 2 (64 mL/min) contained more than 50% platelets and red cells and low numbers

Table III. Enrichment of pDC by CCE. Quantities of pDC in the IP and MP were measured by CD123 and BDCA-2 immunofluorescence staining and flow cytometry. Results are expressed as mean, SD and CV.

N = 39 elutriations	Mean	SD	CV
Number of PBMC	2241×10^6	626×10^6	0.28
% pDC in PBMC	0.44	0.13	0.29
Number of cells in intermediate pool	260×10^6	103×10^6	0.40
% pDC in intermediate pool	1.25	0.56	0.45
% pDC in monocyte pool	0.82	0.3	0.36

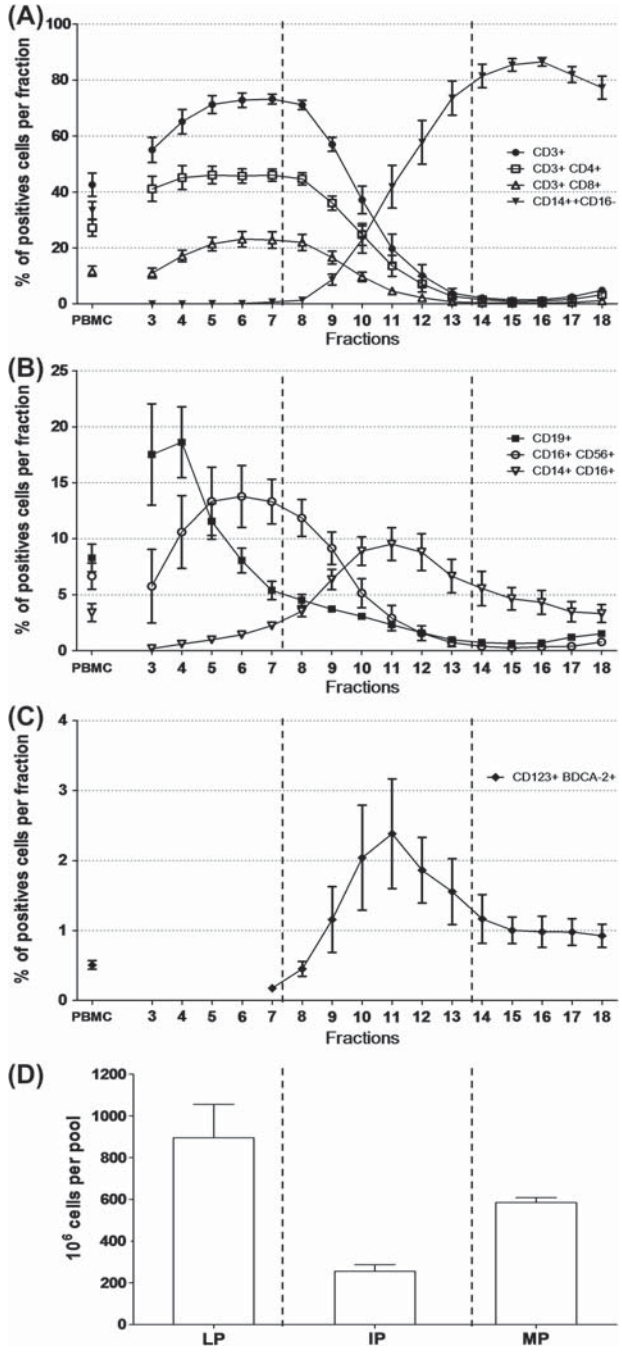


Figure 2. Cellular composition analysis of fractions obtained after CCE. Frequencies of (A) $CD3^+$ T lymphocytes ($CD4^+$ and $CD8^+$), $CD14^{++}$ $CD16^-$ monocytes, (B) $CD19^+$ B lymphocytes, $CD16^+$ $CD56^+$ NK cells, $CD14^+$ $CD16^+$ monocytes and (C) $CD123^+$ BDCA-2^+ pDC were measured in each CCE fraction and in PBMC by immunofluorescence and flow cytometry. (D) The number of cells per pool was measured. Results (mean ± SEM) of five elutriations are reported.

Table IV. Comparison of pDC purification from PBMC or IP. Negative immunomagnetic selection of pDC was performed using 100×10^6 PBMC or IP. The results of three experiments are expressed as mean, SD and CV.

N = 3 elutriations		Mean	SD	CV
from PBMC:	measured of pDC	0.47×10^6	0.05×10^6	0.10
	sorted pDC	0.25×10^6	0.01×10^6	0.06
	recovery (%)	52.9	6.0	0.11
from IP:	measured pDC	1.41×10^6	0.64×10^6	0.45
	sorted pDC	0.55×10^6	0.26×10^6	0.47
	recovery (%)	39.2	9.6	0.24

of other cells, they were not retained for further analysis.

The LP contained approximately 70% $CD3^+$ T lymphocytes, with a majority of $CD3^+$ $CD4^+$ cells (Figure 2). We also detected $CD19^+$ B lymphocytes in the LP, with a 2.25-fold enrichment in the earliest lymphocyte fractions (F3 and F4). $CD16^+$ $CD56^+$ natural killer (NK) cells were also enriched in the LP, by up to 2.1-fold in the latest lymphocyte fractions and earliest intermediate fractions (F5–F8). The MP contained a majority of $CD14^{++}$ $CD16^-$ cells, which are specialized in the production of interleukin (IL)-10 after stimulation by lipopolysaccharide (LPS) (13). We also detected approximately 5% $CD14^+$ $CD16^+$ cells, which are also known as pro-inflammatory monocytes and which produce tumor necrosis factor (TNF)-α in response to LPS. The MP contained small, detectable amounts of NK, B and T cells, with a frequency $< 1\%$. It probably also contained polynuclear cells, which are known to have no significant effect on monocyte differentiation (14), but we did not measure the frequency of these cells.

The cell subset composition of IP (F8–F13) was very heterogeneous. It contained a combination of T and B lymphocytes, NK cells and monocytes. Interestingly, in this pool we found a 2.71-fold enrichment of $CD123^+$ BDCA-2^+ cells corresponding to pDC (from $0.51 \pm 0.06\%$ in PBMC to $1.38 \pm 0.37\%$ in IP). This enrichment culminated in fraction 11 (from $0.51 \pm 0.06\%$ in PBMC to $2.4 \pm 0.78\%$ in F11). A small enrichment of pDC was also detected in the MP, with a 1.71-fold increase compared with PBMC (from $0.51 \pm 0.06\%$ in PBMC to $0.87 \pm 0.16\%$ in MP). Despite the fact that the frequency of pDC in IP was higher than in MP (Figure 2C), the estimated quantity of pDC was higher in MP (5.1×10^6 pDC) than in IP (3.5×10^6 pDC), as the MP contained a mean of $583.8 \times 10^6 \pm 24.7 \times 10^6$ cells and the IP a mean of $255.6 \times 10^6 \pm 31.9 \times 10^6$ cells (Figure 2D). We also observed an enrichment of $CD14^+$ $CD16^+$ monocytes, also known as pro-inflammatory monocytes, in IP, with a maximum of 2.8-fold in fraction 11. pDC can, thus, be enriched mechanically from PBMC using CCE, as they exhibited a higher sedimentation velocity than lymphocytes and a slightly lower velocity than monocytes.

Purification of pDC from PBMC or IP by magnetic bead depletion

In subsequent experiments, we compared the purification of pDC directly from PBMC (PBMC-pDC) with that from IP (IP-pDC) obtained by CCE using negative-magnetic bead sorting. We first measured the frequencies of pDC in PBMC and in the intermediate pool by flow cytometry using antibodies against CD123 and the pDC-specific marker BDCA-2. We found three times more $CD123^+$ BDCA-2^+ pDC in IP than PBMC (Table IV). We then performed negative-bead sorting using the EasySep human plasmacytoid DC enrichment kit from StemcellTechnologies. The purities of the sorted PBMC-pDC or IP-pDC were analyzed. These were similar and were $>97\%$ in all sorting experiments. We recovered 0.25×10^6 and 0.55×10^6 pDC from 100×10^6 PBMC and IP, respectively. Despite the fact that the recovery was inferior using IP, which was probably because of the presence of more monocytes in the IP than in PBMC, we sorted more than twice as many pDC than from PBMC. We also performed this experiment using the plasmacytoid dendritic cell isolation kit from Miltenyi Biotec and obtained similar results (see Supplementary Figure 1 to be found online at http://www.informahealthcare.com/doi/abs/10.3109/14653249.2012.689129). The recovery with the plasmacytoid dendritic cell isolation kit was slightly lower ($32.76 \pm 16.13\%$, $n = 5$) than the recovery obtained with the EasySep human plasmacytoid DC enrichment kit ($39.2 \pm 9.6\%$, $n = 3$).

We recovered a mean of 1.4×10^6 pDC from the three negative-magnetic bead sorting procedures performed on IP. A greater yield of pDC could be obtained if fractions 14 and/or 15 from MP were added to IP.

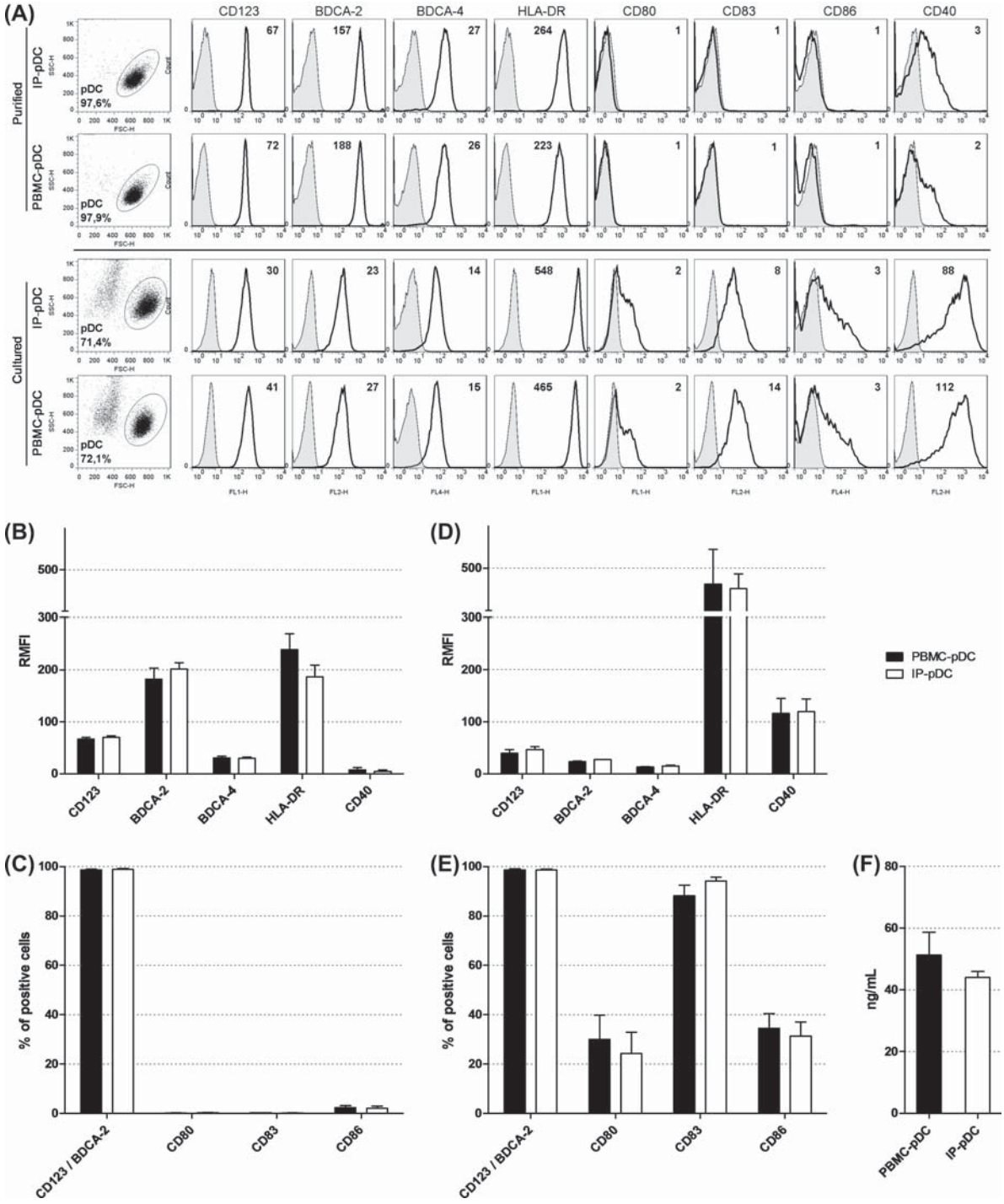


Figure 3. Comparison of pDC purified from PBMC or IP for their phenotype and ability to produce IFN-α. Expression of CD40, CD80, CD83, CD86, CD123, BDCA-2, BDCA-4 and HLA-DR by (A–C) non-cultured pDC purified from PBMC or IP, or (A, D, E) cultured with CpG-A, was analyzed by immunofluorescence and flow cytometry. (F) IFN-α was measured by ELISA in supernatants of pDC purified from PBMC or IP and cultured with CpG-A for 16 h. Results were obtained from three experiments.

CCE does not affect pDC phenotype and function

In order to verify that the CCE procedure does not affect pDC, we first compared the phenotypes of sorted PBMC-pDC and IP-pDC. Both pDC populations exhibited a very similar phenotype with no significant differences. As expected, both pDC populations expressed the same quantity of markers known to be on the surface of pDC: CD123, BDCA-2, BDCA-4 and HLA-DR (Figure 3A,B). They also both exhibited an immature phenotype characterized by lack of expression of the maturation marker

CD83 and of the co-stimulatory molecules CD80 and CD86 (Figure 3A,C). IP-pDC were cultured with IL-3 for 16 h (see Supplementary Figure 2). They maintained an immature phenotype similar to that of IP-pDC directly after purification (Figure 3A), with the exception of a strong induction of CD40 and a small induction of CD80 expression.

To ensure that the CCE procedure had no effect on pDC function, PBMC-pDC and IP-pDC were cultured for 16 h in the presence of CpG-A, an oligodeoxynucleotide known to induce pDC maturation by triggering Toll-like receptor-9 (15). pDC maturation is characterized by a modification of the phenotype and a strong production of type I IFN, such as IFN-α. No phenotypic difference was observed between PBMC-pDC and IP-pDC cultured for 16 h in the presence of CpG-A (Figure 3A,D,E). Both pDC populations similarly increased the expression of molecules involved in antigen presentation to T cells and in co-stimulation, such as HLA-DR, CD40, CD80 and CD86. We also noted a down-regulation of the expression of CD123 and the C-type lectin receptors BDCA-2 and BDCA-4, as reported by Dzionek *et al.* (16). The amounts of IFN-α measured in the supernatants of PBMC-pDC and IP-pDC were not significantly different (*c.* 50 ng/mL) (Figure 3F). Thus pDC collected from IP were similar to those in PBMC, and CCE had no impact on the phenotype and IFN-α secretion capacity of pDC in response to CpG-A.

pDC can be purified from cryopreserved IP

In the final experiments, we wanted to determine whether pDC purification by magnetic beads could be performed on cryopreserved IP. We succeeded in purifying pDC from cryopreserved IP, with a purity of $CD123^+$ $BDCA\text{-}2^+$ cells $>97\%$ (Figure 4). We found that the survival of pDC immediately after the sorting from cryopreserved IP was comparable to the survival of non-cryopreserved IP-pDC (see supplementary Figure 3A). However, after a 16-h culture with IL-3 or CpG-A, we observed a 15% decrease in survival for the pDC purified from frozen IP.

We found that the surface phenotype of pDC obtained from cryopreserved IP was similar to that obtained from non-frozen IP (Figures 3 and 4; and see supplementary Figure 3B). We also observed that pDC maturation following exposure to CpG-A was equivalent between the two pDC populations tested,

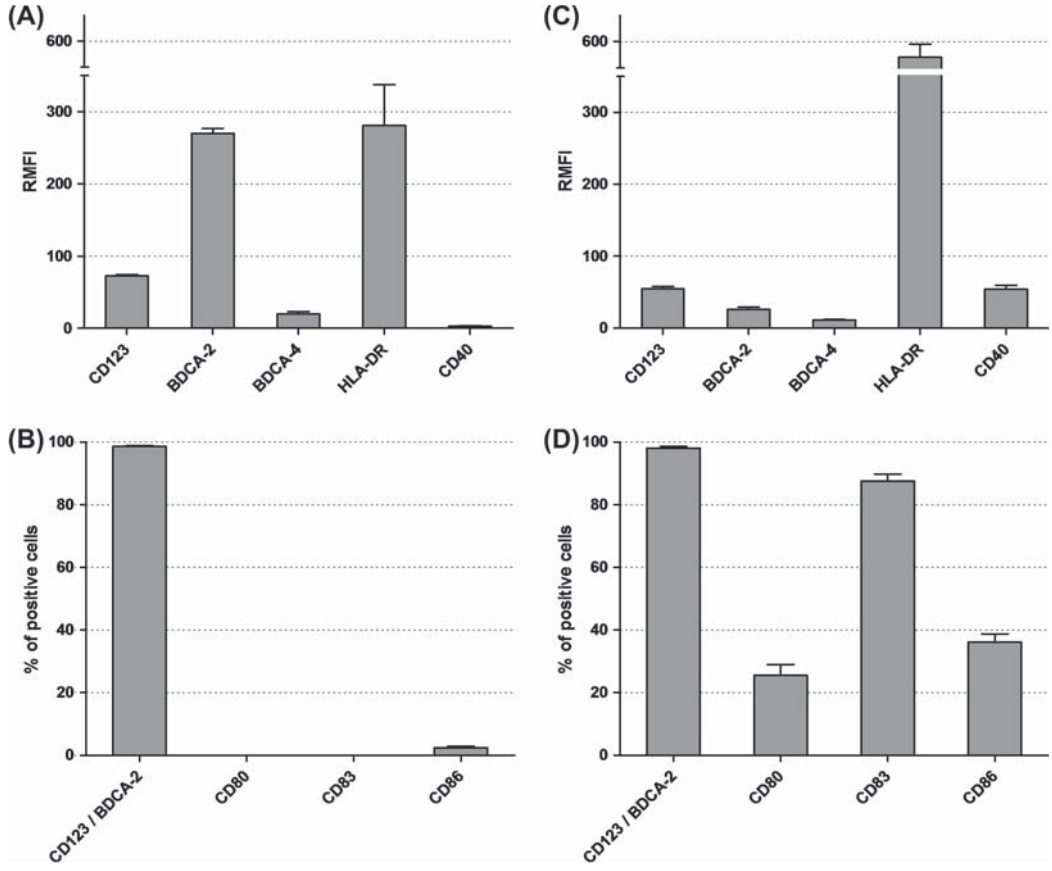


Figure 4. Cryopreservation of IP does not affect recovery, phenotype and maturation of pDC. Expression of CD40, CD80, CD83, CD86, CD123, BDCA-2, BDCA-4 and HLA-DR by (A, B) non-cultured pDC purified from a cryopreserved IP or (C, D) cultured with CpG-A was analyzed by immunofluorescence and flow cytometry. Results were obtained from three experiments.

with a similar level of induction of the maturation marker CD83, a comparable up-regulation of co-stimulation molecules (CD80, CD86 and CD40) and a similar down-regulation of BDCA-2 and BDCA-4.

We concluded that IP obtained after CCE could be frozen and could be used after thawing to purify pDC, but the cryopreservation decreased the survival of pDC by 15%. Cryopreservation did not affect the capacity of pDC to mature after exposure to CpG-A. This result confirmed the findings of Gerrits *et al.* (17), which showed that blood pDC survived PBMC cryopreservation.

Discussion

In this study we have shown that CCE allows the enrichment of pDC from PBMC, without affecting their phenotype and their ability to produce IFN-α. pDC can then be purified using commercially available immunomagnetic bead-sorting kits. The main advantage of this method is that CCE allows the enrichment of major blood cell subsets without staining them with magnetic beads, which may affect their functions (see Supplementary Table I). These major blood cell subsets obtained by CCE can then be further negatively purified using magnetic beads or other sorting methods. Thus the method described in our study should be useful for obtaining or comparing different blood cell subsets without staining them with magnetic beads. Furthermore, for laboratories that routinely use CCE to purify different cell subsets from PBMC, our method reduces by half the amount of immunomagnetic reagents required for pDC purification (see Supplementary Table I). Our method should, then, be useful for the purification of these cells for experimental research and may also be adaptable for clinical use in immunotherapy.

Our study also showed that pDC exhibit a higher sedimentation velocity than lymphocytes, and a slightly lower velocity than monocytes. However, the fractions containing pDC overlap with those containing monocytes. Thus a proportion of pDC are not separable from monocytes by CCE. Indeed, our results show that monocytes purified by CCE contain some pDC; 0.82% in our study. This result must be taken into account in the experimental and clinical use of monocytes purified by CCE (8,9). The fact that more than half of the pDC remain in the MP fraction is a disadvantage of our method (see Supplementary Table I). It is, therefore, preferable to use purification directly from PBMC when a maximum number of pDC is required and when untouched PBMC are not needed.

Finally, we have shown that the intermediary pool can be frozen and that magnetic bead sorting of pDC can be performed later, after thawing, without significant loss of functions but with a 15% decrease in survival. This result confirms the findings of Gerrits *et al.* (17), which showed that blood pDC survived PBMC cryopreservation. This observation may be useful, for example, for studies where pDC are compared with monocyte-derived DC, the IP can be stored while monocyte-derived DC are prepared, a procedure that usually takes 1 week. It may also be interesting for the development of an immunotherapy procedure requiring multiple preparations of pDC, monocyte-derived DC and lymphocytes from the same donor or patient.

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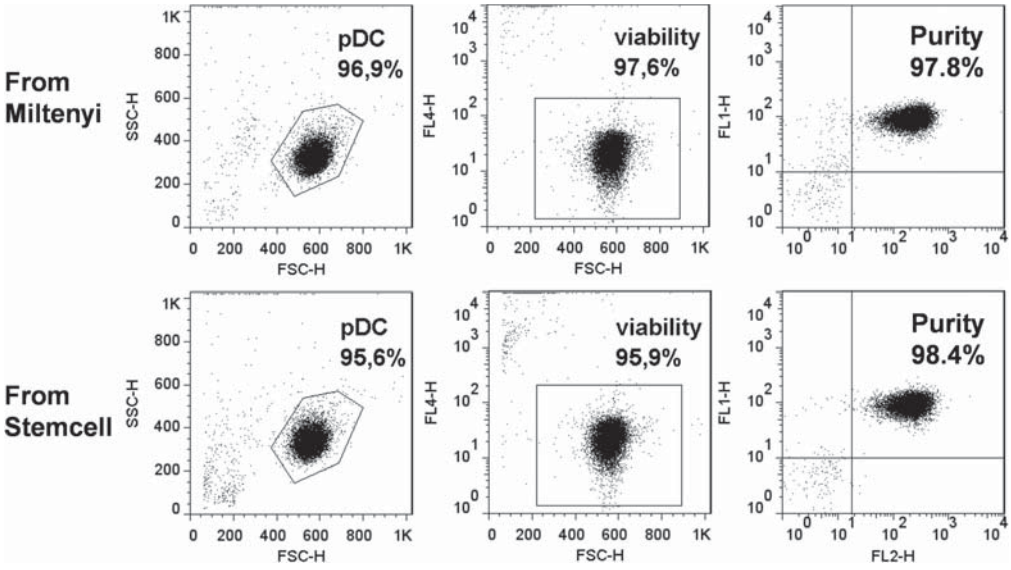
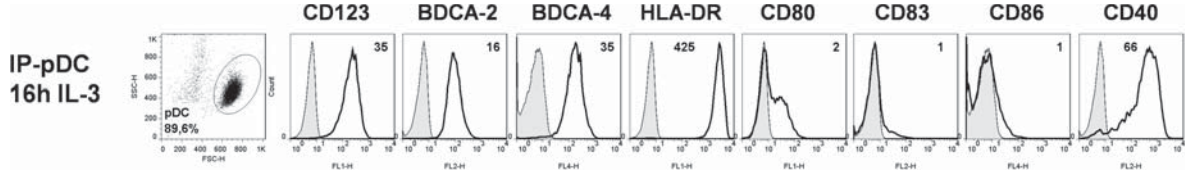
Supplementary material available online

Supplementary Figures 1–3
Supplementary Table I

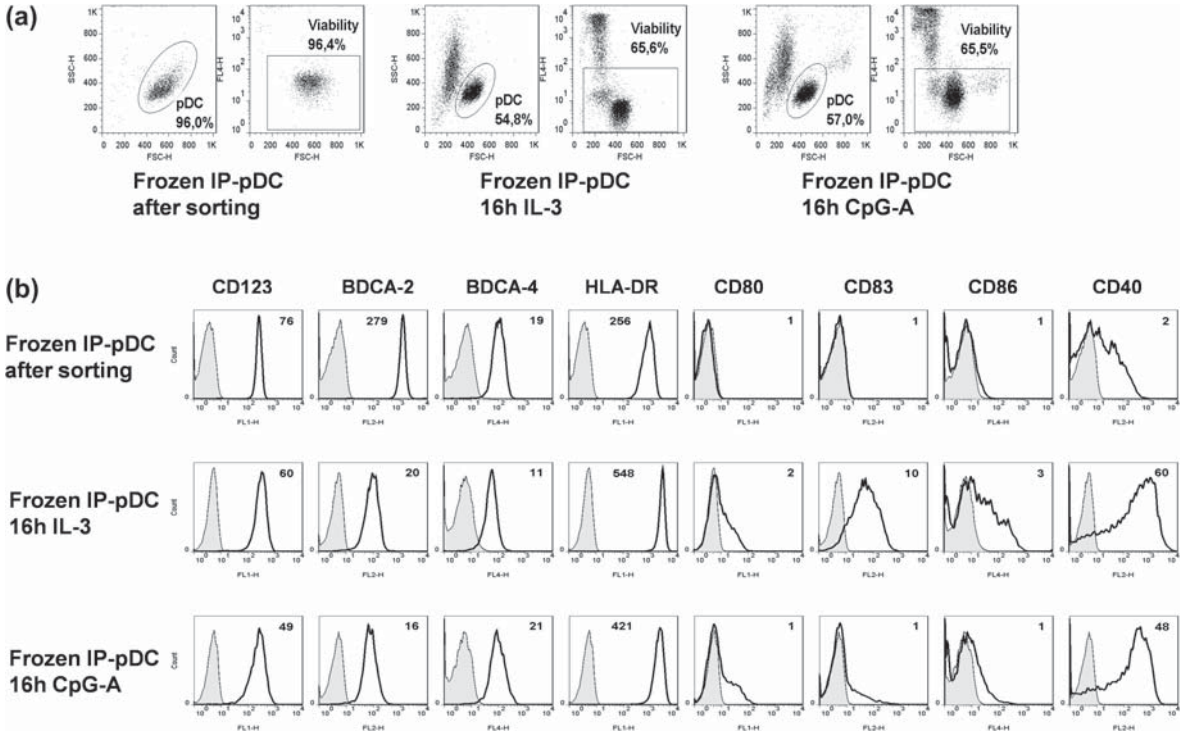
Supplementary material for Coulais D *et al.* Purification of circulating plasmacytoid dendritic cells using counterflow centrifugal elutriation and immunomagnetic beads, *Cytotherapy*, *2012;* doi: 10.3109/14653249.2012.689129.



Supplemental Figure 1: Comparison of survival and purity of pDC obtained from IP using either Miltenyi or Stemcell purification Kit. pDC were purified from IP using either the Miltenyi or Stemcell purification Kit. Viability was determined by TO PR0®3 staining with no gate on FSC/SSC. Purity was determined by a FITC conjugated CD123 and a PE-conjugated BDCA-4 specific mAb staining with a gate on pDC on the FSC/SSC dot plot.



Supplemental Figure 2: Phenotype of pDC purified from IP with stemcell kit after 16h culture with IL-3. pDC were purified from IP using the Stemcell purification Kit. They were cultured for 16 h in the presence of IL-3. They were then stained with mAb specific for CD123, BDCA-2, BDCA-4, HLA-DR, CD80, CD83, CD86 or CD40. Fluorescence was analyzed by flow cytometry with a gate on pDC on the FSC/SSC dot plot.



Supplemental Figure 3: Survival and phenotype of pDC obtained from cryopreserved IP using Stemcell purification Kit. pDC were purified from cryopreserved IP using the Stemcell purification Kit. pDC were studied immediatly or cultured for 16h with IL-3 or CpG-A. A Viability was determined by TO-PR0®3 staining with no gates on FSC/SSC. 8 They were stained with mAb specific for CD123, BDCA-2, BDCA-4, HLA-DR, CD80, CD83, CD86 or CD40. Fluorescence was analyzed by flow cytometry with a gate on pDC on the FSC/SSC dot plot.

Supplemental Table I: Comparison of pDC purification from IP or PBMCs with the same cell input.

	Purification from IP	Purification from PBMCs
Total cell input (N = 39)	$2.241 \times 10^9 \pm 626 \times 10^6$ PBMCs	
Number of cells after CCE (N = 39)	$0.260 \times 10^9 \pm 103 \times 10^6$ cells	
% of pDC in IP and PBMCs (N = 39)	1,25% ± 0.56%	0.44% ± 0.13%
% of recovered pDC (N = 3)	39.2% ± 9.6%	52.9% ± 6%
Total number of purified pDC (estimated)	1.25×10^6 pDCs	5.2×10^6 pDCs
Number of pDC depletion kits used	0.13 kit 0.104 kit/10^6 pDC	1.12 kit 0.215 kit/10^6 pDC
Time spent	CCE procedure (90 min) Magnet sorting procedure (10 min)	No CCE procedure Magnet sorting procedure (60 min)
Other cells	598×10^6 untouched monocytes (purity > 91.7%) 880×10^6 untouched lymphocytes (purity > 80%)	2.235×10^9 of pDC depleted magnetic beads positive PBMCs.