Efficient generation of CD34+ progenitor-derived dendritic cells from G-CSF-mobilized peripheral mononuclear cells does not require hematopoietic stem cell enrichment

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Abstract: As a result of their potent antigen-presentation function, dendritic cells (DC) are important tools for cell therapy programs. In vitro-generated DC from enriched CD34+ hematopoietic stem cells (HSC; enriched CD34 DC) have already proven their efficiency in Phase I/II clinical trials. Here, we investigated whether enrichment of CD34+ HSC before the onset of culture was absolutely required for their differentiation into DC. With this aim, we developed a new two-step culture method. PBMC harvested from G-CSF-mobilized, healthy patients were expanded for 7 days during the first step, with early acting cytokines, such as stem cell factor, fetal liver tyrosine kinase 3 ligand (Flt-3L), and thrombopoietin. During the second step, expanded cells were then induced to differentiate into mature DC in the presence of GM-CSF, Flt-3L, and TNF-α for 8 days, followed by LPS exposure for 2 additional days. Our results showed that the rate of CD34+/CD38+/lineage^{neg} cells increased 19.5 ± 10-fold (mean±SD) during the first step, and the expression of CD14, CD1a, CD86, CD80, and CD83 molecules was up-regulated markedly following the second step. When compared with DC generated from enriched CD34+ cells, which were expanded for 7 days before differentiation, DC derived from nonenriched peripheral blood stem cells showed a similar phenotye but higher yields of production. Accordingly, the allogeneic stimulatory capacity of the two-step-cultured DC was as at least as efficient as that of enriched CD34 DC. In conclusion, we report herein a new two-step culture method that leads to high yields of mature DC without any need of CD34+ HSC enrichment. *J. Leukoc. Biol.* 81: 957–967; 2007.

Key Words: *ex vivo expansion · immunotherapy · cell vaccine · lipopolysaccharide*

INTRODUCTION

Dendritic cells (DC) represent a rare population of highly potent APC. They are derived from CD34+ bone marrow stem cells and can be generated in vitro from blood monocytes in the presence of GM-CSF and IL-4 (Mo-DC) [1] or from CD34+ hematopoietic stem cells (HSC) in the presence of GM-CSF and TNF-α (CD34 DC) [2]. They play a major role in the processing and presentation of antigens to different immune cells, and they have the unique capacity to prime naïve T lymphocytes. To play this role, DC up-regulate the expression of CD1a, HLA Class II, CD80, CD86, and CD83 molecules. This differentiation and maturation process promotes the polarization of naïve T cells and activates antigen-specific T lymphocytes [3].

Generation of DC from CD34+ HSC gives rise to two DC subpopulations: CD14+CD1a– cells, which are precursors of interstitial DC, and CD1a+CD14– cells, which are precursors of Langerhans cells [2, 4–8]. DC can also be generated in vitro from monocyte precursors. Mo-DC represent a homogeneous population of immature DC, resembling interstitial DC [1, 9–11]. Although Mo-DC require exposure to additional factors to mature fully, CD34 DC partially express low levels of CD83, which is a maturation marker. The presence of TNF-α, an activation factor of DC [2], from the onset of culture could account for the presence of this marker.

Vaccines against cancer aim to induce tumor-specific effector T cells, which can reduce tumor mass, as well as tumor-specific memory T cells, which can control tumor relapse. Owing to their capacity to regulate T cell immunity, DC are being used increasingly as adjuvants for vaccination, and the immunogenicity of antigens delivered by these DC has now

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been shown in patients with cancer. In particular, CD34 DC have proven to be efficient adjuvants in a Phase I/II trial, which resulted in clinical and immunological responses in 10 out of 17 patients suffering from metastatic melanoma [12–14].

Recently, two different studies have demonstrated that high yields of functional DC from human umbilical cord blood or bone marrow CD34+ cells can be generated by using a two-step culture method. Enriched CD34+ hematopoietic progenitor cells were expanded during the first step of culture [15, 16] using reported expansion protocols [17, 18] and then induced to differentiate into DC during the second step. However, this methodology requires high numbers of CD34+ HSC and costly enrichment protocols. Therefore, here, we addressed whether enrichment of CD34+ HSC before expansion was absolutely required for getting high numbers of DC. Reported herein is a new two-step culture method, whereby nonenriched PBMC, harvested from G-CSF-mobilized patients, are expanded and then differentiated into DC. The efficacy of this process was evaluated by comparing, at phenotypical and functional levels, these two-step-cultured DC with the classical, enriched CD34 DC reported by Caux et al. [2] and with enriched, two-step, cultured DC.

MATERIALS AND METHODS

Culture media and cytokines

Complete medium (CM) used was RPMI 1640 (Eurobio, France), supplemented with 2 mM L-glutamine and 100 IU/ml penicillin-streptomycin (Gibco, Grand Island, NY, USA) and 10% heat-inactivated FBS (Gibco). IMDM (Gibco), supplemented with 10% heat-inactivated FBS, was used for expansion. Recombinant human stem cell factor (rhSCF), thrombopoietin (TPO), fetal liver tyrosine kinase 3 ligand (Flt-3L), GM-CSF, and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA).

Enrichment of CD34+ HSC from G-CSF-mobilized peripheral blood

After obtaining informed consent, healthy donors received rhG-CSF (Granocyte® or Neupogen®), 10 μg/kg/day s.c., for 5 days for peripheral blood stem cell mobilization and then underwent apheresis for 2 consecutive days to collect mobilized CD34+ HSC. Mononuclear cells (MNC) were isolated from mobilized peripheral blood by Ficoll density gradient centrifugation and were split in two parts. One part was used to purify CD34+ cells by using anti-CD34-coated magnetic beads (Miltenyi Biotec, Inc., Germany), relative to Miltenyi protocol. The purity of the CD34+ fractions was controlled. CD34+-enriched HSC were then used immediately in the two-step culture method or frozen until use in the one-step culture method.

Differentiation of enriched CD34 HSC into DC in the classical, one-step culture method

As described previously by Caux et al. [2] and Banchereau et al. [12], enriched CD34 HSC, issued from the same healthy donors, were thawed and cultured in parallel with Day 7-expanded, nonenriched HSC. Cells (2×10^6) were seeded in a 25-cm^2 flask in 5 ml CM supplemented with 50 ng/ml GM-CSF, 10 ng/ml TNF-α, and 100 ng/ml Flt-3L. At Day 3, cultures were fed by adding 1.5 ml fresh medium with cytokines. At Day 5, cultures were split into two flasks with fresh medium and cytokines. At Day 8, half of the cells were harvested for analyses, and half of the cells were induced to mature by exposure for 48 h to 100 ng/ml LPS (Sigma Chemical Co., St. Louis, MO, USA).

Expansion of nonenriched HSC and enriched CD34 HSC in the first culture step

The unfractionated MNC (nonenriched HSC) or enriched CD34 HSC from the same healthy donors were expanded in parallel. Nonenriched, G-CSF-mobilized MNC (3×10^6) or 2×10^5 enriched CD34 HSC were seeded in a 25-cm^2 flask and cultured in IMDM media supplemented with 10% heat-inactivated FBS. The cells were expanded for 7 or 14 days in the presence of 50 ng/ml SCF, 10 ng/ml TPO, and 50 ng/ml Flt-3L. The medium was refreshed regularly by partial depletion throughout the culture period.

Differentiation of expanded, nonenriched HSC or expanded, enriched CD34 HSC into DC in the second culture step

Day 7-expanded, enriched CD34 HSC, issued from the same donor, were cultured in parallel with Day 7-expanded, nonenriched HSC. Cells (2×10^6) were seeded in a 25-cm^2 flask in 5 ml CM supplemented with 50 ng/ml GM-CSF, 10 ng/ml TNF-α, and 100 ng/ml Flt-3L. At Day 3, cultures were fed by adding 1.5 ml fresh medium with cytokines. At Day 5, cultures were split into two flasks with fresh medium and cytokines. At Day 8, half of the cells were harvested for analyses, and half of the cells were induced to mature by exposure for 48 h to 100 ng/ml LPS (Sigma Chemical Co.).

Flow cytometry

The antibodies used for flow cytometry were FITC- or PE-conjugated mouse antihuman CD34, lineage (Lin; including CD3, CD14, CD16, CD19, CD20, CD56), CD1a, HLA-DR, CD14, CD80, CD86, and CD83. The mAb against CD34, CD38, Lin, and CD1a were purchased from BD PharMingen (San Diego, CA, USA). The mAb against HLA-DR, CD14, CD80, CD86, and CD83 were purchased from Immunotech (Marseille, France). Cultured cells were collected, washed twice, and then resuspended in 200 μl PBS containing 0.1% BSA (PBSA). Cells were stained with specific labeled antibodies or appropriate isotypic controls. They were incubated on ice for 30 min and washed with PBSA and then fixed with 1% paraformaldehyde solution. Analyses were performed using a FACScan (BD Medical System, Sandy, UT, USA) and the WinMDI software (Version 2.8, Scripps Institute, La Jolla, CA, USA). Data were expressed as percent mean positivity ± SD.

MLR

Cells were collected at the end of the second period of culture and used to stimulate fully mismatched T lymphocytes. Graded doses of irradiated, stimulating cells were seeded with 1×10^5 allogeneic T lymphocytes in round-bottom microwell tissue-culture plates in complete RPMI 1640 with 10% human AB serum. Thymidine incorporation was measured at Day 5, following an 18-h pulse with 1 μCi ^{3}H-thymidine.

Statistical analysis

Data were expressed as percent mean positivity ± SD. The paired Student's *t*-test was performed to compare two or more mean values. A probability of null hypothesis less than 5% ($P \leq 0.05$) was considered statistically significant.

RESULTS

Expansion of HSC from G-CSF-mobilized MNC without prior CD34+ enrichment and characterization of the best period for DC generation

We performed three different experiments to analyze the kinetics of expansion of nonenriched CD34+/CD38+/Lin^{neg} cells. Nonenriched, G-CSF-mobilized MNC were cultured in the presence of SCF, TPO, and Flt-3L for 7 and 14 days, respectively. Nonenriched, G-CSF-mobilized MNC were cultured in the presence of SCF, TPO, and Flt-3L for 7 and 14 days, respectively. The phenotype of the expanded cells was analyzed by staining them for the CD34, CD38, and Lin markers to detect the CD34+/CD38+/Lin^{neg} cells, which represent short-term, hematopoietic precursors in human. A

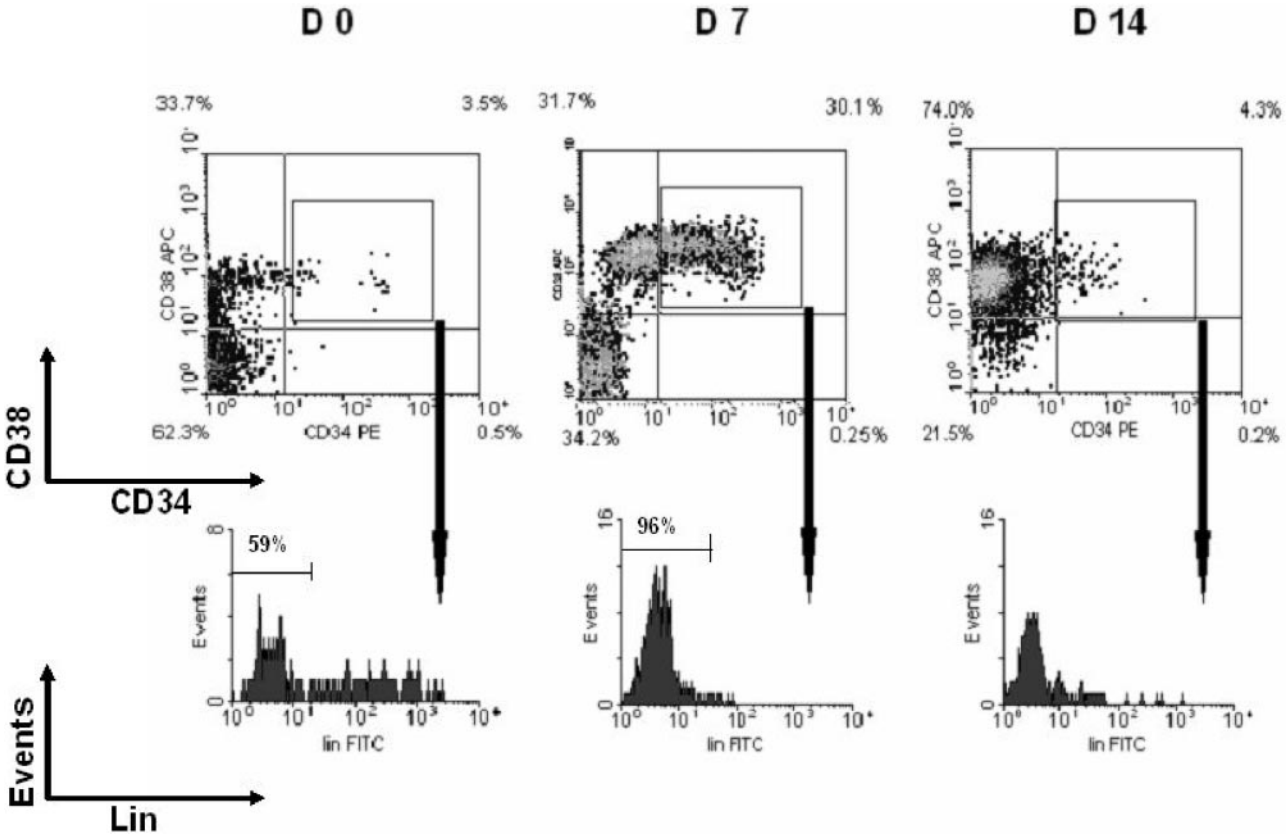

Fig. 1. CD34+CD38+/Lin^{neg} phenotype of Day 7- or Day 14-expanded HSC from nonenriched, G-CSF-mobilized MNC, which were expanded for 7 and 14 days, respectively, by culture with SCF, TPO, and Flt-3L. Expanded HSC were labeled with CD34-APC, CD38-PE, and Lin-FITC antibodies and analyzed in a three-color flow cytometer. Quadrants were set based on the isotype-matched control dot plot, and 10,000 events were acquired. The percentage of positive CD34+CD38+ cells is shown in each dot-plot profile. The CD34+CD38+ population was then gated to analyze Lin expression in these cells. This phenotype is representative of three individual experiments.

representative experiment is shown in **Figure 1**, and the three experiments are reported in **Table 1A**. As shown in Figure 1, we observed that the percentage of CD34+/CD38+/Lin^{neg} cells increased markedly from 3.5% at Day 0 to 30% at Day 7. However, this increase was transient, as the rate of short-term hematopoietic precursors decreased drastically at Day 14. Accordingly, the CD34+ absolute cell number increased strongly during the first week of culture to reach a 19.5 ± 10-fold increase at Day 7 but subsequently dropped at Day 14 (Table 1A). Therefore, we defined the optimum period for expansion of G-CSF-mobilized HSC to be at Day 7 of the first culture step. As shown in Table 1A and **Table 1B,** this time-frame was used in the following experiments, unless otherwise stated.

TABLE 1A. Expansion Rates of HSC ($\times 10^6$) and CD34+ Cells ($\times 10^4$) from Nonenriched or CD34-Enriched, G-CSF-Mobilized MNC

	Day 0 first culture step			Day 7 first culture step			Day 14 first culture step		
No.	MNC ($\times 10^6$)	% of CD34+	Absolute number of CD34+ ($\times 10^4$)	HSC ($\times 10^6$)	% of CD34+	Absolute number of CD34+ ($\times 10^4$)	HSC ($\times 10^6$)	% of CD34+	Absolute number of CD34+ ($\times 10^4$)
Nonenriched Ia-1	3	3.50%	10.5	5.8	32%	186	6.1	1.50%	9.1
Nonenriched Ia-2	3	1.80%	5.4	2.6	30%	78	16.9	4.30%	72.7
Nonenriched Ia-3	3	2.30%	6.9	3.3	26%	86	11.1	ND	ND
Mean ± SD	3	2.5 ± 0.9%	7.6 ± 2.6	3.9 ± 1.7	29 ± 3%	117 ± 60	11.4 ± 5.4	2.9 ± 1.9%	40.9 ± 44.7
CD34-enriched Ia-1	0.2	67.50%	13.5	1.8	73.30%	131.9	7.2	5.3	38.2
CD34-enriched Ia-2	0.2	98%	19.6	2	72.20%	144.4	5.8	ND	ND
Mean ± SD	0.2	83 ± 22%	16.6 ± 4.3	1.9 ± 0.14	72.8 ± 0.8%	138.2 ± 8.8	6.5 ± 1	5.3%	38.2

Data are from three individual experiments with nonenriched (n=3) or enriched CD34+ (n=2) cells issued from the same G-CSF-mobilized patients. Total MNC, percentage of CD34+ cells, and absolute number of CD34+ cells during the first culture step are analyzed at Days 0, 7, and 14. ND, Not done.

TABLE 1B. Expansion of HSC ($\times 10^6$) and CD34+ Cells ($\times 10^4$) from Nonenriched and from Enriched CD34, G-CSF-Mobilized MNC in the First Culture Step for DC Generation

	Day 0 first culture step			Day 7 first culture step		
No.	MNC ($\times 10^6$)	% of CD34+	Absolute number of CD34+ ($\times 10^4$)	HSC ($\times 10^6$)	% of CD34+	Absolute number of CD34+ ($\times 10^4$)
Nonenriched Ib-1	3	2%	6	8	21.70%	173.6
Nonenriched Ib-2	3	2.10%	6.3	8	32%	256
Mean ± SD	3	2 ± 0.1%	6.1 ± 0.2	8	26.9 ± 7.3%	215 ± 58
CD34-enriched Ib-1	0.2	65%	13	0.5	72.70%	36.5
CD34-enriched Ib-2	0.2	85%	14	0.5	73.30%	36.7
Mean ± SD	0.2	75 ± 14%	13.5 ± 0.7	0.5	73 ± 0.4%	36.6 ± 0.1

Data are from two individual experiments with nonenriched ($n = 2$) or enriched CD34+ HSC ($n = 2$) cells issued from the same G-CSF-mobilized patients. Total MNC, percentage of CD34+ cells, and absolute number of CD34+ cells during the first culture step are measured at Days 0 and 7.

Generation of DC from expanded HSC by the two-step culture method results in more differentiated DC as compared with the standard method

We then investigated whether the HSC enrichment step was required before differentiating HSC into DC. Therefore, Day 7-expanded cells from nonenriched cultures were washed extensively and further cultured with GM-CSF, Flt-3L, and TNF-α for 8 days during a second step of culture. Enriched CD34 HSC from the same donor were cultured in the same conditions in parallel, to compare the phenotypes and functions of the DC obtained by these two methods. As shown in a representative experiment (**Fig. 2**), cells obtained by both methods expressed CD1a and CD14, two markers of CD34 DC.

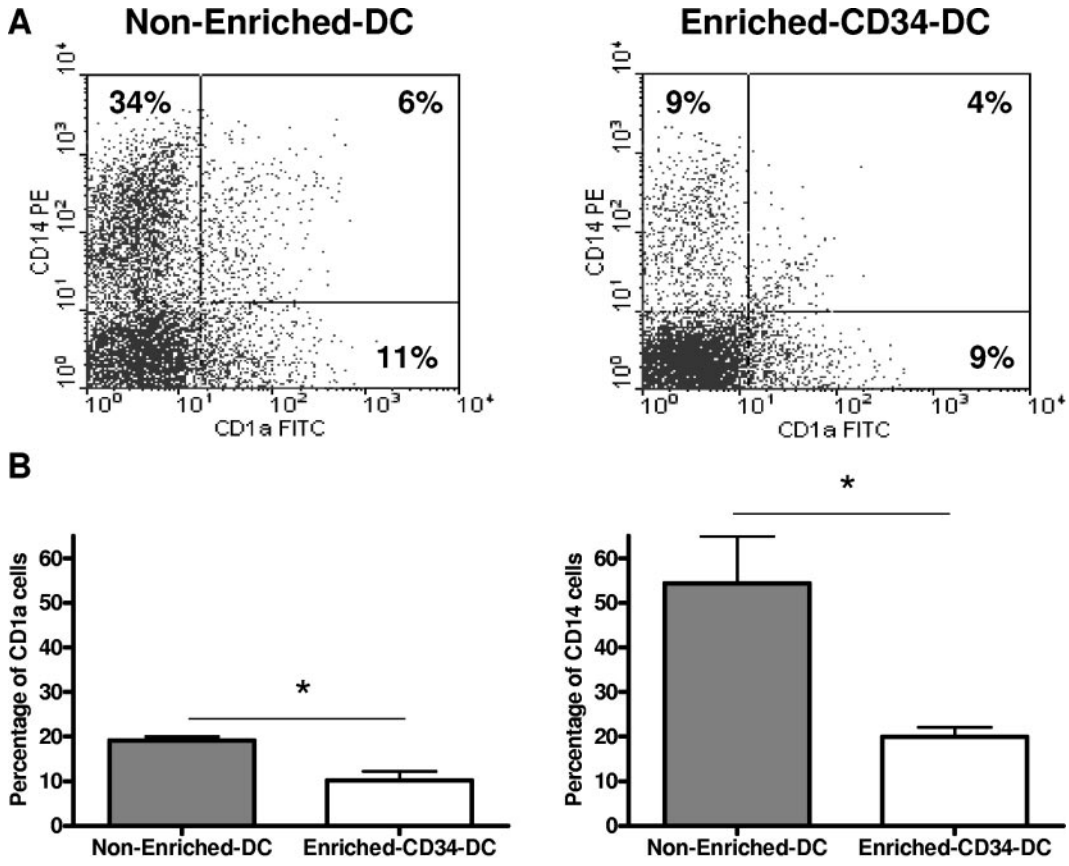

Fig. 2. Comparison of the phenotype of DC generated by the nonenriched, two-step culture method with that of classical, enriched CD34 DC in a one-step culture method. At the end of the second step of culture, DC were stained for CD1a and CD14 expression. The levels of expression of these molecules were compared by cytofluorometry with that of enriched CD34 DC. The subpopulation of CD14+CD1a– cells represents precursors of interstitial DC, whereas the subpopulation of CD1a+CD14– represents precursors of Langerhans cells. This phenotype is representative of five individual experiments. Quadrants were set up on the isotype-matched control dot plot, and 10,000 events were acquired. The percentage of nongated cells is shown in the dot-plot profiles. (B) The percentages of CD1a+ cells and CD14+ cells, which were obtained from the two different culture methods, were calculated. Results are represented as mean percent ± SD of five separate experiments. *, $P < 0.05$.

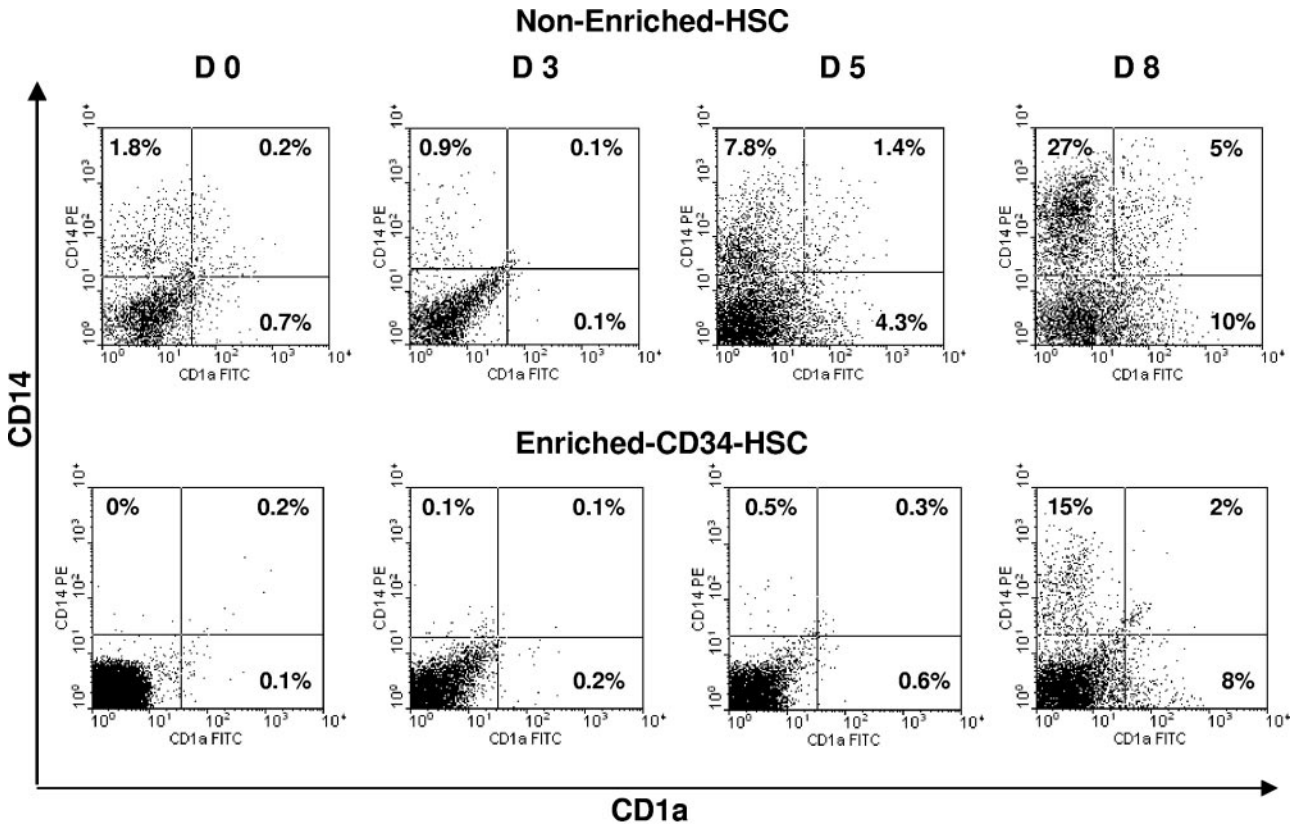

Fig. 3. Kinetics of DC generation by the nonenriched, two-step culture method, as compared with that of one-step, cultured, enriched CD34 DC. Cells harvested at Day 7 of the first culture step or enriched CD34+ HSC were induced to differentiate into DC in the presence of the appropriate cytokines. They were then harvested at Days 3, 5, and 8 of the DC differentiation step and evaluated for their expression of CD1a and CD14 on their cell surface. These data are from one experiment representative of two individual experiments.

It is interesting that the percentage of CD14+ cells or CD1a + cells was higher when DC were generated by the nonenriched, two-step culture method, as compared with the standard one (Fig. 2). Few cells were double-positive for these two markers. Nevertheless, the percentage of double-positive cells was higher in the nonenriched, two-step, cultured DC (4.3–14.1%) than the enriched CD34 DC population (0.9–5.1%). Altogether, these data show that the nonenriched, two-step, culture method allows efficient generation of cells with a DC phenotype. As the only difference between the two cultures was the first step of HSC expansion, our results demonstrate that the enrichment step could be replaced by an expansion step.

Cells generated by the nonenriched, two-step culture method differentiate into DC faster than those from the classical, one-step culture method

The higher levels of CD14+ and CD1a+ DC, which were generated by the nonenriched, two-step culture method, led us to question whether the first step of culture could have influenced the capacity of HSC to differentiate into DC during the second step or whether CD14+ and CD1a+ cells were already present at Day 0 of the differentiation step. Therefore, a time-course of DC generation was performed. Cells were harvested at Days 3, 5, and 8 of the second step of culture and were analyzed for their levels of CD1a and CD14 molecule expression. As shown in **Figure 3**, cells derived from enriched CD34+ HSC did not express any of these two DC markers at Days 0, 3, and 5 of the DC differentiation step. Indeed, cell surface expression of CD1a and CD14 molecules on these cells did not appear until Day 8 of the second culture step (Fig. 3). In contrast, cells, which were expanded previously during a first step of culture, already showed some nonsignificant levels of CD14 and CD1a surface molecule expression (1.9% and 0.8% of ungated cells, respectively) at Day 7 of the first step or Day 0 of the differentiation step. At Day 5 of the differentiation step or Day 12 of the DC total culture, multiple, nonadherent cell aggregates were observed and expanded progressively up to Day 8 of the differentiation step. These cell aggregates were floating and showed typical dendrites under an inverted microscope. In accordance with these observations, CD1a- and CD14-positive cells appeared as early as Day 5, and their rates increased up to Day 8 of the second culture step (Fig. 3). These CD1a+ and CD14+ cells did not express CD34 or CD38 (data not shown), assessing that these cells were not HSC precursors. Therefore, this kinetics study suggests an earlier differentiation of HSC into DC with the nonenriched, two-step culture method as compared with a classical method using CD34-enriched cells.

The nonenriched, two-step culture method leads to higher rates of mature DC than the enriched CD34 standard method

As DC appeared as soon as Day 5 of the second step of culture and showed dendrites under an inverted microscope, we then

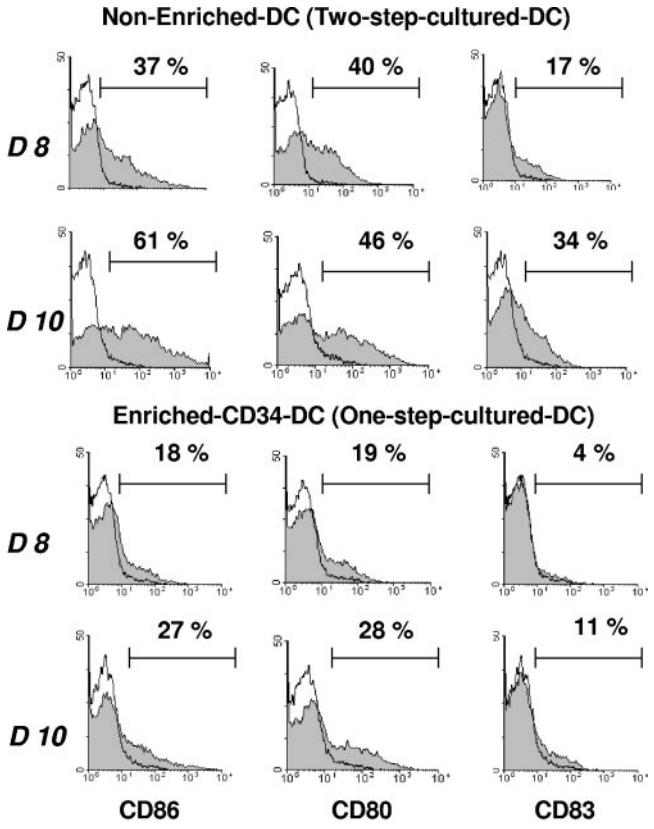

Fig. 4. DC generated by the nonenriched, two-step culture method are more differentiated and matured faster than one-step, cultured, enriched CD34 DC. At Day 8, cells were induced to mature by exposure to 100 ng/ml LPS for 48 h. Maturation of DC was phenotypically characterized by the up-regulation of T cell costimulatory molecules (CD80, CD86) and the maturation marker (CD83). These phenotypes are representative of five individual experiments. The percentage of cells is shown above the histogram profile. Open histograms represent negative control; solid histograms represent cells stained with the indicated, relevant mAb.

investigated whether the two-step culture method could lead to generation of higher rates of mature DC, as compared with the CD34-enriched standard method. Therefore, we harvested DC at Day 8 of the second step, washed them, and cultured them for 48 h with LPS to induce their maturation. As shown in **Figure 4**, the levels of T cell costimulatory molecules (CD80, CD86) at Day 8 of culture, before induction of maturation, were higher in immature DC generated by the nonenriched, two-step culture method than in enriched CD34 DC. In addition, we observed the presence of significant levels of CD83 molecule expression in these cells (Fig. 4, upper rows). Following LPS exposure, CD80, CD86, and CD83 molecules were up-regulated in both DC subtypes, but overall, CD80, CD86, and CD83 molecules were expressed at higher levels in the DC obtained by the nonenriched, two-step culture method as compared with enriched CD34 DC. Thus, up to 34% of the nonenriched, two-step, cultured DC were positive for the CD83 marker, as compared with 11% for enriched CD34 DC (Fig. 4, lower rows). The mean of mature DC in the nonenriched, two-step culture method was of 25% versus 16% in the enriched, one-step culture method ($P<0.05$, $n=5$; **Table 2A**). Therefore, these results suggest that the nonenriched, two-step culture method leads to more mature DC than the enriched CD34, one-step culture method.

The allostimulatory capacity of DC generated by the nonenriched, two-step culture method is at least as high as that of enriched CD34 DC

We then evaluated the allostimulatory capacity of the DC generated by the nonenriched, two-step culture method, in comparison with that of one-step, cultured, enriched CD34 DC. The mean value of four independent MLR assays (four at Day 8 and four at Day 10) showed a trend toward higher prolifer-

TABLE 2A. Differentiation of DC Generated from Two-Step, Cultured, Nonenriched HSC ($\times 10^6$) or from One-Step, Cultured, Enriched CD34 HSC ($\times 10^6$)

	Day 0 second culture step	Day 8 second culture step					Day 10 second culture step	
		imDC					mDC	
No.	HSC ($\times 10^6$)	Absolute number ($\times 10^6$)	% CD14	% CD1a	% CD80	% CD86	% CD86	% CD83
Nonenriched IIa-1	2	5.4	40	17.6	36	21	49	26
Nonenriched IIa-2	2	2.8	63	20.5	50	47	65	34
Nonenriched IIa-3	2	5.3	72	17.7	86	77	69	24
Nonenriched IIa-4	2	10	78	21.7	63	69	84	29
Nonenriched IIa-5	2	10.1	24	18.3	66	73	43	14
Mean ± SD	2	6.7 ± 3.2	55.4 ± 23	19.2 ± 1.8	60 ± 18.7	57.4 ± 23.4	62 ± 16.4	25.4 ± 7.4
		imDC					mDC	
No.	HSC ($\times 10^6$)	Absolute number ($\times 10^6$)	% CD14	% CD1a	% CD80	% CD86	% CD86	% CD83
CD34-enriched IIa-1	2	5.1	13	13	23	28	31	16
CD34-enriched IIa-2	2	34.6	27	13.6	87	90	67	11
CD34-enriched IIa-3	2	33.3	18	6.5	66	75	77	17
CD34-enriched IIa-4	2	30	22	13.7	52	56	35	13
CD34-enriched IIa-5	2	5.2	18.4	4	28	88	65	22
Mean ± SD	2	21.6 ± 15.1	19.7 ± 5.2	10.6 ± 4.6	51.2 ± 26.6	67.4 ± 26	55 ± 20.6	15.8 ± 4.2

Data are from five individual experiments with nonenriched or enriched CD34+ cells issued from the same G-CSF-mobilized patients. Absolute number of HSC (Day 0 of the second-step culture) and DC (Day 8 and Day 10 of the second culture step) is shown. imDC, Immature DC; mDC, mature DC.

TABLE 2B. Differentiation of DC Generated from Two-Step, Cultured, Nonenriched HSC ($\times 10^6$) or from Two-Step, Cultured, Enriched CD34 HSC ($\times 10^6$)

	Day 0 second culture step	Day 8 second culture step					Day 10 second culture step	
		imDC					mDC	
No.	HSC ($\times 10^6$)	Absolute number ($\times 10^6$)	% CD14	% CD1a	% CD80	% CD86	% CD86	% CD83
Nonenriched IIb-1	2	4.8	73	13	52	8	65	26
Nonenriched IIb-2	2	2.8	88	5	80	62	71	8
Mean ± SD	2	3.8 ± 1.4	80 ± 10.6	9 ± 5.7	60 ± 19.8	35 ± 38	68 ± 4.2	17 ± 12.7
		imDC					mDC	
No.	HSC ($\times 10^6$)	Absolute number ($\times 10^6$)	% CD14	% CD1a	% CD80	% CD86	% CD86	% CD83
CD34-enriched IIb-1	2	4.8	70	11	30	51	65	11
CD34-enriched IIb-2	2	5.2	82	3	56	5	43	22
Mean ± SD	2	5 ± 0.2	76 ± 8.5	7 ± 5.7	43 ± 18.4	28 ± 32.5	54 ± 15.6	16.5 ± 0.5

Data are from two individual experiments with nonenriched or enriched CD34+ cells issued from the same G-CSF-mobilized patients. Absolute number of HSC (Day 0 of the second-step culture) and DC (Day 8 and Day 10 of the second-step culture) is shown.

ation rates with the nonenriched, two-step, cultured DC, particularly at the immature stage, as compared with enriched CD34 DC (**Fig. 5, A** and **B**). Thus, the allostimulatory capacity of DC generated by the nonenriched, two-step culture method was at least as potent as that of purified CD34 DC. It must be pointed out that the two types of DC induced much higher rates of proliferation than peripheral mononuclear cells, even when added at a 100-fold less ratio to responder T cells (Fig. 5B).

Generation of DC from nonenriched HSC by the two-step culture method results in DC that express similar phenotype than DC generated from enriched CD34 HSC in a two-step culture method

As HSC expansion during the first culture step requires the presence of Flt-3L, which is a major cytokine in DC differentiation [21–23], we then asked whether the faster differentiation of HSC into DC could be related to the presence of this cytokine for 15 days as opposed to 8 days only when no expansion was performed. Therefore, to answer this question, we compared the differentiating efficiency, starting from nonenriched HSC versus enriched CD34 HSC and using the same two-step culture method. As shown in Table 1B and similarly to the results obtained in Table 1A, following expansion, the percentage of CD34+ cells increased markedly from 2% at Day 0 to 26.9% at Day 7 in the nonenriched HSC culture while staying at a high percentage (75%) in the enriched HSC culture. In the second culture step, cells obtained by both methods expressed similar levels of CD1a and CD14 (**Fig. 6** and **Table 2B**). Accordingly, the levels of T cell costimulatory molecules (CD80, CD86) were comparable in both cells (Fig. 6 and Table 2B). Finally, after LPS maturation, the CD86 and

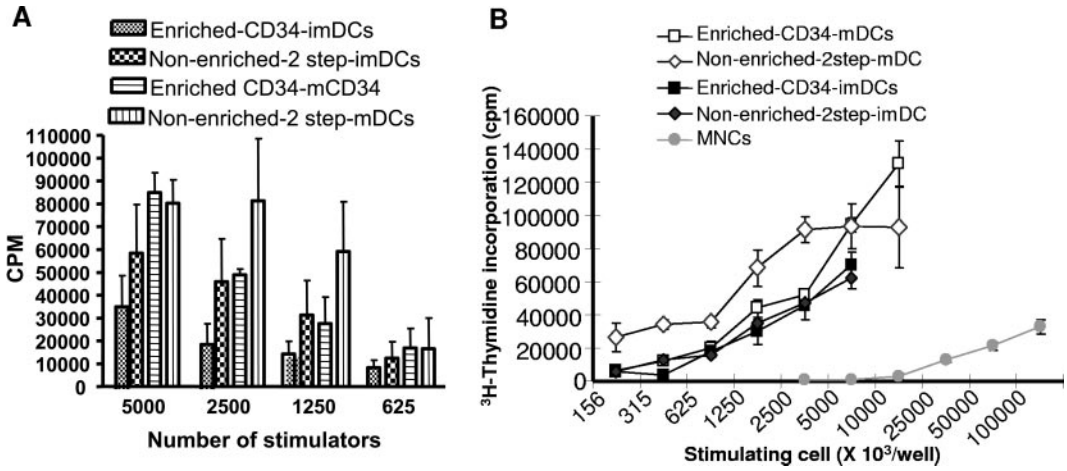

Fig. 5. Allogeneic stimulatory capacity of DC generated with the nonenriched, two-step culture method is comparable with that of one-step, cultured, enriched CD34 DC. T cells were stimulated in a primary MLR assay with graded doses of allogeneic DC generated from nonenriched but expanded HSC or enriched CD34 HSC. The number of allogeneic T cells per well was constant. The ratio of in vitro-generated DC to T cells varied from 1/20 to 1/160. Data are representative of four independent MLR assays (four at Day 8 and four at Day 10). The denomination "imDC" corresponds to DC at Day 8 of the differentiated step, whereas "mDC" corresponds to DC treated with LPS for 2 additional days. Data are represented as mean ± SD of these four MLR assays (A). Data show one MLR representative of four individual experiments (B).

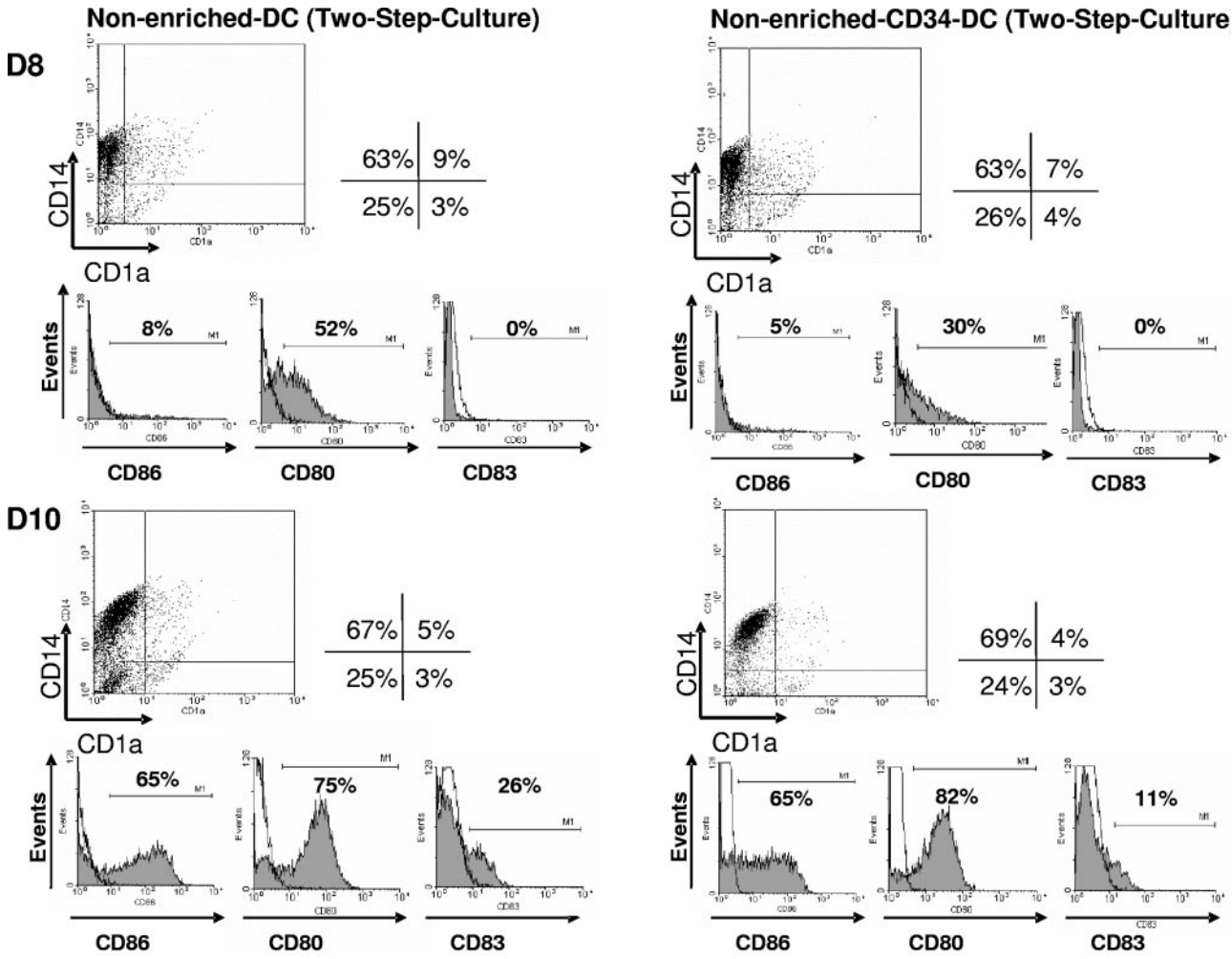

Fig. 6. DC generated by the nonenriched, two-step culture method present a similar phenotype than two-step, cultured, enriched CD34 DC. At the end of the second step of culture, DC were stained for CD1a and CD14 expression. The levels of expression of these molecules were compared by cytofluorometry with that of enriched CD34 DC in two-step culture. This phenotype is representative of two individual experiments. Quadrants were set up on the isotype-matched control dot plot, and 10,000 events were acquired. The percentage of nongated cells is shown in the dot-plot profiles. At Day 8, cells were induced to mature by exposure to 100 ng/ml LPS for 48 h. These phenotypes are representative of two individual experiments. The percentage of cells is shown above the histogram profile. Open histograms represent negative controls; solid histograms represent cells stained with the indicated, relevant mAb.

CD83 maturation marker was expressed at almost similar levels in DC obtained by the nonenriched, two-step culture method, as compared with enriched, two-step, cultured CD34 DC (Fig. 6 and Table 2B).

Generation of two-step, cultured DC from nonenriched HSC results in higher yields of DC as compared with two-step, cultured, enriched CD34 DC

As the phenotype of DC derived from nonenriched HSC at Day 8 was at least as differentiated as that of enriched CD34 DC, we compared the yields of DC obtained from both cultures by calculating the absolute number of DC obtained per HSC. As shown in **Figure 7**, a mean of 24.3 mature DC was obtained from one CD34 HSC using the nonenriched HSC culture method as compared with 3.6 mature DC/HSC, starting from enriched CD34 HSC. Therefore, these results indicate that the nonenriched HSC culture method is the most efficient in generating mature DC from HSC precursors.

DISCUSSION

In the current study, we developed a new culture method for generating large numbers of DC from hematopoietic progenitor cells without prior HSC enrichment. We divided the culture method into two steps. The first step aimed to initially expand HSC harvested from G-CSF-mobilized blood without prior purification of hematopoietic progenitors. Therefore, MNC were separated by gradient density and were cultured in the presence of cytokines known to favor the expansion of hematopoietic progenitors such as SCF, Flt-3L, and TPO. The second step was a DC differentiation phase in the presence of cytokines such as GM-CSF, Flt-3L, and TNF-α. This new, nonenriched, two-step culture method resulted first in a marked amplification of CD34+/CD38+/Lin^{neg} HSC after 7 days of culture and then in the differentiation of the expanded HSC into functional DC. In addition, these DC were able to mature by signaling through TLR4, which recognizes LPS.

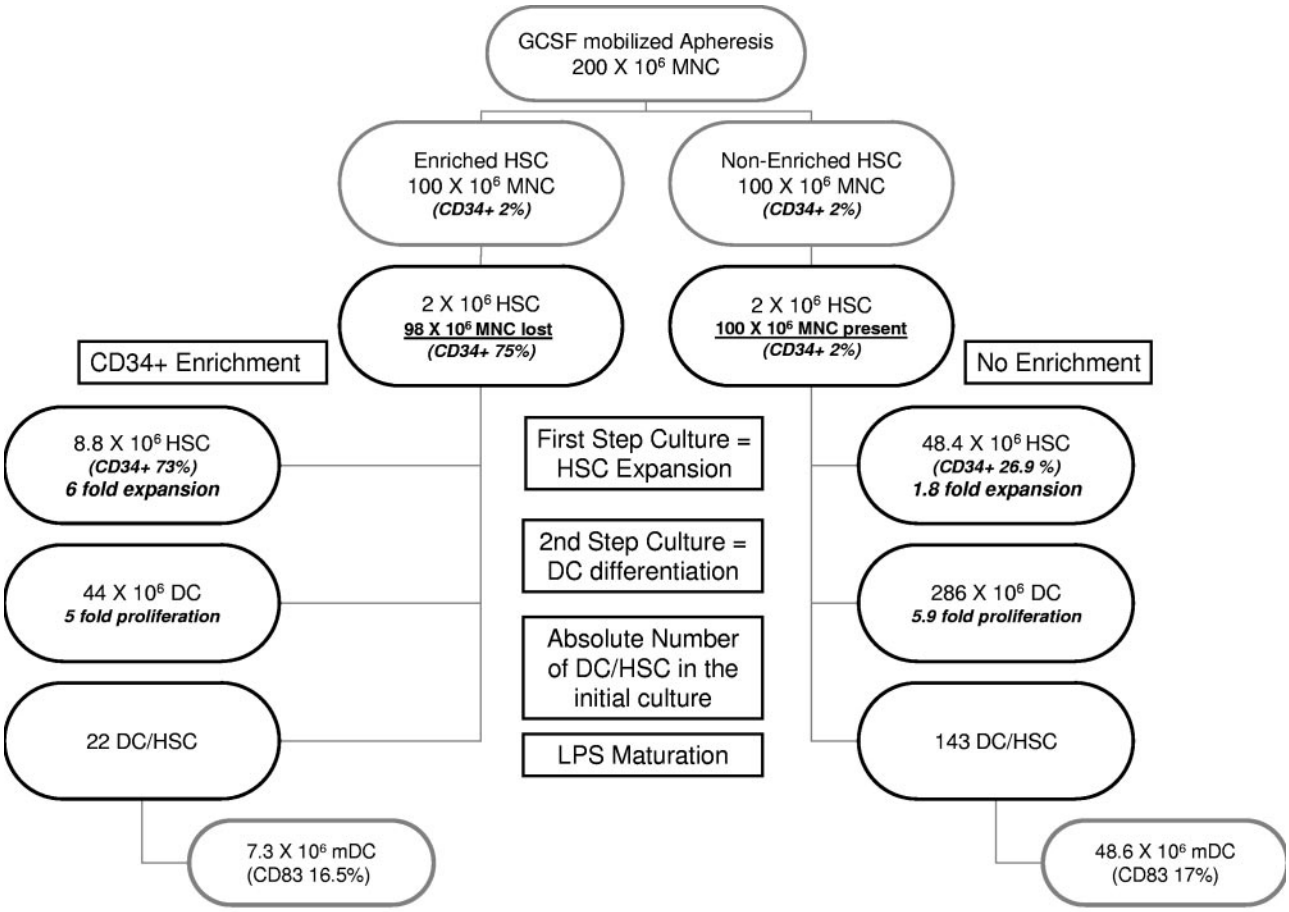

Fig. 7. DC generated by the nonenriched, two-step culture method results in larger yields than two-step, cultured, enriched CD34 DC. This figure represents a scheme of the different steps used to culture enriched CD34 DC and nonenriched DC, respectively. Starting from 200×10^6 G-CSF-mobilized MNC apheresis, two batches of 2×10^6 HSC (after CD34+ enrichment by a Miltenyi column) or 100×10^6 HSC for the enriched culture or the nonenriched culture method were seeded, respectively. Absolute numbers of HSC were calculated at the end of the first culture step by multiplying the mean of expansion during the first culture step (six for enriched HSC and 1.8 for nonenriched HSC, respectively) with the percentage of CD34+ cells (73% for enriched HSC and 26.9% for nonenriched HSC, respectively). Absolute numbers of DC were calculated at the end of the second-step culture method by multiplying the mean of expansion during the second culture step (five for enriched HSC and 5.9 for nonenriched HSC, respectively) with the absolute number of HSC. Then, ratios of DC per HSC were calculated, as well as ratio of mature DC per HSC.

G-CSF mobilizes hematopoietic progenitor cells from bone marrow to the peripheral blood and also promotes the maturation of myeloid cells. G-CSF-mobilized MNC comprise a major fraction of committed hematopoietic cells and a minor fraction of hematopoietic progenitor cells, which are mostly CD34+/CD38+/Lin^{neg}. Hence, using G-CSF-mobilized MNC as a direct source for expanding HSC circumvents the need for stem cell enrichment prior to expansion, thereby simplifying the process substantially in terms of efficiency and cost. In this study, only 2% of CD34+ cells were present in G-CSF-mobilized MNC, but the percentage of CD34+ cells increased up to 29% after 7 days of culture with early acting cytokines. However, this high percentage of CD34+ cells was transient, as it decreased from Day 7 to Day 14 of the first step of culture. Therefore, to take advantage of this rapid and strong CD34+/CD38+/Lin^{neg} HSC expansion, we defined the optimum period for differentiating HSC into DC to be at Day 7 of the first step of culture.

The well-established, standard, one-step method of DC generation from CD34+ precursors gives rise, after 8 days of culture, to two distinct populations of DC: the CD1a+CD14– cells, which are precursors of Langerhans cells, and the CD14+CD1a– cells, which are precursors of interstitial DC [1, 9–11]. In this study, DC generated from nonenriched, G-CSF-mobilized MNC or from enriched CD34+ cells were obtained from the same donors and were thus comparable. During the differentiation step of this new method, DC were generated earlier and at higher rates from expanded, nonenriched HSC as compared with the enriched CD34, one-step culture method. Indeed, DC generated from purified-CD34+ HSC did not express CD1a and CD14 markers before Day 8 of culture. On the contrary, DC generated from expanded but nonenriched HSC showed CD14- or CD1a-positive cells on the fifth day of the second culture step, and the percents increased up to Day 8. It has been reported by Caux et al. [2] that the appearance of a double-positive CD14+CD1a+ population correlates with the progressive disappearance of the CD14+CD1a– population, demonstrating that double-positive cells arise from CD14+ cells, which then acquire the CD1a marker. In our nonenriched, two-step culture method, the percentage of dou-

ble-positive cells was higher (4.3–14.1%) than that of enriched, one-step, cultured CD34 DC (0.9–5.1%), suggesting that the nonenriched, two-step, cultured DC were more differentiated.

CD34+ HSC have been shown to proliferate during differentiation into DC [2]. The fold increase during the differentiation step of enriched CD34 DC was of 10.8 ± 7.5 (mean±SD) and 2.5 ± 0.1 (mean±SD) for enriched, two-step CD34 DC versus 2.9 ± 1.5 (mean±SD) for DC generated by the nonenriched, two-step culture method and starting from the same amount of MNC in the initial culture. These data indicate thus that this new, nonenriched, two-step culture method leads to generation of higher yields of cells expressing a DC phenotype, as compared with the one-step culture method using enriched CD34 HSC. As the percentage of mature DC was higher with the nonenriched HSC two-step culture method as compared with the enriched HSC classical method, 34% versus 11%, respectively, our data suggest that this new, nonenriched, two-step culture method should lead to generation of more mature DC.

This rapid differentiation of HSC into mature DC, even without prior enrichment, could be related to the expansion step, as Flt-3L was present for 15 days during culture of nonenriched, two-step-generated DC, as compared with 8 days only during culture of enriched, one-step, cultured DC. Indeed, Flt-3L is known to trigger stimulation of Flt-3 receptor tyrosine kinase, which leads to expansion of early hematopoietic progenitors, but also of DC in human and mice [19–21]. In particular, in vitro or in vivo administration of Flt-3L drives Flt-3+ DC development from Flt-3+ progenitors [19–21]. To test this hypothesis, we performed a two-step culture comparing enriched and nonenriched cells in parallel. The results showed indeed that the phenotype of DC from enriched or nonenriched cells, obtained after 15 days of two-step culture, was comparable for CD14, CD1a, CD80, and CD86 markers, suggesting that the cytokines used in the expansion step are helping DC differentiation as well. It is interesting that the CD83 marker was expressed at similar percentage in both cells, assessing thus that Flt-3L may play an important role in the facilitation of DC generation and maturation.

TNF-α participates in the maturation process but is not sufficient by its own to stimulate the terminal differentiation of DC generated from CD34+ HSC. In previous studies, low or undetectable levels of CD83 expression in Day 8-generated CD34 DC have been described [22–26]. Accordingly, we observed that the CD83 maturation marker was expressed at low levels at the surface of enriched CD34 DC in one-step culture. Using the nonenriched, two-step culture method, we have been able to generate more differentiated DC, as up to 17% of the cells expressed CD83 prior to exposure to LPS, as compared with up to only 4% with the standard, enriched, one-step culture method. When we attempted to induce maturation of these DC by using LPS, the levels increased up to 34% with the nonenriched, two-step culture method, as compared with 11% with the standard, enriched, one-step culture method. In most instances, TLR4 stimulation by LPS favors the development of a Th1 response, particularly through IL-12 secretion [27]. In the particular case of enriched CD34 DC, LPS has been shown to mobilize MHC Classes I and II molecules from late endosomes to the cell surface, following activation of CD34+ Langerhans cells [28–29]. In addition, the efficacy of TLR4-mediated DC maturation was shown in a murine antitumor vaccination study [30]. Last, Blander and Medzhitov [31] have shown recently that phagocytosed particles with LPS contained stable MHC Class II molecules with indications of advanced stages of antigen processing, which suggests usefulness of LPS in the design of new vaccines.

Generation of higher amounts of DC by a two-step culture method has been reported already, using cord blood or bone marrow HSC [15, 16]. However, these protocols required enrichment of CD34+ cells and a 2-week expansion step before differentiating them into DC. Here, we found that expansion during 7 days only is sufficient to allow production of marked levels of HSC, even when starting from low percentages of CD34+ cells and that these cells differentiate into mature DC at high rates. Therefore, as compared with the other methods, our protocol is of particular interest when the amount of CD34+ cells harvested from G-CSF-mobilized blood is too low to allow enrichment. Indeed, in patients who undergo multiple chemotherapies, mobilization of HSC is known to be less efficient and thus, does not easily allow CD34+ HSC purification. Thus, when we addressed the possibility of obtaining DC, using cells from a patient who has been treated intensively with multiple chemotherapies for lymphoma, we have been able to generate significant amounts of DC from nonenriched, G-CSF-mobilized MNC, although at a lower yield than that usually obtained with healthy donors (data not shown). Therefore, as the range of DC injected per kg body weight varies from 0.1 to 1×10^6/kg body weight in clinical settings, this new method should allow sufficient amounts of DC to be obtained for cell therapy programs, even when poor grafts are obtained. Further experiments are planned to compare different media, particularly serum-free medium, to optimize the culture method.

In conclusion, this study provides a novel strategy for easily generating large numbers of DC from hematopoietic progenitors without any need of HSC purification. DC were generated from nonenriched, G-CSF-mobilized MNC by a two-step culture method and were shown to differentiate and mature efficiently at the phenotypical and functional levels. These findings might have implications into the use of DC as adjuvants for cell immunotherapy.

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