CD34+ Hematopoietic Progenitors from Human Cord Blood Differentiate Along Two Independent Dendritic Cell Pathways in Response to GM-CSF+TNFα

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Summary

Human dendritic cells (DC) can now be generated in vitro in large numbers by culturing CD34+ hematopoietic progenitors in presence of GM-CSF+TNFα for 12 d. The present study demonstrates that cord blood CD34+ HPC indeed differentiate along two independent DC pathways. At early time points (day 5–7) during the culture, two subsets of DC precursors identified by the exclusive expression of CD1a and CD14 emerge independently. Both precursor subsets mature at day 12–14 into DC with typical morphology and phenotype (CD80, CD83, CD86, CD58, high HLA class II). CD1a+ precursors give rise to cells characterized by the expression of Birbeck granules, the Lag antigen and E-cadherin, three markers specifically expressed on Langerhans cells in the epidermis. In contrast, the CD14+ progenitors mature into CDla+ DC lacking Birbeck granules, E-cadherin, and Lag antigen but expressing CD2, CD9, CD68, and the coagulation factor XIIIa described in dermal dendritic cells. The two mature DC were equally potent in stimulating allogeneic CD45RA+ naive T cells. Interestingly, the CD14+ precursors, but not the CD1a+ precursors, represent bipotent cells that can be induced to differentiate, in response to M-CSF, into macrophage-like cells, lacking accessory function for T cells.

Altogether, these results demonstrate that different pathways of DC development exist: the Langerhans cells and the CD14+-derived DC related to dermal DC or circulating blood DC. The physiological relevance of these two pathways of DC development is discussed with regard to their potential in vivo counterparts.

Dendritic cells (DC)1 are professional antigen-presenting cells that are required for the initiation of immune responses (1). Present at trace levels in all organs, DC are believed to function as sentinels of the immune system. Many types of DC with subtle differences in phenotype have been described in peripheral blood, skin, and lymphoid organs. In peripheral blood three different subsets of DC can be distinguished according to the expression of the surface antigens CD11c (2) or CD33 (3, 4) and CD83 (5). In the skin, epidermal Langerhans cells (LC) and the so-called dermal dendrocytes (Birbeck granule−, factor XIIIa+) add some diversity to the family of DC (6, 7). In peripheral lymphoid organs, populations with different localization and phenotype have been described in spleen (8) and Peyer’s patches (9). Although each of these DC subsets display the ability to activate naive T cells, it is not clear whether they represent different stages of maturation of a unique DC lineage or whether they stem from different progenitors. In this respect, thymic DC in mice appear to originate from a hematopoietic progenitor with lymphoid but no myeloid potential (10). This subset might have been identified also in humans (11). In contrast, DC related to LC generated in vitro from hematopoietic progenitors, using GM-CSF in mice (12–14) or GM-CSF+TNFα in human (15–19) appear to originate from a progenitor common to monocytes and granulocytes. Thus, different pathways of DC development may exist.

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1Abbreviations used in this paper: DC, dendritic cells; HPC, hematopoietic progenitor cells; LC, Langerhans cells.

Preliminary results were presented at the Third International Symposium on Dendritic Cells, held in Annecy (France) in June 1994; and at the Keystone Meeting on Dendritic Cells, held in Taos (New Mexico) in March 1995.
In this study we demonstrate that cord blood CD34+ HPC cultured with GM-CSF+TNFα develop along two independent DC pathways that, after 5–7 d of culture, can be identified at an immature stage according to CD1a and CD14 expression. CD1a+ precursors yield, at day 12–14, DC related to LC characterized by the expression of CDla, E-cadherin, Lag antigen and Birbeck granules. In contrast, CD14+ precursors yield, at day 12–14, DC related to dermal dendrocytes or peripheral blood DC, characterized by the lack of Birbeck granules, E-cadherin, and Lag antigen and the expression of CD1a, CD2, CD9, CD68, and factor XIIIa.

Materials and Methods

Hematopoietic Factors. rhGM-CSF (specific activity: 2 × 10^6 U/mg; Schering-Plough Research Institute, Kenilworth, NJ) was used at a saturating concentration of 100 ng/ml (200 U/ml). rhTNFα (specific activity: 2 × 10^6 U/mg; Genzyme, Boston, MA) was used at an optimal concentration of 25 ng/ml (50 U/ml) (20) rhSCF (specific activity 4 × 10^5 U/mg; R&D, Abington, UK) and rhM-CSF (specific activity: 2 × 10^5 U/mg; R&D) were used at optimal concentrations of 25 ng/ml.

Collection and Puriﬁcation of Cord Blood CD34+ HPC. Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen were isolated from mononuclear fractions (21, 22) through positive selection, using anti-CD34 mAb (Immuno-133.3, Immunotech, Marseille, France) and goat anti-mouse IgG-coated microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Isolation of CD34+ progenitors was achieved using Minimag's separation columns (Miltenyi Biotech GmbH) (23). In all experiments the isolated cells were 80–99% CD34+ as judged by staining with anti-CD34 mAb. After purification, CD34+ cells were cryopreserved in 10% DMSO.

Puriﬁcation of Cord Blood and Adult Peripheral Blood CD45RA+ T Cells. Mononuclear cells were isolated from adult peripheral blood or cord blood and depleted of adherent cells by overnight adherence to plastic, in complete medium, at 1 × 10^6 cells per ml. CD45RA+ T lymphocytes were then purified by immunomagnetic depletion using a cocktail of mAbs IOM2 (CD14), ION16 (CD16), and ION2 (HLA-DR) (Immunotech, Marseille, France); NKH1 (CD56) and OKT8 (CD14) (Becton Dickinson) mAbs directly labeled with FITC. Negative controls were performed with biotinylated mAbs revealed by PE-conjugated streptavidin (Becton Dickinson) or PE-conjugated mAbs. For double staining, after saturation in 5% mouse serum, cells were stained with OKT6 (anti-CD1a) (Ortho) or Leu-M3 (anti-CD14) (Becton Dickinson) mAbs directly labeled with FITC. Negative controls were performed with unrelated murine mAbs. Fluorescence analysis was determined with a FACSscan® flow-cytometer (laser setting: power, 15 mW; excitation wavelength, 488 nm; Becton Dickinson), 5,000 events and 10,000 to 50,000 events were collected for single and double staining, respectively.

Immunostainings. Cells were cytospin centrifuged for 4 min at 400 rpm on a microscope slide. Some slides were used for May Grünwald-Giemsa staining and the others were fixed in methanol/acetone at 20°C for 1 min for immunocytochemistry. Slides were washed in PBS for 5 min and incubated with mAbs anti-CD68 (IgG3, Mo876, Dako) anti-Lag (IgG1, (25)) and anti-S10013 (IgG1, Dako) and mAbs anti-CD14 (Ortho) or PE-conjugated Leu-M3 (CD14) (Becton Dickinson) or PE-conjugated mAbs. For double staining, after saturation in 5% mouse serum, cells were stained with OKT6 (anti-CD1a) (Ortho) or Leu-M3 (anti-CD14) (Becton Dickinson) mAbs directly labeled with FITC. Negative controls were performed with unrelated murine mAbs. Fluorescence analysis was determined with a FACSscan® flow-cytometer (laser setting: power, 15 mW; excitation wavelength, 488 nm; Becton Dickinson), 5,000 events and 10,000 to 50,000 events were collected for single and double staining, respectively.

Electron Microscopy Procedures. Cells were recovered from day 6 to day 16. After washes, cells were directly fixed for 18 h with 2% glutaraldehyde in cacodylate buffer, then for 1 h with 1% osmium tetroxide and embedded in epoxy medium after dehydration through a graded series of ethanol. Ultrathin sections were post-stained with uranyl acetate and lead citrate and examined on a JEOL 1200 EX electron microscope (CMEABG, Université de
A minimum of 100 cells of each population was analyzed for the presence of Birbeck granules.

T Cell Proliferation Assay. After 14 d of culture, CDla+ and CD14+-derived cells were collected and, after irradiation (30 Gy), used as stimulator cells for allogeneic CD45RA+ naive adult or cord blood T cells (2 × 10⁴ per well). From 10 to 10⁴ stimulator cells were added to the T cells in 96-well round-bottomed microtiter tissue-culture plates (Nunc, Roskilde, Denmark). Cultures were performed in RPMI 1640 medium supplemented with 10% heat-inactivated human AB+ serum, and glutamine and antibiotics as above. After 5 d of incubation, cells were pulsed with 1 μCi of ³H-TdR (specific activity 25 Ci/mmol) per well, for the last 8 h, harvested and counted. Tests were carried out in triplicate, and results were expressed as mean counts per minute (cpm) ± SD. The levels of ³H-TdR uptake by stimulator cells alone were always below 100 cpm.

Results

Identification of Two DC Precursor Subsets Based on Exclusive Expression of CDla and CD14. In previous studies we have reported that cord blood CD34+ hematopoietic progenitor cells (HPC) cultured for 12 d in presence of GM-CSF+TNFα yield DC characterized by the expression of CD1a (15). As addition of SCF allows a three- to fivefold increase in cell numbers after 6 d of culture without altering cell differentiation (18, and unpublished observations), SCF was added from day 0 to day 6 in all cultures performed along this study. Herein, we have followed, using double color fluorescence, the kinetic of CD1a and CD14 expression during the differentiation of CD34+ HPC cultured in the presence of GM-CSF+TNFα (Fig. 1 A, n >30 experiments). At day 0, CD34+ cells express neither CD1a nor CD14. At early time points (day 3 and 5), two populations characterized by the exclusive expression of CD1a and CD14 emerge independently, the CD14+ population being usually dominant over the CD1a+ population (mean 1.8 ± 0.8-fold, range 0.8–2.5). At day 7, a distinct population of double positive cells can be identified (3–8%), which reaches a maximum of 20–38% between day 8 and day 10. The appearance of this double positive population correlates with the progressive disappearance of the CD14+ CD1a- population, suggesting that the double positive cells stem from the CD14+ cells which acquire CD1a. From day 10 to day 12, the double positive population starts disap-
pearing and at day 14, most cells lack CD14 and express CD1a.

To confirm that CD14+ cells effectively acquired CD1a and lost CD14, the two populations, CD14+CD1a- and CD14-CD1a+, were FACS-sorted at day 5-6. As shown in Fig. 1 B, upon reculture in GM-CSF+TNFα for 7 additional days, the CD14+-derived cells expressed CD1a and lack CD14. The CD1a+-derived cells remained CD14--CD1a+.

To better characterize the two populations, an extensive phenotype was performed by double staining at day 5-6. Both populations are negative for CD5, 7, 8, 15, 16, 21, 24, 25, 34, 35, 45RA, 64, 83. They express comparable levels of CD4, 11a, 11c, 13, 15S, 18, 26, 33, 38, 39, 40, 43, 44, 45RO, 48, 49 (d and e), 50, 54, 58, 59, 74, 78, 80, 86 and HLA class II. CD1b and CD1c were expressed at higher levels on CD1a+ cells than on the CD14+ cells. Conversely, CD11b, CD32, and CD36 were more intensely expressed on CD14+ cells than on CD1a+ cells. Interestingly, while CD72 was expressed on CD1a+ cells, this molecule was not detected on CD14+ cells. On the contrary, only CD14+ cells bore CD2, CD9 and the M-CSF-R. Fig. 2 illustrates the major phenotypic differences observed between the two populations. Altogether, these results demonstrate that the two precursor populations, identified by the exclusive expression of CD1a or CD14, differed by the expression of a set of molecules confirming that the two populations are unique and unrelated entities.

**DC Precursors Do Not Proliferate but Differentiate into Mature DC.** To characterize the properties of the DC precursor populations and of their respective progenies, CD14+ and CD1a+ cells were routinely FACS-sorted after culturing CD34+ HPC with GM-CSF+TNFα+SCF for 5–6 d. First the proliferative capacity of the two populations was assessed by comparison with that of CD14-CD1a- cells. Sorted cells were seeded at 3 × 10⁴ cells per well in round-bottomed microtest tissue culture plates with or without growth factors and the proliferation was assessed 3 d later by ³H-TdR uptake. As shown in Fig. 3, both CD14+CD1a+ and CD14+CD1a- failed to proliferate, while the double negative population strongly proliferated in response to GM-CSF or GM-CSF+TNFα. The lack of proliferative capacity of CD14+CD1a+ and CD14+CD1a- populations was further confirmed in kinetic experiments based on the incorporation of Hoechst 33342 (not shown).
Thus, once the two precursors can be identified based on CD1a and CD14 expression, they no longer can proliferate while the precursors lacking CD1a and CD14 can still proliferate.

The differentiation potential of these two populations was then analyzed according to morphology and phenotype upon reculturing in presence of GM-CSF+TNFα up until day 16. The morphology of the cells at different stages of culture/maturation is shown in Fig. 4. At day 6, the two subsets contained a relatively homogeneous population of medium size with irregular shape (Fig. 4, A and E). The CD1a+ derived DC developed a pronounced dendritic morphology at day 9, maintained at day 12 (Fig. 4, B–D), with small dendrites homogeneously distributed over the cell surface. The dendritic morphology of CD14-derived DC developed at later time points (day 12 to day 16). The CD14+ derived DC are characterized by polarized lamellipodia (day 12, Fig. 4, F–H) and some cells with a veiled

Figure 4. The day 5 DC precursors differentiate at day 12-16 into cells displaying a dendritic morphology. Cord blood CD34+ HPC were cultured in presence of GM-CSF+TNFα. After 5–6 d, cells were FACS®-sorted into CD14+CD1a+ and CD14+CD1a−. Sorted cells were seeded in presence of GM-CSF+TNFα or M-CSF (1–2 × 10⁵ cells per ml) for 6–7 additional days, a last medium change being performed at day 10. Cells were processed for May Grünwald Giemsa (MGG) staining at day 6, 12, and 16. CD1a-derived DC cultured in GM-CSF+TNFα are shown after MGG staining at day 6 (A) and day 12 (B and C) and by phase contrast microscopy at day 12 (D). CD14-derived DC cultured in GM-CSF+TNFα are shown after MGG staining at day 6 (E), day 12 (F and G), and day 16 (I) and by phase contrast microscopy at day 12 (H). CD14-derived DC cultured in M-CSF are shown after MGG staining at day 12 (J). Magnification, ×400. Results are representative of four experiments.
morphology can be observed at certain stages of differentiation (day 12–16, Fig. 4, F-J).

In terms of phenotype (Fig. 5 A), at day 14 both populations yield cells expressing molecules expected on active DC including high levels of MHC class II, CD80, CD86, CD40, and the DC-specific marker CD83 and the intracytoplasmic molecule S100B (Fig. 6, A and B). The two populations also expressed similar levels of CD1c, CD4, CD11a, CD11c, CD13, CD15s, CD18, CD33, CD43, CD44, CD45RO, CD48, CD49e, CD49d, CD50, CD54, CD58, CD59, CD71, CD74, CD78. The phenotype of mature DC was usually observed earlier on the CD1a+--derived DC (day 10–12) than on the CD14+--derived DC (day 14–16) (not shown).

Taken together these results show that the two DC precursor populations isolated at day 5--6 differentiate, at day 12–16, into cells with a characteristic DC morphology and phenotype.

**The Two DC Populations Display Different Characteristics.** Although the two cell populations have a phenotype characteristic of DC, some major phenotypic differences could be observed at day 14. As shown in Fig. 5 B, the CD1a+-derived DC express low levels of CD72 and high levels of the E-cadherin, a molecule expressed on LC and involved in homophilic interactions between keratinocytes and LC in the epidermis (26, 27). In addition, immunostaining (Fig. 6, C and D) show that CD1a+-derived DC (19–34%) express the antigen Lag (Langerhans-associated granule, (25)). On the contrary, CD14+-derived DC lack CD72, the Lag antigen and E-cadherin. Furthermore, in contrast to CD1a-derived cells, CD14-derived cells display CD9, CD2, FCyRII/CD32, and the intracytoplasmic CD68 (see also Fig. 6, E and F). Finally, at day 14, CD14+-derived DC still express the complement receptor CD11b (12–35%) as well as CD36 (all the cells), not detected on CD1a+-derived DC. At day 16–18 these two molecules were lost on CD14-derived DC (not shown). Also expression of Factor XIIIa, a procoagulation factor described on dermal DC but not on epidermal LC, has been detected only on the CD14+-derived DC (Fig. 6, G and H). The restricted expression of Factor XIIIa on the CD14+-derived DC has been confirmed by RT-PCR (not shown).

The two cell populations were also analyzed by electron microscopy. At day 13, the two cell populations had a characteristic dendritic cell shape and a typical multilobed nucleus. However, as shown in Fig. 7, only the CD1a+-derived DC were found to develop Birbeck granules. Between 12 and 14% of cells in the total population express Birbeck granules against 31–56% in the CD1a+-derived DC population and 0–1% in the CD14+-derived DC population. In all experiments, and at any time point tested beyond day 6 (from day 13 to 16), the CD1a+-derived DC
population always contained cells with Birbeck granules while the CD14+-derived DC population never contained cells with Birbeck granules (≤1%).

Thus, CD1a+ precursors yield typical epidermal LC characterized by the expression of CD1a, Birbeck granules, Lag antigen, and E-cadherin, while the CD14+ precursors homogeneously differentiate into DC with features of dermal DC and blood DC such as Factor XIIIa, CD68, CD9, and CD2 expression.

**The CD14+CD1a- Precursors Differentiate into Macrophage-like Cells in Response to M-CSF.** As shown in Fig. 2, CD14+ precursors express the M-CSF-R while CD1a+ precursors do not. Thus, the effects of M-CSF on the differentiation of the two precursor populations was analyzed in comparison to that of GM-CSF+TNFα. After 7-8 additional days of culture with M-CSF, CD14+ cells remained alive while CD1a+ cells were lost. As shown in Fig. 8 A, CD14+ precursors cultured in presence of M-CSF differentiate into
CD14^+CD1a^- cells contrasting with the CD14^-CD1a^+ cells generated in presence of GM-CSF+TNFα. Furthermore, the M-CSF-derived cells were characterized by the lack of CD2, CD40, CD80, and CD83 expression, lower levels of CD86, HLA-DR and HLA-DQ (not shown) and higher levels of CD9 and CD11b. In term of morphology, as shown in Fig. 4 J, the M-CSF-derived cells, displayed a macrophage-like morphology with numerous vacuoles, contrasting with the dendritic morphology of the GM-CSF+TNFα-derived cells. These differentiation events occurred without proliferation as shown in Fig. 3.

Thus, the CD14^+ precursors display a double differentiation potential as they can be induced to differentiate into either DC in response to GM-CSF+TNFα or macrophage-like cells in response to M-CSF. In contrast, the CD1a^- precursors have a unique differentiation potential towards LC.

The Two DC Populations Are Equally Potent in Stimulating Naive T Cell Proliferation. Activation of naive T cells being the functional characteristic of dendritic cells, the two mature DC populations were analyzed with regard to their ability to induce allogenic CD45RA^- naive T cells proliferation. CD45RA^- naive T cells were isolated either from adult peripheral blood (Fig. 9 A) or from cord blood (Fig. 9 B). At day 14, comparable stimulatory capacities of the two DC populations were observed. Half maximal proliferation of CD45RA^- T cells from either adult or cord blood was obtained with 100-200 CD1a^- or CD14^-derived DC. As few as 10 cells of any populations were able to induce significant naive T cell proliferation (>100-fold background values). In contrast CD14^-derived cells recovered after M-CSF culture lacked stimulatory capacity for naive T cell proliferation (Fig. 9 C). Between 3 × 10^3–10^4 cells were required to induce a significant T cell proliferation, 1,000-fold more M-CSF-derived cells were required to induce levels of T cell proliferation observed with GM-CSF+TNFα-derived cells. Thus in addition to morphologic and phenotypic characteristics of dendritic cells CD1a^- and CD14^-derived cells have the functional property of dendritic cells, i.e., potent induction of naive T cell proliferation.

Discussion

Previous studies in humans and mice have demonstrated the central role of GM-CSF in the generation of DC from hematopoietic progenitor cells. Here, we demonstrate that under a unique culture condition (GM-CSF+TNFα), CD34^-HPC are induced to differentiate along two distinct DC pathways. After 3–7 d of culture, two precursor popu-
lations, characterized by the reciprocal expression of CD1a or CD14 antigens, emerge independently. Each precursor population expresses a unique pattern of surface molecules: CD1a+ precursors specifically bearing CD72 while CD14+ precursors express CD9, CD11b, CD36, and the M-CSF-R. In response to GM-CSF+TNFα, both populations mature at day 12-16 into DC characterized by a dendritic morphology, a phenotype of DC (high HLA class I and II, CD1a+, CD83+, CD86+, CD40+, CD14-) and the capacity to activate naive CD45RA+ cord blood T cells. The CD1a-derived DC mature, at day 12, into typical LC according to the expression of Birbeck granules, intracytoplasmic Lag molecule and of surface E-cadherin, all borne by epidermal LC (25, 26, 27). The CD14+-derived cells mature, at day 14–16, into DC that lack the LC antigens Lag and E-cadherin as well as Birbeck granules but express Factor XIIIa, CD11b, and CD36, markers of dermal (interstitial) dendritic cells (6, 7). CD14+ derived DC, in contrast to CD1a-derived DC, do not express CD72.

Our study challenges the concept that the DC populations observed in vivo may indeed represent different stages of maturation/activation of the same DC lineage and raises the question about the relationship that exists between these early hematopoietic progenitors. Our results suggest that LC may represent an epithelial specific cell directly arising from precursors such as circulating CD34+ cells that could mature locally. By contrast, the circulating peripheral blood DC might represent cells in migration from the bone marrow to the peripheral organs (e.g., dermis, liver, kidney . . .) or from the peripheral organs to secondary lymphoid organs (e.g., spleen, lymph nodes). Our present findings would be consistent with the observations in rel-B knock-out mice which have normal LC but lack interdigitating cells (30, 31).

The CD1a+-derived DC (epithelial DC type) may represent an independent pathway of development unrelated to monocytes. In contrast, the CD14+-derived DC are likely linked to the monocyte lineage for the following reasons: (a) at certain stage of maturation, they share with monocytes, a number of molecules such as CD14, CD11b, CD36, and CD68; (b) they transiently display non-specific-esterase activity, a marker of scavengers while CD1a-derived DC do not (manuscript in preparation); (c) upon M-CSF activation, CD14+ precursors differentiate into macrophage-like cells lacking accessory function for T cells, as recently described (32), while CD1a+ precursors do not respond to M-CSF, presumably because of the absence of M-CSF-R expression. In line with this hypothesis, peripheral blood monocytes which differentiate into macrophages in re-
Figure 9. The two DC subpopulations display overlapping naive T cell activation properties. Cord blood CD34⁺ HPC were cultured in presence of GM-CSF+TNFα. After 5-6 d, cells were FACS⁺-sorted into CD14⁻CD1a⁺ and CD14⁺CD1a⁻. Sorted cells were seeded (1-2 × 10⁶ cells per ml) in presence of GM-CSF+TNFα (A and B), or M-CSF (C) for 7-8 additional days, a last medium change being performed at day 10. (A and B) Cells were recovered at day 14, after culture in GM-CSF+TNFα, and used after irradiation (30 Gy) as stimulator cells for adult (A) or cord blood (B) naive CD45RA⁺ T cells (2 × 10⁴ cell per well). (C) CD14⁺-derived cells were recovered at day 14, after culture with GM-CSF+TNFα or with M-CSF and used after irradiation (30 Gy) as stimulator cells for adult naive CD45RA⁺ T cells (2 × 10⁴ cell per well). The proliferation was revealed by ³H-TdR uptake after 5 d of culture. Results are expressed as mean cpm ± SD of triplicate cultures. Results of each panel are representative of three experiments or more.

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