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 CD1a$^+$ 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.

[1] *Abbreviations used in this paper:* DC, dendritic cells; HPC, hematopoietic progenitor cells; LC, Landerhans 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 CD1a, 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 × 10^7 U/mg; Genzyme, Boston, MA) was used at an optimal concentration of 2.5 ng/ml (50 U/ml) (20) rhSCF (specific activity 4 × 10^5 U/mg; R&D, Abington, UK) and rhM-CSF (specific activity: 2 × 10^6 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 (Immu-133.3, Immunotech, Marseille, France) and goat anti–mouse IgG-coated microbeads (Miltenyi Biotec GmBH, Bergish Gladbach, Germany). Isolation of CD34$^+$ progenitors was achieved using Minimacs separation columns (Miltenyi Biotec 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 (CD8)(Ortho Diagnostic System, Raritan, NJ); and 4G7 (CD19), mAb 89, (CD40)(24), and UCHL-1 (CD45RO). After two rounds of bead depletion, the purity of CD45RA$^+$ T cells was routinely higher than 95%.

Liquid Cultures for Dendritic Cell Generation. Cultures were established in the presence of SCF, GM-CSF, and TNFα, as described (15, 18) in endotoxin-free medium consisting of RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Flow Laboratories, Irvine, UK), 10 mM Hepes, 2 mM L-glutamine, 5 × 10^{-5} M 2-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) (referred to as complete medium).

After thawing, CD34$^+$ cells were seeded for expansion in 25 to 75 cm^2 falcons (Corning, New York; NK) at 2 × 10^4 cells/ml. Optimal conditions were maintained by splitting these cultures at day 4 with medium containing fresh GM-CSF and TNFα (cell concentration: 1–3 × 10^5 cells/ml). For most experiments cells were routinely collected after 5–6 d of culture for FACS®-sorting.

Isolation of CD1a and CD14 DC Precursors by FACS®-Sorting. After 5–6 d of culture in presence of SCF, GM-CSF, TNFα, cells were collected and labeled with FITC-conjugated OKT6 (CD1a) (Ortho) and PE-conjugated Leu-M3 (CD14) (Becton Dickinson & Co., Mountain View, CA). Cells were separated according to CD1a and CD14 expression into CD14$^+$CD1a$^-$, CD14$^-$CD1a$^+$, and eventually CD14$^-$CD1a$^-$ fractions using a FACStarplus® (laser setting: power, 250 mW; excitation wavelength, 488 nm; Becton Dickinson). All the procedures of staining and sorting were performed in presence of 5 mM EDTA in order to avoid cell aggregation. Reanalysis of the sorted populations showed a purity higher than 98%.

Sorted cells were seeded in the presence of GM-CSF+TNFα (1–2 × 10^5 cells per ml) for 6–7 additional days, a last medium change being performed at day 10. Cells were routinely collected between day 11 and day 14. Eventually adherent cells were recovered using a 5 mM EDTA solution.

Proliferation Assay. Cells at day 5–6 (CD14$^-$CD1a$^+$, CD14$^+$CD1a$^-$, CD14$^-$CD1a$^-$) and total populations were seeded at 3 × 10^4 cells per well in round-bottomed microtest tissue culture plates in presence of medium alone, GM-CSF, TNFα, GM-CSF+TNFα or M-CSF. After 3 d of culture, cells were pulsed with 1 μCi of ^{3}H-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

Cytofluorimetric Cell-Surface Phenotyping. Cells were processed for double staining (day 6) or single staining (day 12 sorted populations) using either uncoupled mAbs revealed by phycoerythrin (PE)-conjugated sheep F(ab')$_2$ anti–mouse immunoglobulin (Ortho) or 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 FACScan® 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 cytocentrifuged 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 immunocytology. Slides were washed in PBS for 5 min and incubated with mAbs anti-CD68 (IgG3, Mo876, Dako) anti-Lag (IgG1, (25)) and anti-S100β (IgG1, S2532, Sigma) and a rabbit polyclonal anti-Factor XIIIa (Nordic Immunological Laboratories, Tilburg, Netherlands) and mAb and polyclonal matched controls for 60 min. The slides were washed twice in PBS. For CD68, slides were incubated with biotinylated sheep anti–mouse IgG3 for 30 min (PB276u; Binding Site, Birmingham, UK). For S100β and Lag, slides were incubated with biotinylated sheep anti-mouse IgG1 (PB273; Binding Site) for 30 min. For Factor XIIIa, slides were incubated with biotinylated goat anti–rabbit Ig (E432; Dako) for 30 min. All stainings were then incubated with streptavidin coupled to alkaline phosphatase for 30 min. The slides were washed three times in PBS and the enzyme activity was developed by the fast red substrate (Dako).

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 ethanols. Ultrathin sections were post-stained with uranyl acetate and lead citrate and examined on a JEOL 1200 EX electron microscope (CMEABG, Université de

Lyon, Lyon, France). 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, CD1a$^+$- 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 (2×10^4 per well). From 10 to 10^4 stimulator cells were added to the T cells in 96-well round-bottomed microtest 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 ^{3}H-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 ^{3}H-TdR uptake by stimulator cells alone were always below 100 cpm.

Results

Identification of Two DC Precursor Subsets Based on Exclusive Expression of CD1a 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-

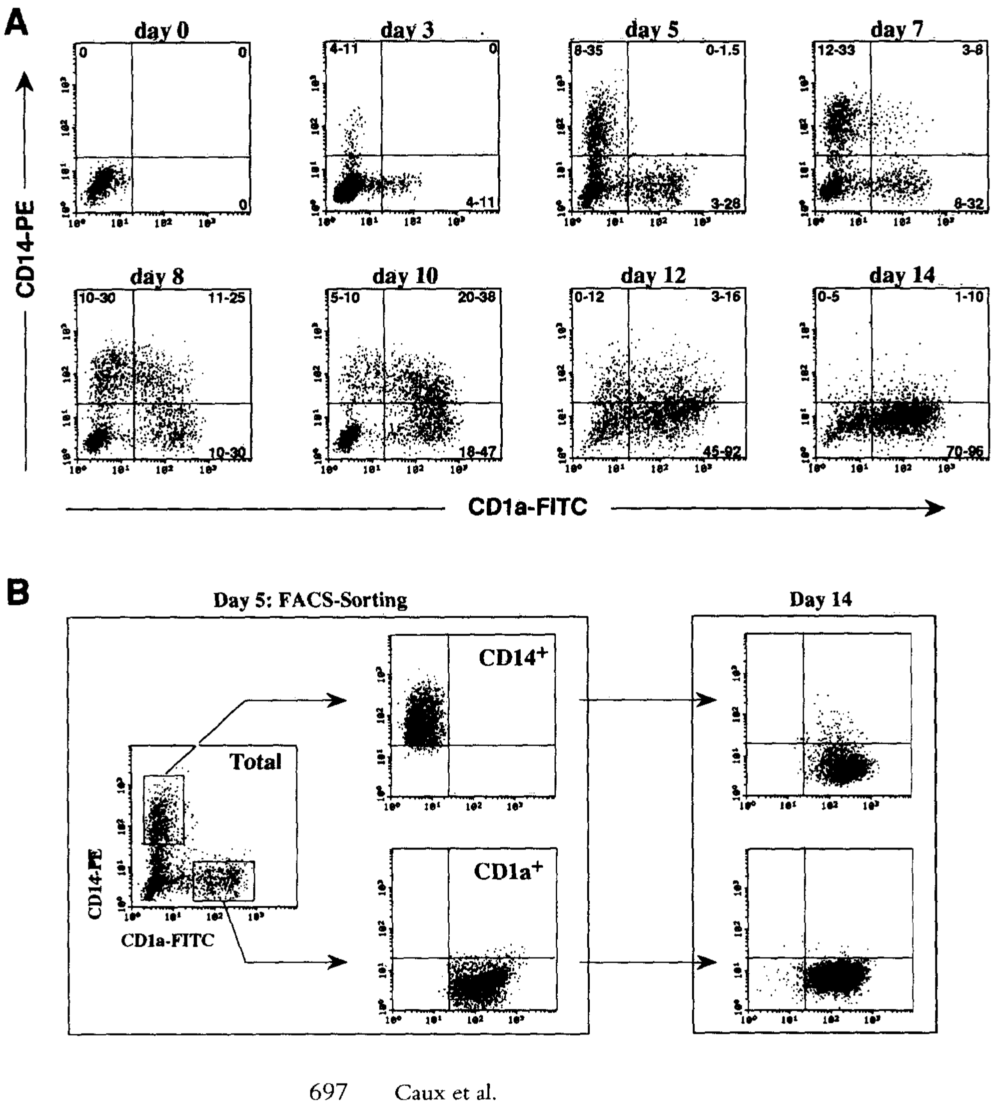

Figure 1. Expression of CD1a and CD14 on 5–6 d cultured CD34$^+$ HPC delineate two subpopulations of dendritic cells. (*A*) Kinetics of CD1a and CD14 expression during culture of CD34$^+$ HPC in GM-CSF+TNFα. Cord blood CD34$^+$ HPC were cultured in presence of GM-CSF+TNFα for 14 d. At the time point indicated, independent aliquots of cells were recovered and cells were processed for double staining using anti-CD14-PE and anti-CD1a-FITC. Quad were set up on the isotype matched control dot plot, 10,000 events were acquired. Numbers represents ranges from more than 30 experiments. (*B*) Cord blood CD34$^+$ HPC were cultured for 5–6 d in presence of GM-CSF+TNFα. Then the cells were collected, processed for double staining using anti-CD14-PE and anti-CD1a-FITC and FACS®-sorted into CD1a$^+$CD14$^-$ and CD14$^+$-CD1a$^-$ (*left panel*). Sorted cells were seeded in presence of GM-CSF+TNFα ($1–2 \times 10^5$ cells per ml) for 6–7 additional days, a last medium change being performed at day 10. At day 12, cells were reanalyzed for CD1a and CD14 expression by double color fluorescence (*right panel*). Quad were set up on the isotype matched control dot plot, 10,000 events were acquired. Results are representative of more than 30 experiments.

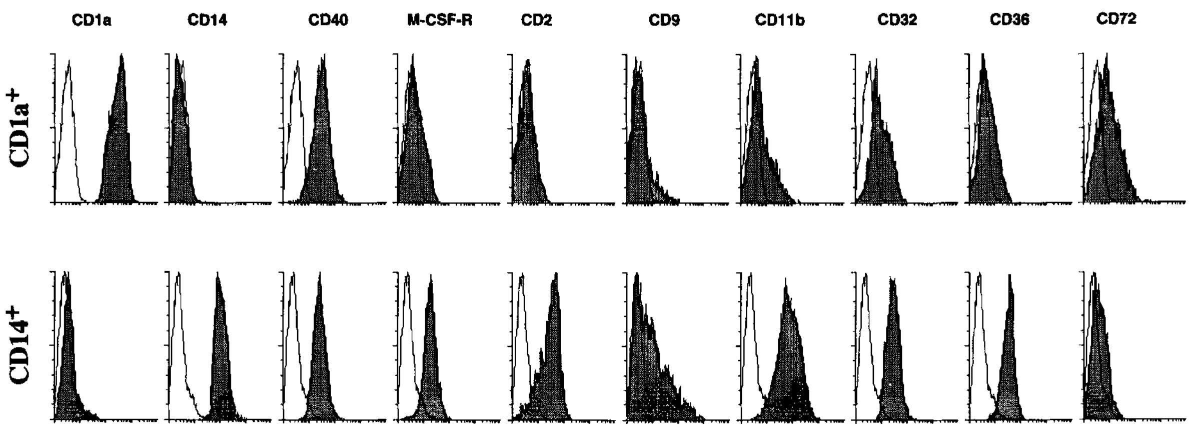

Figure 2. The day 5 precursors display a discrete phenotype. Cord blood CD34$^+$ HPC were cultured for 5–6 d in presence of GM-CSF+TNFα. The phenotype of the different populations was determined by two-color analysis. Cells were processed for double staining using either uncoupled mAbs revealed by PE-conjugated anti-mouse Ig or biotinylated mAbs revealed by PE-conjugated streptavidin or PE-conjugated mAbs. Then, after saturation in 5% mouse serum, cells were stained with anti-CD14-FITC or anti-CD1a-FITC. 20,000–50,000 events were acquired. Histograms show PE staining gated either on CD1a$^+$ cells (*top*) or on the CD14$^+$ cells (*bottom*). White histograms show the isotype matched controls. Results are representative of more than six experiments. mAbs were purchased as follows: CD2-PE (S5.2; Becton Dickinson), CD9-PE (CBL-162; Cymbus Bioscience LTD, Southampton, UK), CD11b-PE (D12; Becton Dickinson), CD32-PE (B73-1; Caltag Labs.), CD36-biotin (SMO; Ancell Corporation, Bayport, MN), CD40 (mAb 89; Immunotech), CD72 (BU040; Binding Site), c-*fms* (AB-2; Oncogene-Science, Inc., Cambridge, MA).

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.

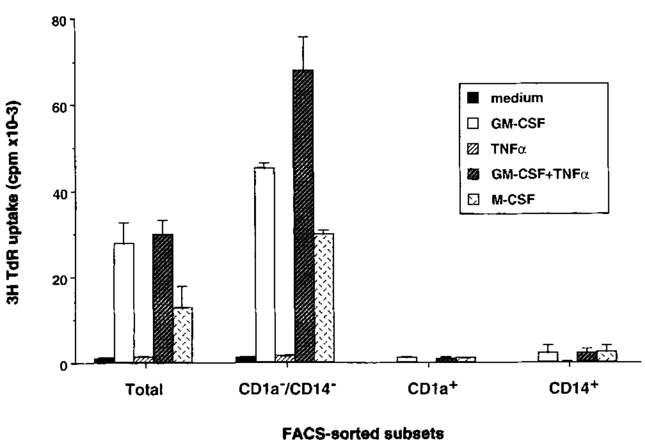

Figure 3. The isolated day 5–6 dendritic cell precursors lack proliferative capacity. Cord blood CD34$^+$ HPC were cultured in presence of GM-CSF+TNFα. After 5–6 d, cells were collected, processed for double staining using anti-CD14-PE and anti-CD1a-FITC and FACS®-sorted into CD14$^-$CD1a$^+$, CD14$^+$CD1a$^-$, and CD14$^-$CD1a$^-$. Sorted cells were seeded at 3 × 10^4 cells per well in round-bottomed microtest tissue culture plates in presence of medium alone, GM-CSF, TNFα, GM-CSF+TNFα, or M-CSF. Proliferation was measured by ^{3}H-TdR uptake after 3 d of culture. Results are representative of four experiments.

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 × 10^4 cells per well in round-bottomed microtest tissue culture plates with or without growth factors and the proliferation was assessed 3 d later by ^{3}H-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

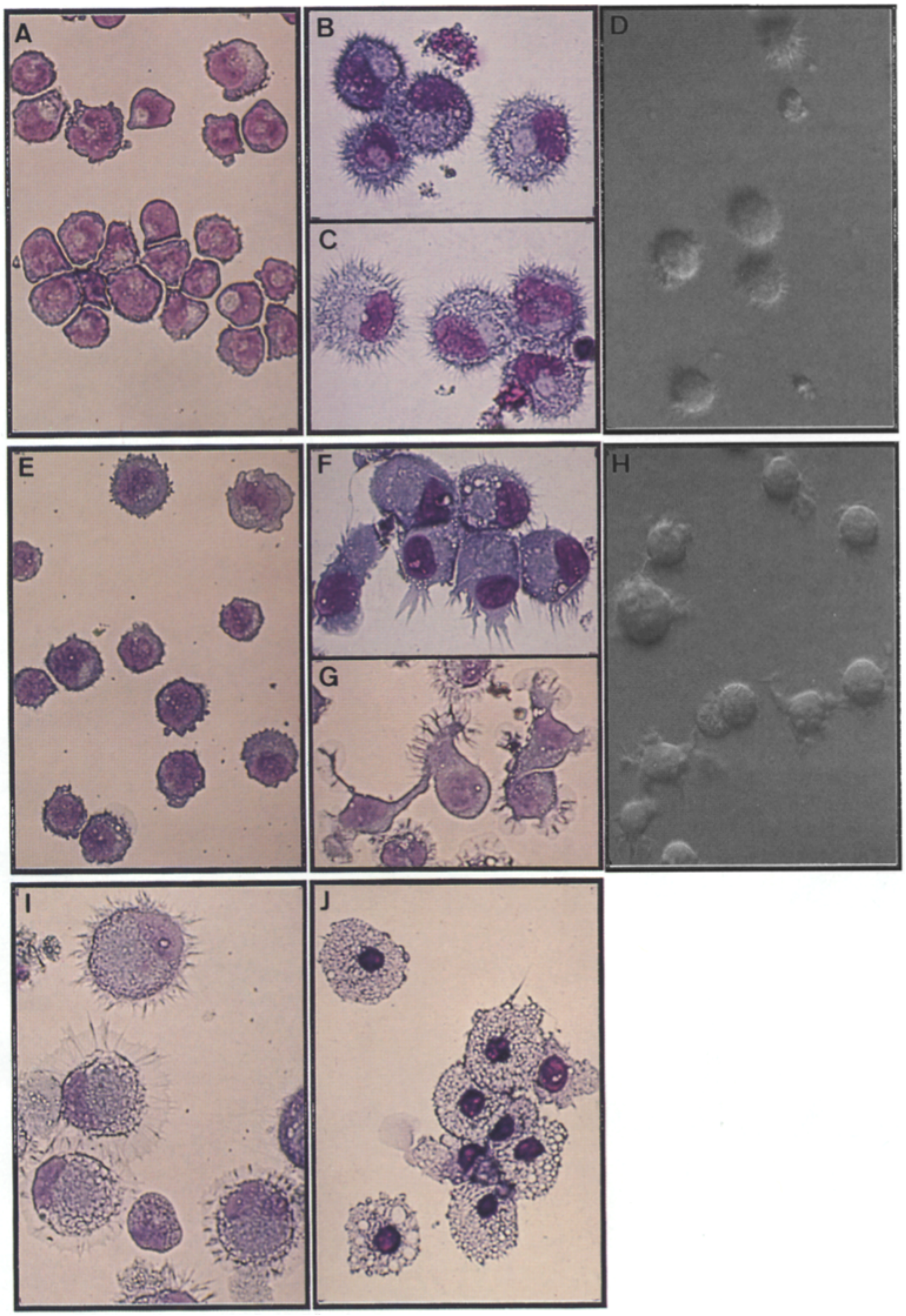

Figure 4. The day 5 DC precursors differentiate at day 12–16 into cells displaying a dendritic morphology. Cord blood $CD34^+$ HPC were cultured in presence of GM-CSF+TNFα. After 5–6 d, cells were FACS®-sorted into $CD14^-CD1a^+$ and $CD14^+$-$CD1a^-$. Sorted cells were seeded in presence of GM-CSF+TNFα or M-CSF (1–2 × 10^5 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–I*).

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 S100β (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, FCγRII/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

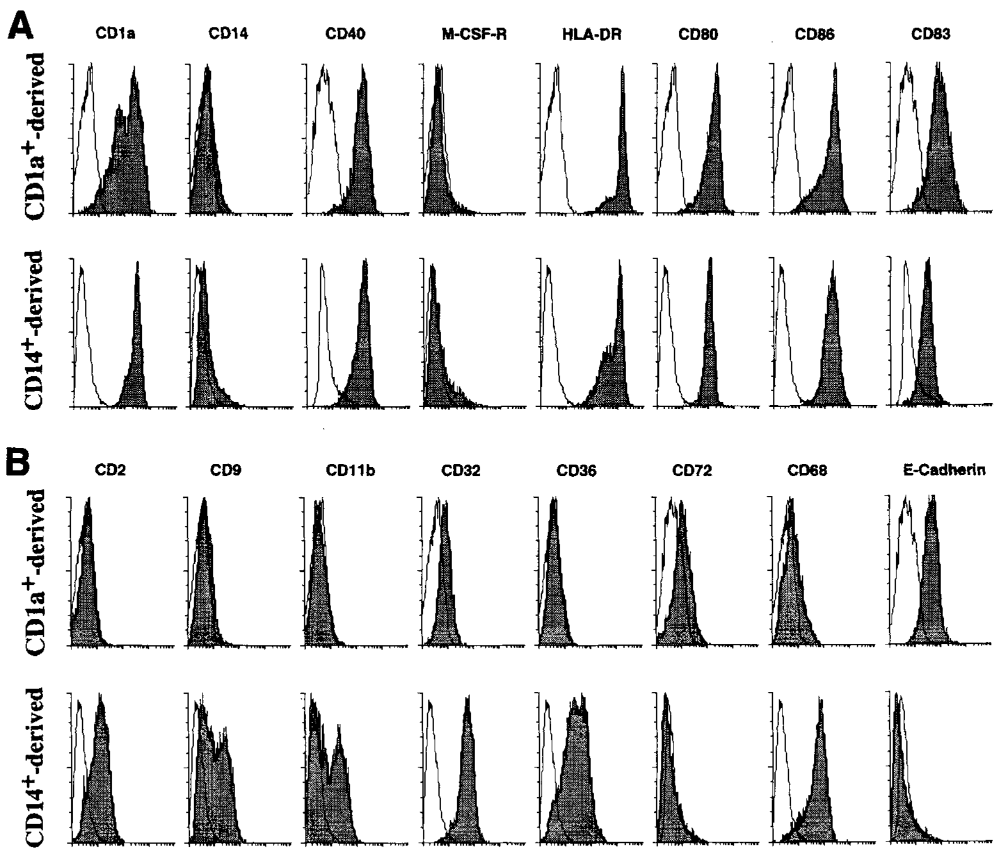

Figure 5. The day 5 dendritic cell precursors differentiate at day 14 into cells with typical dendritic cell antigens as well as discrete markers. Cord blood $CD34^+$ HPC were cultured in presence of GM-CSF+TNFα. After 5–6 d cells were FACS®-sorted into $CD14^-CD1a^+$ and $CD14^+CD1a^-$. Sorted cells were seeded in presence of GM-CSF+TNFα (1–2 × 10^5 cells per ml) for 6–7 additional days, a last medium change being performed at day 10. At day 14 the phenotype of the two populations was determined by single color analysis. Cells were stained using either uncoupled mAbs revealed by PE-conjugated anti-mouse Ig or biotinylated mAbs revealed by PE-conjugated streptavidin or PE-conjugated mAbs. 5,000 events were acquired. White histograms show the isotype matched controls. Results are representative of more than six experiments. (*A*) Dendritic cell antigens. (*B*) Discrete markers. mAbs were purchased as followed: CD68, (EMB11; Dako), CD80-PE (L307.4; Becton Dickinson), CD83 (HB15a; kindly provided by Zhou and Tedder), CD86-PE (IT2.2; PharMingen), E-cadherin (Hecd-1; Takara).

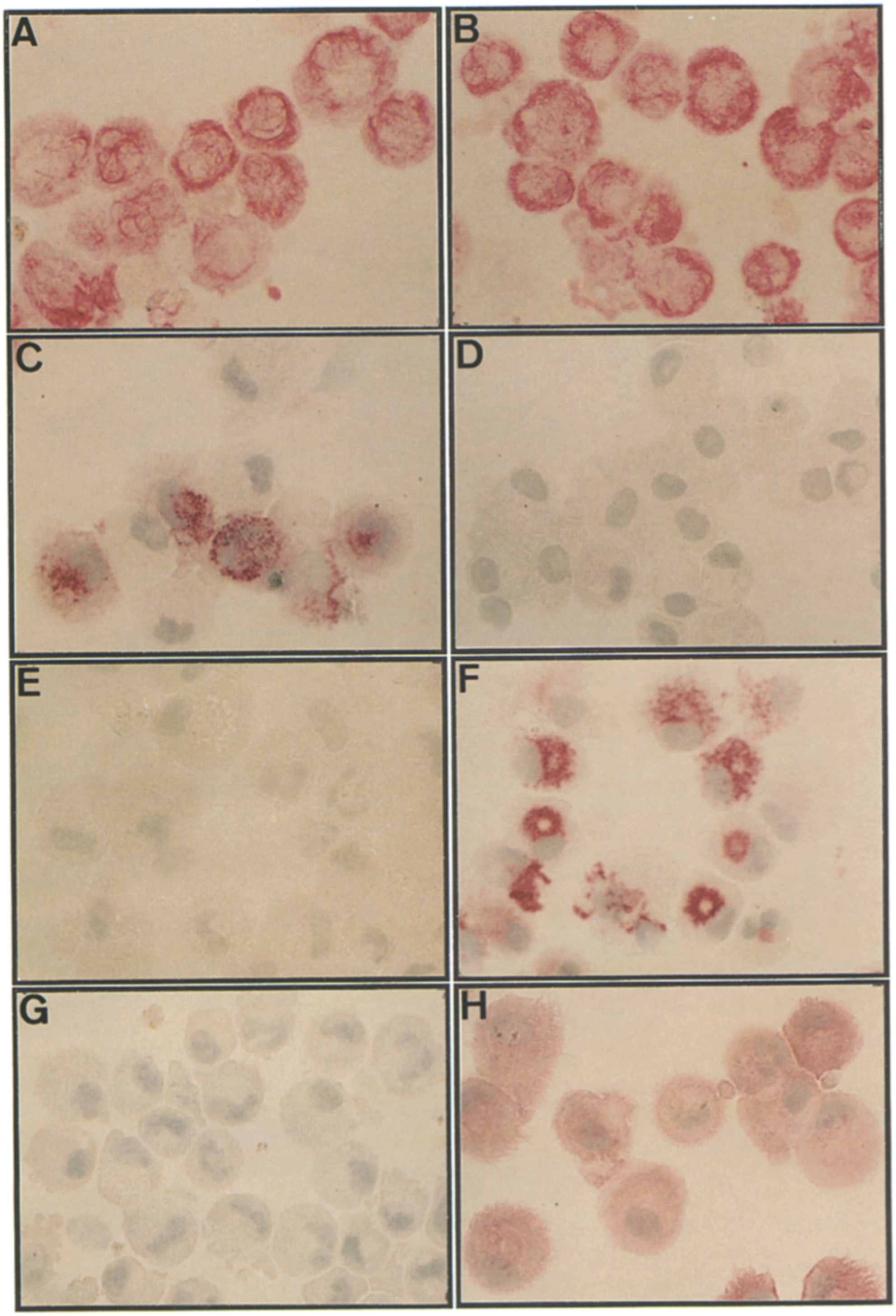

Figure 6. Immunostainings for intracytoplasmic molecules revealed phenotypic differences between the two DC populations. CD1a- and CD14-derived DC subpopulations were obtained as described in Materials and Methods and legends of Figs. 2 and 5. At day 12, cells were fixed and processed for immunostaining. Cells were stained with mAbs anti-S100β (*A* and *B*), anti-Lag (*C* and *D*), and anti-CD68 (*E* and *F*) and polyclonal anti-Factor XIIIa (G and *H*). CD1a-derived DC are shown in *A, C, E, G,* and CD14-derived DC in *B, D, F,* and *H.* No staining was detected with mAb and polyclonal matched controls (not shown). Magnification, ×400. Results are representative of three experiments.

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

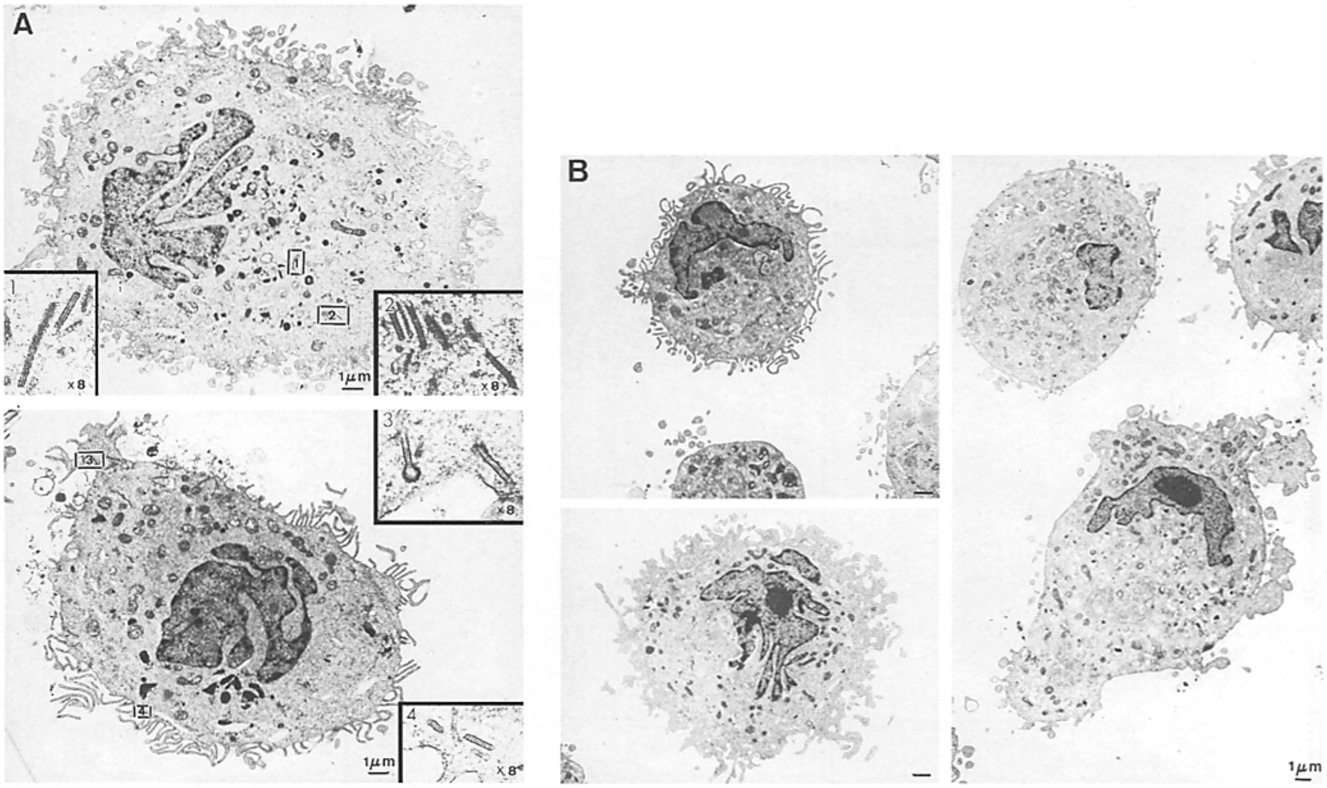

Figure 7. Only CD1a$^+$-derived DC develop Birbeck granules at day 13. CD1a- and CD14-derived DC subpopulations were obtained as described in Materials and Methods and legends of Figs. 2 and 5. At day 13, cells were fixed and processed for electron microscopy observation. *A* represents CD1a-derived DC and *B* represents CD14-derived DC. Bars represent 1 μm. Insets in *A* represent higher magnification (×8) of Birbeck granules. Between 100 and 200 cells were observed for each condition. At day 6, Birbeck granules were never detected. At day 13, the cells expressing Birbeck granules represent 12–14%, 31–56%, and 0–1% of bulk, CD1a$^+$ and CD14$^+$ populations, respectively. Percentages represent ranges from more than four experiments.

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 allogeneic 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-

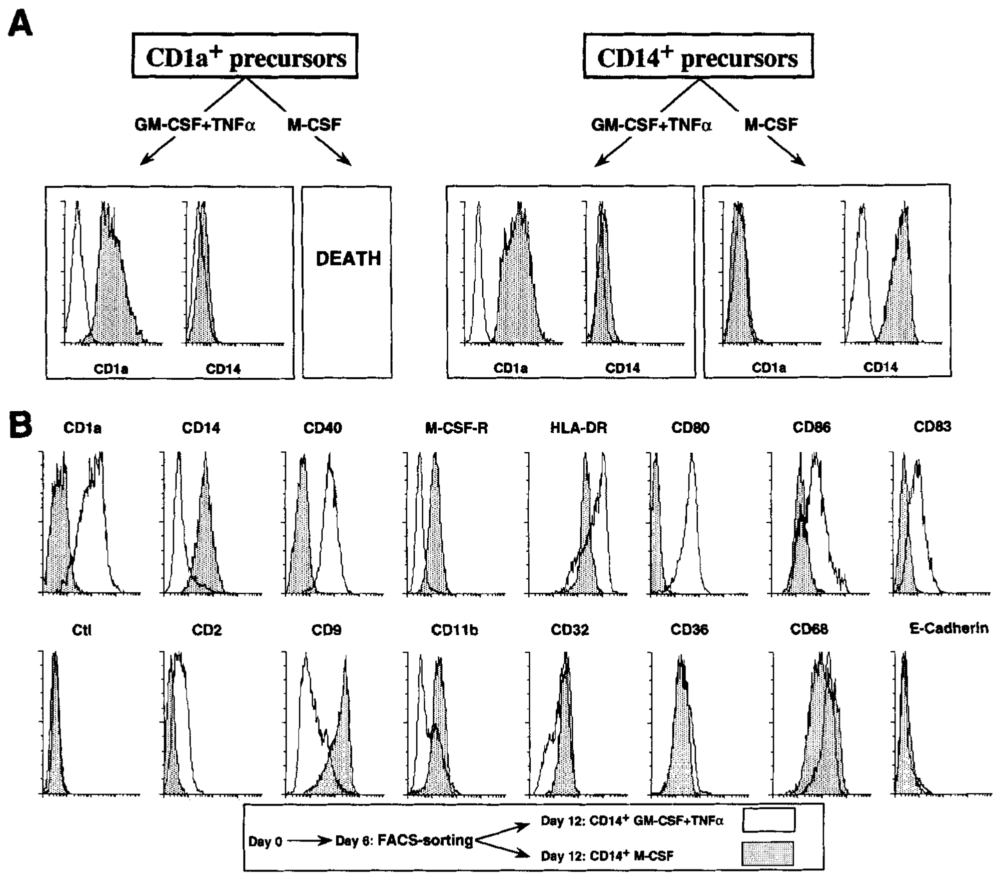

Figure 8. Only CD14 precursors cultured in presence of M-CSF differentiate into monocyte/macrophage-like cells. (*A*) Cord blood CD34$^+$ HPC were cultured in presence of GM-CSF+TNFα. After 5–6 d cells were FACS®-sorted into CD14$^-$-CD1a$^+$ and CD14$^+$CD1a$^-$. Sorted cells were seeded in presence of GM-CSF+TNFα or M-CSF (1–2 × 10^5 cells per ml) for 7–8 additional days, a last medium change being performed at day 10. At day 14, cells were reanalyzed for CD1a and CD14 expression by single-color fluorescence using anti-CD1a-PE and anti-CD14-PE. 5,000 events were acquired. White histograms show the isotype match controls. Results are representative of five experiments. (*B*) CD14 precursors isolated at day 5–6 (see legends to Figs. 2 and 5) were cultured in presence of GM-CSF+TNFα or M-CSF. At day 14, the phenotype of the cells was determined by single color analysis (see legend to Fig. 5. 5,000 events were acquired. The phenotype of CD14 precursors cultured in M-CSF (*gray*) or in GM-CSF+TNFα (*white*) is shown. Results are representative of three experiments.

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, also express CD9 and CD2 molecules, which have been identified on circulating peripheral blood DC (2, 28, 29).

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 in vitro DC populations, the in vivo ones and their specific 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 responses to M-CSF (33), have been shown to differentiate into DC upon activation with GM-CSF+IL-4 (34-36).

The existence of different pathway of DC development is indeed supported by the description, in mice, of thymic DC originating from hematopoietic precursors with a lymphoid differentiation potential contrasting with myeloid precursors yielding splenic DC (10, 14, 37). In this context, a CD10$^+$ CD34$^+$ HPC able to differentiate into DC, T, B, and NK cells but not into myeloid cells has also been identified in humans (11), thereby supporting the notion that one type of DC is developmentally more closely related to the lymphoid lineage than to the myeloid lineage. These cells might actually correspond to the presently described CD1a$^+$-derived DC which originates from early CD38^{low} CD34$^+$ HPC (not shown). This CD1a$^+$-derived DC probably corresponds to the CFU-DC progenitor yielding pure DC colonies identified by Young et al. (38). Further experiments are required to formally establish this hypothesis. On the contrary, the CD14$^+$-derived DC are clearly developmentally linked to the myeloid lineage as illustrated by the isolation of CD14$^+$CD1a$^-$ common precursors that can yield either macrophages in response to M-CSF or dendritic cells in response to GM-CSF+TNFα. In summary, this study demonstrates that CD34$^+$ HPC yield two separate precursors, CD14$^-$CD1a$^+$ and CD14$^+$CD1a$^-$, that can differentiate without proliferating into phenotypically distinct dendritic cells respectively corresponding to epidermal and interstitial/dermal DC.

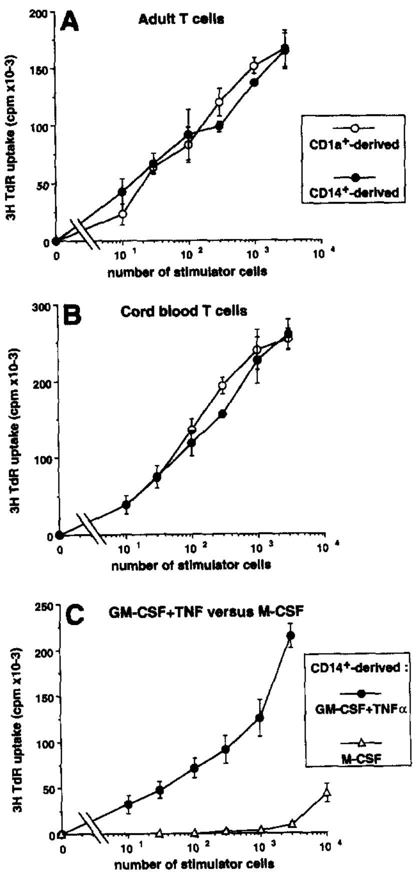

Figure 9. The two DC subpopulations display overlapping naive T cell activation properties. Cord blood CD34$^+$ HPC were cultured in presence of GM-CSF+TNFα. After 5–6 d, cells were FACS®-sorted into CD14$^-$-CD1a$^+$ and CD14$^+$CD1a$^-$. Sorted cells were seeded (1–2 × 10^5 cells per ml) in presence 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 × 10^4 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 × 10^4 cell per well). The proliferation was revealed by ^{3}H-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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References

1. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271–296.
2. O'Doherty, U., M. Peng, S. Gezelter, W.J. Swiggard, M. Betjes, N. Bhardwaj, and R.M. Steinman. 1994. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology.* 82: 487–493.
3. Thomas, R., L.S. Davis, and P.E. Lipsky. 1993. Isolation and characterization of human peripheral blood dendritic cells. *J. Immunol.* 150:821–834.
4. Thomas, R., and P.E. Lipsky. 1994. Human peripheral blood dendritic cell subsets. Isolation and characterization of precur-

sor and mature antigen-presenting cells. *J. Immunol.* 153: 4016–4028.
5. Weissman, D., Y. Li, J. Ananworanich, L.-J. Zhou, J. Adelsberger, T.F. Tedder, M. Baseler, and A.S. Fauci. 1995. Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA.* 92: 826–830.
6. Lenz, A., M. Heine, G. Schuler, and N. Romani. 1993. Human and murine dermis contain dendritic cells. *J. Clin. Invest.* 92:2587–2596.
7. Nestle, F.O., X.-G. Zheng, C.B. Thompson, L.A. Turka, and B.J. Nickoloff. 1993. Characterization of dermal dendritic cells obtained from normal human skin reveals phenotypic and functionally distinctive subsets. *J. Immunol.* 151:6535–6545.
8. Agger, R., M. Witmer-Pack, N. Romani, H. Stossel, W.J. Swiggard, J.P. Metlay, E. Storozynsky, P. Freimuth, and R.M. Steinman. 1992. Two populations of splenic dendritic cells detected with M342, a new monoclonal to an intracellular antigen of interdigitating dendritic cells and some B lymphocytes. *J. Leukocyte Biol.* 52:34–42.
9. Kelsall, B.L., and W. Strober. 1996. Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *J. Exp. Med.* 183:237–247.
10. Ardavin, C., L. Wu, C.L. Li, and K. Shortman. 1993. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature (Lond.).* 362:761–763.
11. Galy, A., M. Travis, D. Cen, and B. Chen. 1995. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity.* 3:459–473.
12. Inaba, K., R.M. Steinman, M.W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* 175:1157–1167.
13. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693–1702.
14. Inaba, K., M. Inaba, M. Deguchi, K. Hagi, R. Yasumizu, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1993. Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc. Natl. Acad. Sci. USA.* 90:3038–3042.
15. Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Banchereau. 1992. GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature (Lond.).* 360:258–261.
16. Reid, C.D.L., A. Stackpoole, A. Meager, and J. Tikerpae. 1992. Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent $CD34^+$ progenitors in human bone marrow. *J. Immunol.* 149:2681–2688.
17. Santiago-Schwarz, F., E. Belilos, B. Diamond, and S.E. Carsons. 1992. TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J. Leukocyte Biol.* 52:274–281.
18. Szabolcs, P., M.A.S. Moore, and J.W. Young. 1995. Expansion of immunostimulatory dendritic cells among the myeloid progeny of human $CD34^+$ bone marrow precursors cultured with c-kit-ligand, GM-CSF, and TNFα. *J. Immunol.* 154:5851–5861.
19. Gluckman, J.C., M. Rosenzwajg, and B. Canque. 1996. Human dendritic cell differentiation pathway from $CD34^+$ hematopoietic precursor cells. *Blood.* 87:535–544.
20. Caux, C., I. Durand, I. Moreau, V. Duvert, S. Saeland, and J. Banchereau. 1993. TNFα cooperates with IL-3 in the recruitment of a primitive subset of human $CD34^+$ progenitors. *J. Exp. Med.* 177:1815–1820.
21. Caux, C., S. Saeland, C. Favre, V. Duvert, P. Mannoni, and J. Banchereau. 1990. Tumor necrosis factor-alpha strongly potentiates interleukin-3 and granulocyte-macrophage colony-stimulating factor-induced proliferation of human $CD34^+$ hematopoietic progenitor cells. *Blood.* 75:2292–2298.
22. Caux, C., C. Favre, S. Saeland, V. Duvert, I. Durand, P. Mannoni, and J. Banchereau. 1991. Potentiation of early hematopoiesis by tumor necrosis factor-α is followed by inhibition of granulopoietic differentiation and proliferation. *Blood.* 78:635–644.
23. Miltenyi, S., W. Müller, W. Weichel, and A. Radbruch. 1990. High gradient magnetic cell separation with Macs. *Cytometry.* 11:231–238.
24. Vallé, A., C.E. Zuber, T. Defrance, O. Djossou, M. de Rie, and J. Banchereau. 1989. Activation of human B lymphocytes through CD40 and interleukin 4. *Eur. J. Immunol.* 19: 1463–1467.
25. Kashihara, M., M. Ueda, Y. Horiguchi, F. Furukawa, M. Hanaoka, and S. Imamura. 1986. A monoclonal antibody specifically reactive to human Langerhans cells. *J. Invest. Dermatol.* 87:602–607.
26. Tang, A., M. Amagai, L.G. Granger, J.R. Stanley, and M.C. Udey. 1993. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature (Lond.).* 361:82–85.
27. Blauvelt, A., S.I. Katz, and M.C. Udey. 1995. Human Langerhans cells express E-cadherin. *J. Invest. Dermatol.* 104:293–296.
28. Zhou, L.-J., and T.F. Tedder. 1995. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J. Immunol.* 154:3821–3835.
29. O'Doherty, U., R.M. Steinman, M. Peng, P.U. Cameron, S. Gezelter, I. Kopeloff, W.J. Swiggard, M. Pope, and N. Bhardwaj. 1993. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J. Exp. Med.* 178:1067–1078.
30. Burkly, L., C. Hession, L. Ogata, C. Reilly, L.A. Marconi, D. Olson, R. Tizard, R. Cate, and D. Lo. 1995. Expression of *rel*B is required for the development of thymic medulla and dendritic cells. *Nature (Lond.).* 373:531–536.
31. Weih, F., D. Carrasco, S.K. Durham, D.S. Barton, C.A. Rizzo, R.-P. Ryseck, S.A. Lira, and R. Bravo. 1995. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family. *Cell.* 80:331–340.
32. Szabolcs, P., D. Avigan, S. Gezelter, D.H. Ciocon, M.A.S. Moore, R.M. Steinman, and J.W. Young. 1996. Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-CFU intermediate. *Blood.* 87:4520–4530.
33. Young, D.A., L.D. Lowe, and S.C. Clark. 1990. Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture. *J. Immunol.* 145:607–615.
34. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation

of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* 179:1109–1118.

35. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down-regulation by cytokines and bacterial products. *J. Exp. Med.* 182:389–400.
36. Romani, N., S. Gruner, D. Brang, E. Kämpgen, A. Lenz, B. Trockenbacher, G. Konwalinka, P.O. Fritsch, R.M. Steinman, and G. Schuler. 1994. Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* 180:83–93.
37. Wu, L., D. Vremec, C. Ardavin, K. Winkel, G. Suss, H. Georgiou, E. Maraskovsky, W. Cook, and K. Shortman. 1995. Mouse thymus dendritic cells: kinetics of development and changes in surface markers during maturation. *Eur. J. Immunol.* 25:418–425.
38. Young, J.W., P. Szabolcs, and M.A.S. Moore. 1995. Identification of dendritic cell colony-forming units among normal human $CD34^+$ bone marrow progenitors that are expanded by c-*kit*-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. *J. Exp. Med.* 182:1111–1120.