RAPID COMMUNICATION

Dendritic Cells and Macrophages Can Mature Independently From a Human Bone Marrow–Derived, Post–Colony-Forming Unit Intermediate

By Paul Szabolcs, David Avigan, Stuart Gezelter, David H. Ciocon, Malcolm A.S. Moore, Ralph M. Steinman, and James W. Young

CD34" precursors in normal human bone marrow (BM) generate large numbers of dendritic cells alongside macrophages and granulocytic precursors when cultured for 12 to 14 days in c-kit ligand, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α). This study reports an intermediate cell type that develops by day 6, and has the potential to differentiate into either macrophages or dendritic cells. When the d6 progeny are depleted of mature macrophages and residual CD34" precursors, a discrete CD14" HLA-DR" population persists in addition to immunostimulatory CD14" HLA-DR"++"+++ dendritic cells. Half of the CD14" HLA-DR" population is in cell cycle (Ki-67"), but colony-forming units (CFUs) are no longer detectable. The cells are c-fms", but lack myeloperoxidase and nonspecific esterase. They also possess substantial phagocytic and allostimulatory activity. These post-

From the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, NY; the Bone Marrow Transplantation Service, the Department of Pediatrics; James Ewing Laboratory of Developmental Hematoepoiesis, Cell Biology and Genetics Program, Sloan-Kettering Institute for Cancer Research; and the Allogeneic Bone Marrow Transplantation and Clinical Immunology Services, the Division of Hematologic Oncology, Department of Medicine; Memorial Sloan-Kettering Cancer Center, Cornell University Medical College, New York, NY.

Submitted January 29, 1996; accepted March 18, 1996.

Supported by Grants No. AI-01254 (P. Sz.), HL-46546 (M.A.S.M.), CA-08748 (M.A.S.M.), AI-24775 (R.M.S.), CA-23766 (J.W.Y.), and AI-26875 (J.W.Y.) from the National Institutes of Health; the Bone Marrow Transplantation Service, the Department of Pediatrics; James Ewing Laboratory of Developmental Hematoepoiesis, Cell Biology and Genetics Program, Sloan-Kettering Institute for Cancer Research; and the Allogeneic Bone Marrow Transplantation and Clinical Immunology Services, the Division of Hematologic Oncology, Department of Medicine; Memorial Sloan-Kettering Cancer Center, Cornell University Medical College, New York, NY.

Address correspondence to James W. Young, MD, Box 176, The Rockefeller University, 1230 York Ave, New York, NY 10021-6399.

© 1996 by The American Society of Hematology.

MATERIALS AND METHODS

Isolation of human CD34" BM progenitors. Small aliquots of BM were obtained from normal donors already undergoing harvesting for allogeneic transplantation, in accordance with institutional guidelines. CD34" cells were isolated by positive selection with an immunomagnetic bead system.13,14,16

Culture media and cytokines. CD34" BM progenitors were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL, Grand Island, NY) with 50 μg/mL gentamicin (Sigma Chemical Co, St Louis, MO), monothioglycerol 7.3 × 10⁻⁴ mol/L (Sigma), 20% fetal calf serum (FCS; Gemini Bioproducts, Calabasas, CA), and exogenous cytokines as indicated. Assays for evaluating the function of differentiated progeny used RPMI 1640 (Gibco-BRL) with 10 mmol/L HEPES (Sigma), 1 mmol/L glutamine (Gibco-BRL), 5 × 10⁻⁵ mol/L 2-mercaptoethanol (Eastman Kodak, Rochester, NY), penicillin (100 U/mL)-streptomycin (100 μg/mL) (Gibco-

Blood, Vol 87, No 11 (June 1), 1996: pp 4520-4530
Bipotent CD14+ HLA-DR+ Intermediate for Dendritic Cells and Macrophages

BRL), and 10% heat-inactivated normal human serum (NHS). All media were endotoxin free. The recombinant human cytokines were: c-kit ligand (KL: or stem cell factor, SCF; Amgen, Thousand Oaks, CA) 20 ng/mL; GM-CSF (gift of Kirin Brewery, Maebashi, Gunma, Japan) 100 ng or 1,000 U/mL; TNF-α (R&D Systems, Minneapolis, MN) 10 ng or 1,000 U/mL; M-CSF (Cetus, Oakland, CA) 1,000 U/mL.

Culture of human CD34+ progenitors in suspension and colony-forming assays. Suspension cultures of CD34+ BM cells were initiated with complete IMDM-20% FCS at an initial concentration of 2×10^5 cells/mL in 35-mm tissue-culture wells (#25810-6; Corning Costar Corp, Cambridge, MA), supplemented with c-kit ligand, GM-CSF, and TNF-α. Intermediate populations were isolated as described below. Cells were recultured at ~1×10^6/mL in IMDM-20% FCS with the cytokines indicated for a particular experiment, but without c-kit ligand. Lower yields were scaled down to proportionally smaller volumes and wells for reculture. Medium and cytokines were neither changed nor replenished at any time, except when the sorted populations were recultured.

Colony-forming assays were performed in triplicate in 0.36% agarose (FMC Bioproducts, Rockland, ME) and complete IMDM-20% FCS, starting with 1 to 2×10^5 CD34+ cells in 1-mL cultures; or in microwells with cells seeded at limiting dilution in IMDM-20% FCS. CFUs were quantified by counting colonies of ≥50 cells.

All cultures were maintained at 37°C in humidified 5% CO₂.

Monoclonal antibodies (MoAbs), cell phenotype analysis, and cytochemical staining. Murine antihuman MoAbs were used as either purified Igs or as hybridoma supernatants (Table 1). In some cases the MoAbs were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Otherwise, FITC-conjugated F(ab); goat-anti-FITC (BD); and PE-conjugated F(ab); goat-anti-PE (BD) were used as second-step reagents for indirect staining. For three-color cytofluorographic evaluations, PerCP-conjugated anti–HLA-DR (Becton Dickinson [BD], San Jose, CA) was used. Stained cells were analyzed by cytofluorography on a FACScan (BD). Nonreactive MoAbs of the same isotype and subclass were used as controls. Cytospins were made for Wright-Giemsa and cytochemical staining for nonspecific esterase (NSE; α-naphthyl butyrate esterase; Sigma), specific esterase (naphthol AS-D chloroacetate esterase; Sigma), and myeloperoxidase (Sigma). Ki-67 and c-fms were detected on cytospins using a standard immunoperoxidase staining method.14

Isolation of CD14+ HLA-DR+ intermediates from suspension cultures of normal human CD34+ BM precursors. The bulk progeny were vigorously harvested from the original culture wells, including three washes in original medium. Cells were resuspended with anti–CD34 MoAb (IgG1 clone 11.1.6, under license by Dr Malcolm A.S. Moore to Oncogene Science, Uniondale, NY) and then panned at 4°C on goat-IgG-antimouse-IgG (Fcγ-chain–specific, #55459; Cappel, Division of Organon Teknika, Durham, NC) coated bacteriologic Petri dishes. Nonadherent cells were recovered by gentle washing with cold medium. Pan nonadherent cells were stained with HLA-DR-FITC (BD) and CD14-PE (BD) and sorted cytofluorographically on a FACStar Plus (BD) into CD14+ HLA-DR+ and CD14+ HLA-DR+ subsets (see also Fig 1, day 6). The presort panning depletion proved essential to observe dendritic cell development from the sorted CD14+ HLA-DR+ fraction, because macrophages that had already matured also adhered nonspecifically alongside anti-CD34- sorted cells (see also Results).

PB mononuclear cells (PBMC) and enrichment of blood dendritic cells, monocytes, and T lymphocytes. Mononuclear cells were prepared from buffy coats according to previously published procedures to obtain primary leukocyte subpopulations of T cells, monocytes, and dendritic cells.18-21

Allogeneic mixed leukocyte reaction (MLR) and αCD3 polyclonal mitogenesis. Antigen-presenting cells (APCs; blood dendritic cells, blood monocytes, and marrow-derived CD34+ progeny cells) were added in graded doses to primary allogeneic T cells in 96-microwell tissue culture plates (1×10^5 T cells/round-bottomed microwell). APCs expanded from CD34+ BM precursors were thoroughly washed free of cytokine and irradiated 1,500 rads [10^6C]s before addition. Logistically we could not obtain blood dendritic cells and monocytes from the same donors from whom CD34+ progenitors had been obtained. Therefore, the blood dendritic cells and/or monocytes used for comparison of accessory function with the marrow-derived APCs were from a different donor than the BM progeny. T-cell responders were always from a single donor for a given experiment. The capacity for an APC population to support FeR-mediated anti-CD3 polyclonal mitogenesis was assessed in similar MLRs where OKT3 (1 μg/mL final; Orthoclone OKT3, gift of Ortho Biotech, Raritan, NJ) was added.

Cultures were pulsed for 8 to 12 hours with 1 μCi/well of 3H-thymidine (3HTdR; New England Nuclear, Boston, MA) beginning 60 to 72 hours from the start of culture for anti-CD3 mitogenesis, and beginning ~days 4 to 5 for the allogeneic MLRs. To discount any increased proliferation caused by allospecificities in the MLR during anti-CD3 mitogenesis, an allogeneic MLR was cultured and pulsed in parallel with proliferation subtracted from that in the OKT3 plates. The amount of 3HTdR incorporated by the responder T cells was plotted against the dose of stimulator cells as a measure of stimulatory capacity. Responses have been reported as the mean cpm ± SD of triplicates. Wells containing only T cells or only APCs (1,500 rads [10^6C]s) always incorporated less than 500 cpm 3HTdR.

Phagoctosis assays. FITC-conjugated latex particles (Fluoresbrite, 0.2-μm microspheres, 2.5% solids, 100× stock, cat no. 17151; Polysciences, Inc, Warrington, PA) were added to cells in a final volume of 100 μL medium with 10% FCS. The optimal cell concentration was 5 to 10×10^4/100 μL round-bottomed microwell, and the minimum concentration was 2×10^4/microwell. Positive controls were cultured blood monocytes. Negative controls to determine background fluorescence were cells to which FITC-latex beads were not added, as well as nonphagocytic cells (eg, T lymphocytes) with FITC-latex beads. Cells were incubated in the microwell plate for 1 hour at 37°C in humidified 5% CO₂. Cells were then washed five times while still in the microwell plate using cold buffer of choice, with intervening 3-minute centrifugations at 400 g at 4°C. Cells were analyzed by cytofluorography.

RESULTS

Serial phenotype of human CD34+ BM-derived progeny in suspension cultures supplemented with GM-CSF and TNF-α. CD34+ BM precursors were cultured in medium supplemented with 20% FCS and c-kit ligand, GM-CSF, and TNF-α.13 Serial phenotyping indicated that candidate dendritic cells with the CD14+ HLA-DR+** phenotype began to develop between days 2 and 4 of suspension culture and were well-established by day 6 (Fig 1). A subpopulation of CD14H+ HLA-DR+ cells was also identifiable by day 6 (Fig 1). Phenotypically and quantitatively similar populations were present at days 12 through 14. The increasing proportion of CD14+ HLA-DR+ cells in the bulk day 12 progeny represented granulocytic precursors, owing to the effect of c-kit ligand.13 Based on these cytofluorographic studies we presumed that the CD14+ HLA-DR+ cells were macrophages, and the CD14+ HLA-DR+** cells were dendritic cells. As became evident after more detailed evaluation, the latter proved to be the case, but the aforementioned CD14+...
HLA-DR⁺ cells were quite different from mature monocytes/macrophages and actually proved to be bipotential with respect to subsequent terminal differentiation.

**Identification of mature, CD14⁻ HLA-DR⁺⁺⁺, dendritic cells in the day 6 progeny of CD34⁺ precursors.** Cells with the markers of mature dendritic cells, ie, CD14⁻ and HLA-DR⁺⁺⁺, constituted 2.1% ± 1.1% (n = 12, 5 of which were analyzed in duplicate) of the bulk day 6 progeny. The percent yield of these CD14⁻, intensely HLA-DR⁺⁺⁺ cells did not increase with an additional week of culture. The population was sorted using the gate indicated in Fig 1 for more detailed morphologic, cytochemical, and phenotypic characterization. Cytosins of the sorted population showed cells with cytoplasmic veils and processes that typify dendritic cells.

These cells did not stain for myeloperoxidase or specific cytoplasmic veils and processes that typify dendritic cells.

* Fluorescent intensity was scored according to mean log₁₀ fluorescence on a BD FACScan: --, 10⁰ to 10¹; (+), 10¹ to 10²; ++, 10² to 10³; +++, 10³ to 10⁴ (maximum); except where cytopsin were stained by immunoperoxidase as noted (see footnote§).
* Negative controls were nonreactive MoAbs of the same isotype and subclass.
* Sources of MoAbs were Dako, Carpinteria, CA; Dr David Scheinberg, Memorial Sloan-Kettering Cancer Center, New York, NY; ATCC, American Type Culture Collection, Bethesda, MD; Coulter Immunology, Hialeah, FL; Dr Thomas Tedder, Duke University Medical Center, Durham, NC; Oncogene Science, Uniondale, NY; BD, Becton Dickinson, San Jose, CA; Serotec, Harlan Bioproducts for Science, Inc, Indianapolis, IN; Dr Peter Beverley, Imperial Cancer Research Fund, London, UK; Dr Una O’Doherty, The Rockefeller University, New York, NY; Dr Samuel D. Wright, The Rockefeller University; Dr Stephen Shaw, Bethesda, MD.
* Blood dendritic cells and macrophages were each purified from cultured PBMC subsets as previously published. These phenotypic data have also been published.
* Epitope expression was determined by immunoperoxidase staining of cytospin preparations, scored independently by two individuals relative to isotype control.
Fig 1. Phenotypic development of CD34+ BM cells expanded in c-kit ligand, GM-CSF, and TNF-α. Normal CD34+ BM precursors were cultured in IMDM-20% FCS with c-kit ligand, GM-CSF, and TNF-α. At the serial time points indicated, a representative sample of the culture was stained with FITC-anti-HLA-DR and PE-anti-CD14 and analyzed by cyttofluorography. Sort gates are indicated on the day 6 panel. Representative of three experiments.

of the same phenotype.13 This CD14- HLA-DR+++ fraction expressed CD1a, CD83, CD4, CD45RO > CD45RA, CD14, CD18, CD40, CD54, CD80, CD86, and CD74; c-fms was absent (Table 1). These phenotypic criteria distinguished the day 6 CD14- HLA-DR+++ cells as candidate dendritic cells.

The day 6 CD14- HLA-DR+++ progeny were also tested as stimulators in an allogeneic MLR, an assay that highlights the specialized stimulatory properties of dendritic cells. CD14- HLA-DR+++ cells were sorted as above, irradiated, and added in serial doses to resting allogeneic T cells for 4 to 5 days. Stimulatory capacity was potent and comparable to that exerted by blood dendritic cells (Fig 2). At least one log fewer CD14- HLA-DR+++ cells stimulated the same degree of responder T cell proliferation as that effected by the presort population. These morphologic, cytochemical, phenotypic, and functional criteria indicated that mature CD14- HLA-DR+++ dendritic cells had developed by day 6 from human CD34+ BM cells under these culture conditions.

The CD14- HLA-DR+++ cells present at day 6 were also examined for the possibility that these might be less mature dendritic cells. This was not pursued further because this fraction consisted predominantly of immature myeloid cells that differentiated primarily into granulocytes and macrophages. Few if any additional dendritic cells were generated, irrespective of reculture in GM-CSF and TNF-α.

Properties of day 6 CD14- HLA-DR+++ progeny derived from human CD34+ BM precursors. Day 6 progeny of CD34+ BM cells cultured in c-kit ligand, GM-CSF, and TNF-α were depleted of persisting CD34+ HLA-DR+++ precursor populations by panning. Losses exceeded the few CD34+ cells known still to be present at day 6, and averaged 22% ± 17% (n = 10). Panning did not remove the CD14- HLA-DR+++ dendritic cells. Most of the cells that had attached during CD34+ panning were well spread, firmly adherent, and morphologically typical of macrophages. Functional assays were confirmatory because the attached cells supported vigorous FcR-mediated mitogenesis with anti-CD3 but weak allogeneic MLRs (not shown).

Further characterization of the CD14- HLA-DR+++ cells was undertaken after panning. These cells constituted 18% ± 4% (n = 11, 6 of which were analyzed in duplicate) of the pan nonadherent day 6 cultures and were sorted using the gate shown in Fig 1. This population had features that were atypical of either terminally differentiated macrophages or dendritic cells. The cells did not stain for myeloperoxidase or specific (naphthol AS-D chloroacetate) esterase, and were predominantly nonspecific (α-naphthyl butyrate) esterase.
pared with blood dendritic cells and macrophages. The macro-
potent allogeneic stimulators, although intermediate between
the presort population and either blood dendritic cells or the
sorted CD14+ HLA-DR+ dendritic cells derived from the
same CD34+ precursors. Their potency exceeded that of cul-
tured blood monocytes/macrophages by several logs.

In contrast were their phagocytic properties. When ex-
posed to 0.2 μm FITC-conjugated latex particles, the day 6
sorted CD14+ HLA-DR+ cells were comparably phagocytic
to cultured blood monocytes, although their background con-
trol was somewhat higher (Fig 3, top). Mature blood den-
dritic cells are not actively phagocytic.22

The CD14+ HLA-DR+ cells were also examined for a
panel of surface markers (Table I). The bulk day 6 pan-
onadherent cells were analyzed by three-color cytofluoro-
graphy, and sorted cells were cytocentrifuged onto glass slides
for staining. The profile of these cells was notable for the

The day 6 CD14+ HLA-DR+ cells were further evaluated
for allostimulatory function and phagocytic capacity, and com-

Fig 2. Day 6 progeny of CD34+ BM cells cultured in IMDM-20%
FCS, c-kit ligand, GM-CSF, and TNF-α, tested for stimulatory function
as APCs in the allogeneic mixed leukocyte reaction (MLR). CD34+ BM
cells were cultured for 6 days in IMDM-20% FCS with c-kit ligand,
GM-CSF, and TNF-α. The day 6 progeny were obtained from the
original culture wells by vigorous washing, opsonized with anti-
CD34, and banded on goat-antimouse IgG coated bacteriologic Petri
dishes at 4°C. Approximately 20% of the applied cells were depleted,
most of which proved to be firmly adherent macrophages. The re-
mainder nonadherent cells were stained with PE-anti-CD14 and
FITC-anti-HLA-DR, sorted using the gates indicated in Fig 1, and
tested as stimulators (1,500 rads 131Cs) of primary allogeneic T cells
(10⁶ per round-bottomed microwell) in the MLR. Graded doses of
stimulators are indicated along the abscissa. 3HThdr incorporation by
responder T cells during a 9-hour pulse of 1 μCi/well on day 4 of
the MLR is plotted against the ordinate. Note log scales. T cells or
irradiated APCs only, less than 500 cpm 3HThdr. ( ), presort; ( ),
sorted CD14+ HLA-DR+; ( ), sorted CD14+ HLA-DR++. Comparison
APCs were blood dendritic cells ( ) and blood monocytes ( ) iso-
lated by standard methods and cultured in RPMI-10% NHS without
cytokines. Representative of 11 experiments.

negative. The cells did not attach to plastic, but exhibited
many processes, including large lamellipodia or veils. Forty
to 50% of the cells expressed the Ki-67 nuclear antigen of
cycling cells and had limited proliferative potential in the
presence of M-CSF (see below). However, these cells no
longer had colony-forming capacity in clonogenic assays in
the presence of either GM-CSF and TNF-α, or M-CSF alone.

The day 6 CD14+ HLA-DR+ cells were further evaluated
for allostimulatory function and phagocytic capacity, and com-

Fig 3. Phagocytosis assays of CD14+ HLA-DR+ intermediates at
day 6 and after reculture to day 13 in either GM-CSF/TNF-α or
M-CSF. Day 6 CD14+ HLA-DR+ intermediates, isolated as described in
Materials and Methods and Figs 1 and 2, were tested for phagocytic
capacity using FITC-conjugated 0.2 μm latex particles (Day 6, top).
The assay was repeated after reculture in either GM-CSF/TNF-α or
M-CSF from day 6 through day 13 (Day 13, bottom). Cells were ana-
yzed for ingested particles by cytofluorography, yielding the histo-
gram profiles shown. Positive controls were blood monocytes or
CD14+ HLA-DR+ intermediates recultured in M-CSF. Negative con-
trols were cells to which no FITC-latex was added (as shown) and
nonphagocytic T lymphocytes with FITC-latex added (not shown, but
histograms were superimposable on the negative controls depicted
for blood monocytes, top, and for M-CSF reculture, bottom). Repre-
sentative of three day 6 experiments and two day 12 through 14
experiments.
presence of c-fms, the receptor for M-CSF, and the absence of the dendritic cell marker CD83, as well as the rarity of CD1a and the CD80/CD86 B7 costimulator molecules. The cells uniformly expressed readily detectable levels of CD4, CD11a,b,c, CD18, CD32, CD64, CD40, CD54, and CD58.

Therefore, these data indicated that despite their c-fms+ CD14+ HLA-DR+ phenotype and phagocytic activity, these cells differed with respect to a number of important monocyte features. Similarly, these cells could not be classified as mature dendritic cells, as were their CD14+ HLA-DR+++ counterparts. Given this lack of identifiable commitment to either the monocyctic or dendritic cell lineage, it was investigated whether these CD14+ HLA-DR+ cells constituted intermediates whose terminal differentiation could be experimentally altered by selective cytokine exposure during subsequent in vitro reculture.

**Contrasting differentiation of double-positive CD14+ HLA-DR+ intermediates recultured in GM-CSF and TNF-α versus M-CSF.** The day 6 pan nonadherent, sorted, double-positive CD14+ HLA-DR+ cells were recultured in either GM-CSF and TNF-α, or M-CSF, for an additional 6 to 7 days. After a total of 12 to 14 days in suspension culture, including the initial 6 days of culture in KL, GM-CSF, and TNF-α, the progeny of the second week’s reculture were harvested and manually counted to ensure viability by trypan blue exclusion. The cells were then phenotyped and evaluated in functional assays for their stimulatory and phagocytic properties.

Those CD14+ HLA-DR+ cells recultured in GM-CSF and TNF-α were nonadherent, motile, and displayed prominent cytoplasmic vesicles in suspension culture (Fig 4A) and dendrites on cytocentrifuged preparations (Fig 4B). They lost CD14 and c-fms, whereas class II MHC expression was upregulated (Fig 5A). In addition, all acquired CD80 and CD86 (Fig 5B), most expressed CD1a and CD83 (Fig 5B), and a minority had nonspecific (α-naphthyl butyrate) esterase activity (not shown).

The same double-positive cells recultured from day 6 to day 12 through 14 in M-CSF were mostly adherent without cytoplasmic vesicles or processes (Fig 4C and D) and retained CD14, c-fms, and class II MHC expression (Fig 5A). The cells also expressed B7-2/CD86 at a lower intensity than their counterparts recultured in GM-CSF and TNF-α; CD1a, CD83, and B7-1/CD80 were all absent (Fig 5B). Nonspecific (α-naphthyl butyrate) esterase activity was also present (not shown).

In addition to phenotypic alterations, GM-CSF and TNFα fostered the development of stimulatory function that was comparable with that of mature blood dendritic cells (Fig 6). In contrast, double-positive CD14+ HLA-DR+ intermediates lost stimulatory activity for resting allogeneic T cells when recultured in M-CSF.

The converse was true of phagocytosis. CD14+ HLA-DR+ intermediates became less phagocytic after reculture in GM-CSF and TNF-α. CD14+ HLA-DR+ intermediates recultured in M-CSF became more actively phagocytic. In fact, their phagocytic capacity for FITC-conjugated latex beads exceeded that of their counterparts recultured in GM-CSF and TNF-α by at least 10-fold (Fig 3, bottom).

These same CD14+ HLA-DR+ intermediates were also recultured in GM-CSF alone (not shown). In the absence of TNF-α, the maturing cells were more heterogeneous. Fewer cells developed the characteristic veils of dendritic cells, allostimulatory function was weaker although still in excess of intermediates recultured in M-CSF, and phenotypic loss of CD14 was incomplete.

To ensure that differences in functional maturation were not caused by differences in plastic adherence, the double-positive intermediates were recultured in Teflon beakers with the same cytokines. Cells recultured in M-CSF retained all the features of plastic adherent macrophages, including very poor MLR stimulatory activity. Intermediates recultured in GM-CSF and TNF-α were also no different from their counterparts on tissue culture plastic.

The sorted CD14+ HLA-DR+ intermediates were irradiated or not before reculture to investigate whether a proliferating subpopulation had a survival advantage without changing the net yield. However, differentiation along either pathway was not altered by irradiation before reculture in the respective cytokine conditions (Figs 5 and 6).

The expression of the Ki-67 nuclear antigen by 40% to 50% of the day 6 sorted CD14+ HLA-DR+ fraction was indicative that these cells were still in cycle, despite their loss of clonogenic potential. However, no further rounds of cell division occurred when the CD14+ HLA-DR+ intermediates were recultured in GM-CSF and TNF-α. Expansion was maximally 2.5-fold after reculture in M-CSF. Reculture in IMDM-20% FCS in the absence of exogenous cytokines generated cells like those from M-CSF-supplemented cultures (Figs 4 through 6). Unlike the CD14+ HLA-DR+ cells recultured in M-CSF, however, there was no expansion. We therefore considered monocyte development to be the default pathway for terminal differentiation of the day 6 sorted CD14+ HLA-DR+ intermediates.

The CD14+ HLA-DR+ phenotype does not retain bipotential differentiation capacity beyond the first 6 to 7 days in suspension culture. Depletion of already mature monocytes/macrophages by days 6 to 7 of culture in GM-CSF and TNF-α was essential for the subsequent maturation of the sorted CD14+ HLA-DR+ intermediates along a dendritic cell pathway. By days 8 to 9 and beyond, mature monocytes/macrophages were sufficiently developed that CD14+ HLA-DR+ cells could no longer be isolated with the same bipotential differentiation potential as the double-positive intermediates from day 6 cultures. This is illustrated in Table 2 using day 12 through 14 progeny to stimulate allogeneic T cells in the MLR. Similarly, day 12 through 14 macrophages generated in M-CSF from day 6 CD14+ HLA-DR+ intermediates could not become dendritic cells if recultured beyond days 12 through 14 in GM-CSF and TNF-α (not shown). Standard CD14+ HLA-DR+ blood monocytes/macrophages could also not be transformed into a population of dendritic cells by exposure to GM-CSF and TNF-α, as previously reported.

**DISCUSSION**

CD34+ hematopoietic precursors from human BM have been evaluated with a view toward the development of en-
Fig 4. Day 6 CD14+ HLA-DR+ intermediates develop into morphologically distinct dendritic cells or macrophages when recultured in GM-CSF/TNF-α or M-CSF, respectively. Nonadherent (CD34+ and macrophage depleted), sorted day 6 CD14+ HLA-DR+ intermediates were recultured in IMDM-20% FCS with either GM-CSF/TNF-α or M-CSF. After an additional week's culture dendritic cells developed in GM-CSF and TNF-α (A, phase contrast, original magnification 20×, arrows highlight a few of the prominent cytoplasmic veils; B, Wright-Giemsa, original magnification 100×), whereas adherent macrophages developed in M-CSF (C, phase contrast, original magnification 20×; D, Wright-Giemsa, original magnification 40×). Double-positive intermediates recultured in IMDM-20% FCS only without exogenous cytokines were similar to those recultured with M-CSF (C and D). Nonspecific (α-naphthyl butyrate) esterase staining is not shown but was similar to that of progeny of the same phenotypes, as previously published.13,14

In a prior study of CD34+ BM progenitors cultured in these cytokines in semisolid medium,14 two types of colonies developed. One consisted entirely of dendritic cells. The cells in these CFU-DC derived colonies lacked CD14 and myeloperoxidase but expressed high levels of HLA-DR and stimulating activity in the allogeneic MLR. The other colonies were CFU-GM-derived but contained a minor population (~1%) of dendritic cells, only when TNF-α was added to GM-CSF. Dendritic cell colonies did not develop in GM-CSF alone, and GM colonies did not contain even trace numbers of dendritic cells if TNF-α were not used together with GM-CSF. Another study documented the development of mature, terminally differentiated macrophages and dendritic cells when CD34+ BM precursors were cultured for 2 weeks in suspension with these cytokines. In the present experiments an intermediate cell type has been identified that develops by day 6. It can be diverted from a macrophage to a dendritic cell differentiation pathway depending on subsequent cytokine exposure, thus increasing the frequency of dendritic cells among the final progeny.

These experiments have focused on the day 6 progeny of CD34+ progenitors, selecting cells on the basis of CD14 and HLA-DR staining. Progeny that appeared to be mature dendritic cells (CD14− HLA-DR+++ ) and monocytes (CD14+ HLA-DR− ) had developed by day 6. Restricting analysis only to those cells that were intensely HLA-DR positive, CD14− HLA-DR+++ cells represented ~2% of the day 6 progeny; and percent yields of these extremely HLA-DR bright cells did not increase with an additional week of culture. The cells had all the features of terminally differentiated dendritic cells except relatively higher expression of CD1a, CD4, CD11b, and CD32. These were large cells that formed many processes, either large veils in suspension culture or stellate dendrites on cytopsins. The cells were nonadherent, lacked myeloperoxidase, and stained weakly or nega-
tively for nonspecific esterase. The cells were also negative for c-fms, expressed the dendritic cell antigen CD83, and displayed high levels of B7 antigens, CD80 and CD86, which are all characteristic of dendritic cells. Strong T-cell responses were also induced with DC:T ratios of 1/1,000 or less.

However, the pan nonadherent, sorted CD14+ HLA-DR+ cells proved not to be typical monocytes/macrophages. This was despite their expression of c-fms and phagocytic properties that were comparable to cultured blood macrophages. A large fraction was in cell cycle, as 40% to 50% displayed the Ki-67 nuclear antigen, but clonogenic capacity was no longer detectable. Several characteristic monocyte traits were also absent, eg, morphology (including many cells with cytoplasmic dendrites or veils that are not seen on monocytes/macrophages), strong plastic adherence, and positive staining for myeloperoxidase and nonspecific esterase. These cells also uniformly expressed CD40 as well as some B7 costimulators (CD80, CD86). In addition, these cells were much stronger stimulators than monocytes of primary alloreactive T cells in allogeneic MLRs. CD14+ progeny with allostimulatory activity have recently been identified in 7-day BM cultures by Egner and Hart. However, their bipotential differentia-
Once differentiation from the CD14+ HLA-DR+ intermediate cells had proceeded along either the dendritic cell or monocyte/macrophage pathway, it could not be altered again by cytokines. Dendritic cells died when reculrured in M-CSF and did not become macrophages, and macrophages did not become dendritic cells in GM-CSF and TNF-α. Likewise, CD14+ HLA-DR+ cells isolated at days 8 through 9 and beyond, no longer had the same bipotential differentiation capacity and became macrophages regardless of the cytokine milieu. These later CD14+ HLA-DR+ cells specifically could not be induced toward dendritic cell development by GM-CSF and TNF-α.

Therefore, these findings indicate that there is a bipotential CD14+ HLA-DR+ population in the day 6 progeny of human CD34+ BM cells cultured in GM-CSF and TNF-α. The critical question is why does the dendritic cell potential of this intermediate population not develop routinely in the presence of GM-CSF and TNF-α? To observe this dendritic cell pathway, the day 6 cultures had to be depleted of already matured, firmly adherent macrophages and then supplemented with exogenous GM-CSF and TNF-α. Possibly the macrophages exert a suppressive influence on dendritic cell development. Both IL-1027,33 and transforming growth factor-β (TGF-β)12,33 are macrophage products reported to have suppressive effects on dendritic cell function. These and other as yet unidentified cytokines warrant continued investigation with respect to inhibiting dendritic cell growth and differentiation from CD14+ HLA-DR+ intermediates.

Several hypotheses can be formulated to reconcile these findings with clonogenic studies of BM-derived human dendritic cells.14 CD14+ HLA-DR+++ dendritic cells that develop rapidly by day 6 in suspension culture may be equivalent to the progeny of CFU-DC in clonogenic assays.14 CFU-GM have the potential to generate dendritic cells alongside other myeloid progeny, which in the human requires the addition of TNF-α to GM-CSF.14 TNF-α has pleiotropic effects on hematopoiesis with respect to both cytokines and their receptors, enhancing the recruitment and expansion of less differentiated progenitors while inhibiting the growth of more committed precursor populations.38-39 CD14+ HLA-DR+ intermediates may be included among the developing progeny of CFU-GM, but their bipotential differentiation capacity would not be as apparent because of the competing influence of simultaneously developing macrophages. Another possibility is that there are not two different CD34+ progenitors, one for day 6 CD14+ HLA-DR+++ dendritic cells and another for day 6 CD14+ HLA-DR+ intermediates. The potential of a single myeloid progenitor to form dendritic cell colonies may sometimes be blocked by the early development of suppressive macrophages during the stochastic process of lineage commitment from primitive CFUs.

Caux et al.80 have also presented evidence for two pathways of dendritic cell development from human CD34+ progenitors in cord blood. By day 5, two intermediates have developed in the presence of GM-CSF and TNF-α. One is CD14a CD14+ and the other is CD14a CD14+.

Both give rise to CD14a CD14− dendritic cell progeny after a second
week in culture with GM-CSF, irrespective of the presence of TNF-α. The CD1α+ CD14+ intermediates yield Birbeck granule-containing dendritic/Langerhans cells of the same phenotype. The CD1α+ CD14+ intermediates generate progeny characteristic of CD1α+ CD14+ dermal dendritic cells that lack Birbeck granules. CD1α did not prove to be a discriminating marker in marrow, which also differs from cord blood in having a much greater capacity to form macrophages.

The CD1α+ CD14+ cells in cord blood may be analogous to the CD14+ HLA-DR+ intermediates described here in human BM. This intermediate population has been extensively characterized, including similarities and dissimilarities to standard monocytes, irrespective of the CD14+ phenotype. It has also been distinguished from the CD14- HLA-DR+++ dendritic cells that develop in the same cultures by day 6. This post-CFU, CD14+ HLA-DR+ intermediate population retains bipotential differentiation capacity for either dendritic cells or monocytes/macrophages. Dendritic cell differentiation is cytokine dependent but can be altered by other cells, most likely mature macrophages that have already developed by day 6 in cytokine-supplemented cultures of human CD34+ BM precursors.

ACKNOWLEDGMENT

We appreciate the technical assistance of Koji Shido and Judy Adams. We thank Susan McKenzie and her laboratory staff for their assistance with cytochemical staining. We appreciate the helpful advice of Dr Kayo Inaba with respect to the phagocytosis assays. We also acknowledge the physicians, Sharon Bleau, and Dr Nancy Collins of the Allogeneic Bone Marrow Transplantation Service, Memorial Sloan-Kettering Cancer Center, for their help in obtaining the bone marrow samples for these studies.

REFERENCES

24. Egner W, Hart DJN: The phenotype of freshly isolated and
effects of TGFβ and TNFα on the development of dendritic cells. 


Psoriatic skin-derived dendritic cell function is inhibited by exogenous IL-10. J Immunol 154:1280, 1995


Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-colony-forming unit intermediate

P Szabolcs, D Avigan, S Gezelter, DH Ciocon, MA Moore, RM Steinman and JW Young

Updated information and services can be found at:
http://www.bloodjournal.org/content/87/11/4520.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml