The Journal of Immunology

RESEARCH ARTICLE | MARCH 01 2006

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J Immunol (2006) 176 (5): 2924–2932.
https://doi.org/10.4049/jimmunol.176.5.2924

Directed Differentiation of Human Embryonic Stem Cells into Functional Dendritic Cells through the Myeloid Pathway[1]

Igor I. Slukvin,[2]*†‡ Maxim A. Vodyanik,† James A. Thomson,†‡§¶ Maryna E. Gumenyuk,* and Kyung-Dal Choi*

We have established a system for directed differentiation of human embryonic stem (hES) cells into myeloid dendritic cells (DCs). As a first step, we induced hemopoietic differentiation by coculture of hES cells with OP9 stromal cells, and then, expanded myeloid cells with GM-CSF using a feeder-free culture system. Myeloid cells had a $CD4^+CD11b^+CD11c^+CD16^+CD123^{low}HLA\text{-}DR^-$ phenotype, expressed myeloperoxidase, and included a population of $M\text{-}CSFR^+$ monocyte-lineage committed cells. Further culture of myeloid cells in serum-free medium with GM-CSF and IL-4 generated cells that had typical dendritic morphology; expressed high levels of MHC class I and II molecules, CD1a, CD11c, CD80, CD86, DC-SIGN, and CD40; and were capable of Ag processing, triggering naive T cells in MLR, and presenting Ags to specific T cell clones through the MHC class I pathway. Incubation of DCs with A23187 calcium ionophore for 48 h induced an expression of mature DC markers CD83 and fascin. The combination of GM-CSF with IL-4 provided the best conditions for DC differentiation. DCs obtained with GM-CSF and TNF-α coexpressed a high level of CD14, and had low stimulatory capacity in MLR. These data clearly demonstrate that hES cells can be used as a novel and unique source of hemopoietic and DC precursors as well as DCs at different stages of maturation to address essential questions of DC development and biology. In addition, because ES cells can be expanded without limit, they can be seen as a potential scalable source of cells for DC vaccines or DC-mediated induction of immune tolerance. ***The Journal of Immunology,*** **2006, 176: 2924–2932.**

Dendritic cells (DC)[3] are powerful APCs with a unique ability to induce primary T cell responses (1, 2). Cells of DC lineages are generated continuously in the bone marrow and have a heterogeneous phenotype and function. In humans, DCs develop from 1) $CD34^+$ hemopoietic progenitors through two independent pathways (Langerhans cells and interstitial DCs), 2) monocytes (monocyte-derived DCs (moDCs)), and 3) IFN-α-producing cells (plasmacytoid DCs) (3–8). On the basis of cellular origin and phenotype, human and murine DCs have been classified into two major populations: myeloid and lymphoid (8). It has been suggested that myeloid and lymphoid DCs were derived from common myeloid progenitor and common lymphoid progenitor, respectively (9). However, recent studies have provided evidence that both types of DCs in mice and humans could develop from an either common myeloid or common lymphoid progenitor (10–13), and it has been proposed that common DC precursors give rise to all DC lineages (14).

The availability of human immature DCs is critical for the study of Ag processing and presentation, as well as for understanding the mechanisms of inducing immunity and tolerance. Functional analysis of human DC subsets was significantly facilitated by development of an in vitro system for DC differentiation from $CD34^+$ hemopoietic progenitors and monocytes. However, obtaining large numbers of human DC progenitors is still a laborious process and potentially poses risks for donors. Human embryonic stem (hES) cells represent a unique population of cells capable of self-renewal and differentiation (15), and directed hemopoietic differentiation of hES cells reproduces many aspects of embryonic hemopoiesis (16–18). Therefore, hES cells can be used as a unique source of hemopoietic and DC precursors to address essential questions of DC development. In addition, because ES cells can be expanded without limit, they can be seen as a potential scalable source of cells for DC vaccines or DC-mediated induction of immune tolerance.

Recently, functional dendritic cells were generated from mouse ES cells using the embryoid body method or by coculture of ES cells with a mouse M-CSF-deficient bone-marrow stromal cell line, OP9 (19, 20). The OP9 cells efficiently induce multilineage hemopoietic differentiation of mouse as well as nonhuman primate ES cells (21, 22). Using the OP9 system, we were able to generate a substantial number of $CD34^+$ hemopoietic progenitors from hES cells (17). In this study, we demonstrated that hemopoietic precursors generated in hES cell/OP9 coculture could be differentiated to DCs through the myeloid pathway. The hES cell-derived DCs were morphologically, phenotypically, and functionally comparable with myeloid DCs differentiated from bone marrow hemopoietic progenitors (23, 24). The described technique allowed us to

*Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53706; †Wisconsin National Primate Research Center, University of Wisconsin, Madison, WI 53715; ‡WiCell Research Institute, Madison, WI 53707; §Department of Anatomy, University of Wisconsin Medical School, Madison, WI 53706; and ¶Genome Center of Wisconsin, University of Wisconsin, Madison, WI 53706

Received for publication May 6, 2005. Accepted for publication December 28, 2005.

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[1] This work was supported by Defense Advanced Research Projects Agency Grant DRP5-UWM and National Institutes of Health (NIH) Grant P51 RR000167 to the Wisconsin National Primate Research Center, University of Wisconsin-Madison. I.I.S. was supported by NIH Grant HD44067.

[2] Address correspondence and reprint requests to Dr. Igor I. Slukvin, Department of Pathology and Laboratory Medicine, Wisconsin National Primate Research Center, University of Wisconsin, 1220 Capitol Court, Madison, WI 53715. E-mail address: islukvin@wisc.edu

[3] Abbreviations used in this paper: DC, dendritic cell; moDC, monocyte-derived DC; hES, human embryonic stem cell; SCF, stem cell factor; MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase; $PBCD34^+DC$, DC derived from peripheral blood $CD34^+$ hemopoietic progenitor; HAI, hepatocyte growth factor activator inhibitor; PFKP, platelet-type phosphofructokinase; CFC, colony-forming cell; MFI, mean fluorescence intensity; MMP, matrix metalloproteinase; CLA, cutaneous lymphocyte-associated Ag.

0022-1767/06/$02.00

grow up to 4×10^7 DC at a time from 10^7 initially plated hES cells.

Materials and Methods

Cell lines, cytokines, and mAbs

Human ES cell lines H1 (NIH code WA01; passages 32–51) and H9 (NIH code WA09; passages 40–44) were maintained in an undifferentiated state by weekly passages on mouse embryonic fibroblasts as previously described (25). A mouse bone marrow stromal cell line OP9 was obtained from Dr. T. Nakano (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). This cell line was maintained on gelatinized 10-cm dishes (BD Biosciences) in the OP9 growth medium consisting of α-MEM (Invitrogen Life Technologies) supplemented with 20% defined FBS (HyClone). Sterile, recombinant, endotoxin- and pyrogen-free stem cell factor (SCF), FLT3-L, TNF-α, IL-4 were obtained from PeproTech, GM-CSF (Leukine) was obtained from Berlex Laboratories, and IFN-α was obtained from Schering. The following mouse anti-human mAbs, without detectable cross-reactivity with murine cells, were used for flow cytometric analysis: CD1a-PE, CD4-PE, CD11b-FITC, CD16-PE, CD33-FITC, CD80-PE, CD86-PE, HLA-DR-PE, myeloperoxidase (MPO)-FITC, terminal deoxynucleotidyl transferase (TdT)-FITC (Caltag Laboratories); CD9-PE, CD14-FITC, CD40-PE, CD43-FITC, CD45-PE, GM-CSFRα-PE (CD116), CD209 (DC-SIGN)-FITC, cutaneous lymphocyte-associated Ag (CLA)-FITC (BD Pharmingen); CD11c-PE, CD34-PerCP-Cy5.5 (BD Immunocytometry Systems (BDIS)); CD83-FITC, CD208 (DC-LAMP; Beckman Coulter); CD123-FITC, CD15-FITC (Miltenyi Biotec); HLA-ABC-FITC (W6/32; Sigma-Aldrich); M-CSFR-PE (CD115; R&D Systems). Immunocytochemistry was performed using anti-fascin, -CD68, and -Langerin (CD207) mAbs, the Vectastain ABC-peroxidase kit and the Vector NovaRED substrate kit (Vector Laboratories)

Hemopoietic differentiation of hES cells in coculture with OP9 cells

The induction of hES cell differentiation into hemopoietic cells was done as previously described (17). Briefly, undifferentiated hES cells were harvested by treatment with 1 mg/ml collagenase IV (Invitrogen Life Technologies) and added to OP9 cultures at an approximate density of 1.5×10^6/20 ml per 10-cm dish in α-MEM supplemented with 10% FBS (HyClone) and 100 μM monothioglycerol (Sigma-Aldrich). The hES cell/OP9 cocultures were incubated for 9–10 days with a half-medium change on days 4, 6, and 8 without added cytokines.

Generation of hES cell-derived DCs

A schematic diagram of the protocol used for generation of DCs from hES cells is depicted in Fig. 1. On days 9–10 of hES cell/OP9 coculture, differentiated hES cells were harvested by treatment with collagenase IV (Invitrogen Life Technologies; 1 mg/ml in α-MEM) for 20 min at 37°C, followed by treatment with 0.05% trypsin-0.5 mM EDTA (Invitrogen Life Technologies) for 15 min at 37°C. After trypsin inactivation by FBS, cells were resuspended in α-MEM supplemented with 10% FBS (HyClone) and 100 ng/ml GM-CSF, and transferred into tissue culture flasks (BD Biosciences) coated with poly 2-hydroxyethyl methacrylate (Sigma-Aldrich) to prevent cell adherence. Cells were cultured for 8–10 days with a half-medium change every fourth day to expand dendritic cell precursors. To evaluate the effect of SCF and FLT3-L on the expansion of hES cell-derived DC precursors, we cultured the cells in the presence of 1) 100 ng/ml GM-CSF + 20 ng/ml SCF; 2) 100 ng/ml GM-CSF + 50 ng/ml FLT3-L; or 3) 100 ng/ml GM-CSF + 20 ng/ml SCF + 50 ng/ml FLT3-L.

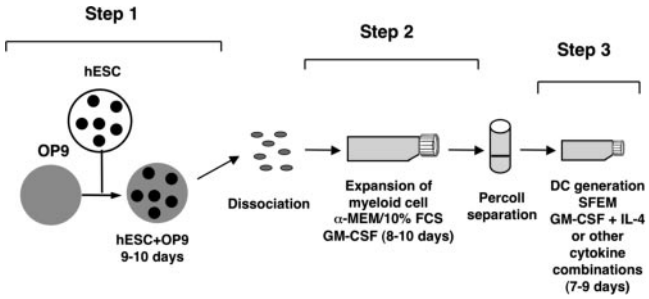

FIGURE 1. Schematic diagram of the protocol used for generation of myeloid precursors and DCs from hES cells.

Subsequently, cells were spun over 20% Percoll (Sigma-Aldrich) to remove dead cells and cell aggregates. As a third step, Percoll-isolated cells were cultured for 7–9 days in 2-hydroxyethyl methacrylate-coated flasks in StemSpan serum-free expansion medium (Stem Cell Technologies) supplemented with lipid mixture 1 (Sigma-Aldrich) and 100 ng/ml GM-CSF, with the addition of the following cytokines: 1) 100 ng/ml IL-4; 2) 20 ng/ml TNF-α; 3) 10^4 U/ml IFN-α; and 4) 100 ng/ml IL-4 + 20 ng/ml TNF-α. Cells were cultured for 7–9 days with a half-medium change every fourth day. To further maturate DCs, we cultured cells obtained in step 3 in serum-free expansion medium with 400 ng/ml A23187 calcium ionophore (Sigma-Aldrich) for 48 h.

Because comparable results were obtained for H1 and H9 hES cell/OP9 cocultures, we reported pooled data for both cell lines in these studies.

Generation of DCs from adult CD34$^+$ progenitor cells (PBCD34$^+$DC) and monocytes (moDC)

Frozen peripheral blood CD34$^+$ cells from healthy donors intended for final disposition were obtained from the University of Wisconsin Hemopoietic Stem Cell Laboratory with approval from the University of Wisconsin Institutional Review Board. Donors had previously signed an Institutional Review Board-approved consent. CD34$^+$ cells (purity >82%) were cultured at 1×10^5/ml for 14 days in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (HyClone) in the presence of 50 ng/ml GM-CSF, 10 ng/ml IL-4, and 2.5 ng/ml TNF-α with a half-medium change every third day. Flow cytometric analysis demonstrated that >40% of the cells generated in these cultures were HLA-DR$^+$CD1a$^+$CD14$^-$ and included CD83-positive cells (10–20% of cells). Adult PBMC were purchased from AllCells. Monocytes were isolated by adherence to plastic and were cultured for 6 days in RPMI 1640 (Invitrogen Life Technologies) containing 1% FBS (HyClone) in the presence of 100 ng/ml GM-CSF and 100 ng/ml IL-4 with half-medium change every 2 days. The moDCs had typical DC morphology and phenotype of immature DCs (HLA-DR$^+$DC-SIGN$^+$CD1a$^{+/-}$CD14$^-$CD83$^-$).

Flow cytometric analysis

Cells were prepared in PBS-FBS (PBS containing 0.05% sodium azide, 1 mM EDTA, and 2% FBS), supplemented with 2% normal mouse serum (Sigma-Aldrich), and labeled with a combination of mAbs. Samples were analyzed using a FACSCalibur flow cytometer (BDIS) with CellQuest acquisition software (BDIS). List mode files were analyzed by FlowJo software (Tree Star). Control staining with appropriate isotype-matched control mAbs (BD Pharmingen) was included to establish thresholds for positive staining and background linear scaled mean fluorescence intensity (MFI) values. The percentage of positive cells was calculated as the percent of positive cells stained with specific mAb − the percent of background staining with corresponding isotype control. ΔMFI was calculated as the MFI of cells stained with specific mAb − the MFI of cells stained with corresponding isotype control. Linear scaled MFI was used as an indicator of relative Ag density on given cells.

RT-PCR

Total RNA was isolated from cells using RNAwiz (Ambion) and treated with DNAfree reagent (Ambion) to remove potentially contaminating DNA. RT-PCR was performed from 1 μg of total RNA using the Omniscript RT kit (Qiagen) and the MasterTaq PCR kit (Eppendorf) and the following human-specific primers: CCL17 forward 5′-ATGGCCCCACT GAAGATGCTT-3′ and reverse 5′-TGAACACCAACGGTGGAGGT-3′ (351 bp); CCL13 forward 5′-ATGACAGCAGCTTTCAACCCC-3′ and reverse 5′-CTCCAAACCAGCAACAAGTCAAT-3′ (451 bp); metalloproteinase 12 (MMP-12) forward 5′-TTTTGCCCGTGGAGCTCAT-3′ and reverse 5′-TTCCCACGGTAGTGACAGCA-3′ (400 bp); cathepsin C forward 5′-TTTCTCAGCTCCCTGCAGCA-3′ and reverse 5′-CATGC ACCCACCCAGTCATT-3′ (400 bp); hepatocyte growth factor activator inhibitor (HAI-2) forward 5′-ATCCACGACTTCTGCCTGGT-3′ and reverse 5′-CGGCAGCCTCCATAGATGAA-3′ (401 bp); platelet-type phosphofructokinase (PFKP) forward 5′-TTTCAAGATGCGGTTCGACT-3′ and reverse 5′-AATCCACCGATGATCAGCAG-3′ (401 bp), PU1 forward 5′-TGGAAGGGTTTCCCCTCGTC-3′ and reverse 5′-TGCTGTC CTTCATGTCGCCG-3′ (546 bp); and pre-Tα forward 5′- AGTACA CAGCCCATGCATCTGTCA-3′ and reverse 5′-AATGCTCCAAGACT GGAGGAAGGA-3′ (446 bp) primers. The following primers were used for amplification of human and mouse β-actin: forward 5′- TGACG GGGTCACCCACACTGTGCCCATCTA-3′ and reverse 5′- CTAGAAG CATTGCGGTGGACGATGGAGGG-3′ (650 bp).

Clonogenic progenitor cell assay

Hemopoietic clonogenic assays were performed in 35-mm low-adherent plastic dishes (StemCell Technologies) using 1 ml/dish MethoCult GF$^+$ H4435 semisolid medium (StemCell Technologies) as previously described (17).

Measurement of OVA uptake and proteolysis

The OVA processing assay was performed using a self-quenched conjugate of OVA (DQ-OVA; Molecular Probes) that exhibits bright-green fluorescence upon proteolytic degradation (26, 27). DCs obtained in step 3 were incubated with 100 μg/ml DQ-OVA for 30 min at 37°C in DMEM/F12 (Invitrogen Life Technologies) containing 2% FBS and 1% nonessential amino acids. Cells incubated at 4°C were used as a control for background fluorescence. OVA proteolysis was evaluated by flow cytometry.

Allogeneic MLR

Adult mononuclear cells were isolated from peripheral blood samples obtained from healthy laboratory volunteers by density gradient centrifugation on Histopaque-1077 or purchased from AllCells. Mononuclear cord blood cells were purchased from Cambrex BioScience. Mononuclear cells were depleted of monocytes by plastic adherence and used as responder cells. Graded numbers (1×10^3 to 3×10^4/well) of irradiated (35Gy) stimulatory cells were cocultured with 1×10^5 responder cells for 6 days in 96-well flat-bottom plates (Corning) in RPMI 1640 containing 5% human AB serum (Sigma-Aldrich). [^{3}H]Thymidine (Sigma-Aldrich) was added (1 μCi/well) during the last 16 h of incubation. Cells were harvested onto glass-fiber filters, and incorporation of [^{3}H]thymidine was measured by scintillation counting. All MLR were performed in triplicate with the mean was calculated for each data point.

Ag presentation assay

An allogeneic HLA-A0201-restricted CD8$^+$ T cell line with specificity to CMV pp65 NLVPMVATV peptide was purchased from Proimmune. Heat-inactivated Towne strain CMV virus was obtained from Biodesign International and NLVPMVATV peptide (> 95% of purity) was synthesized at the Biotechnology Center (University of Wisconsin, Madison, WI). HLA isotyping of the H1 cell line demonstrated that it possesses the *HLA-A02* allele. CD34$^+$ hemopoietic progenitors and monocytes used in the Ag-presenting assay were obtained from HLA-A02-positive donors. DCs obtained in step 3 with GM-CSF and IL-4 were incubated overnight with 40 ng/ml CMV virus in RPMI 1640 medium containing 10% FBS. Control samples were incubated overnight without the virus. Then DCs were washed in medium three times, irradiated, and plated at 3×10^4 in RPMI 1640/10% FBS with 5×10^4 T cells in flat-bottom 96-well plates (Corning). Proliferative response was evaluated after 48 h of incubation. [^{3}H]Thymidine (Sigma-Aldrich) was added (1 μCi/well) during the last 16 h of incubation. In addition, supernatants were collected after 3 days of culture, and IFN-γ release was measured with the human IFN-γ ELISA kit (eBioscience). For peptide presentation, DCs were pulsed with 5 μg/ml NLVPMVATV peptide in the presence of 0.3 μg/ml β_2-microglobulin for 4 h at 37°C, and incubated with T cells as described above. All assays were performed in triplicate with the mean and SD calculated.

Results

hES cell-derived myeloid cells can be expanded with GM-CSF

Recently, we developed an in vitro culture system for hemopoietic differentiation of hES cells using the mouse bone marrow stromal cell line OP9 as a feeder (17). hES cells cocultured with OP9 differentiate into CD34$^+$ cells that are highly enriched in colony-forming cells (CFCs) and contain erythroid and myeloid, as well as lymphoid, progenitors. As we demonstrated earlier, maximal expansion of myeloid CFCs in the OP9 system was observed on days 9–10 of differentiation (17). To induce selective expansion of myeloid lineage, we harvested cells from days 9–10 of hES cell/OP9 coculture and cultured them in nonadherent conditions in the presence of GM-CSF. At the beginning of culture, large cell aggregates formed. Approximately 3 days after initiation of GM-CSF culture, individual cells appeared in suspension and rapidly expanded (Fig. 2*A*). After 9–10 days of culture with GM-CSF and removal of clumps and dead cells by Percoll separation, we obtained a cell population containing ~90% CD45-positive cells. The majority of these CD45$^+$ cells contained intracellular MPO, but not TdT, expressed GM-CSFR (CD116; Fig. 2*D*), and weakly expressed CD33, the marker of myeloid progenitors (data not shown). In addition, hES cell-derived myeloid cells were CD4-positive and weakly expressed IL-3R α-chain CD123. These cells expressed the spectrum of myeloid markers characteristic of bone marrow myeloid lineage cells (28) such as CD16, CD11b, CD11c, and CD15 (Table I, Figs. 2*D* and 3) and included population of M-CSFR (CD115)-positive monocyte-lineage committed cells (29). Morphologically, the GM-CSF-expanded cells had irregularly shaped nuclei with a moderate amount of grayish, occasionally vacuolated, agranular cytoplasm (Fig. 2*B*). Infrequently, cells with very fine cytoplasmic granules could be found. It is interesting to note the presence in step 2 cultures of cells with a pumpkin-shaped

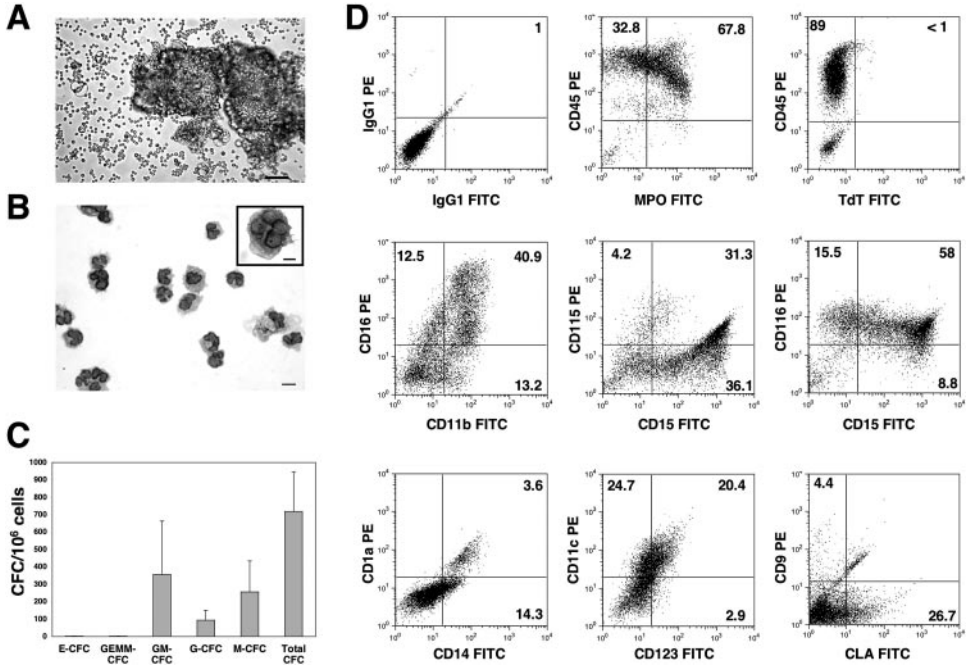

FIGURE 2. Morphologic and phenotypic features and clonogenic potential of myeloid precursors generated from hES cells in step 2. *A*, A phase-contrast micrograph of differentiated H1 cells growing in the presence of GM-CSF (bar is 50 μm). *B*, Wright-stained cytospins of cells obtained in step 2 after culture of differentiated H1 cells with GM-CSF (bar is 20 μm). *Inset*, At high magnification, myeloid cell generated in step 2 (large agranular cell with pumpkin-shaped nucleus containing two nucleoli and moderate amount of grayish cytoplasm; bar is 5 μm). *C*, CFC potential of GM-CSF-expanded cells (results are mean ± SD of five experiments). *D*, Expression of surface and intracellular myeloid markers on GM-CSF-expanded hES cell-derived myeloid cells. The numbers indicate percentages of positive cells (mean of three to four experiments) in corresponding quadrants.

Table I. *Phenotypic analysis of DCs induced by different cytokine combinations*[a]

Cell Subset		Step 2	Step 3			
			GM-CSF + IL-4	GM-CSF + TNF-α	GM-CSF + IL-4 + TNF-α	GM-CSF + IFN-α
R1 gated cells	%	NA	58.8 ± 12.3	45.5 ± 12.1	46.7 ± 14.9	39.9 ± 7.5
CD1a	%	3.3 ± 2.1	82.9 ± 12.4	66.9 ± 24.0	78.2 ± 7.7	30.3 ± 27.1
	ΔMFI		750.2 ± 700.7	74.8 ± 60.8	148.3 ± 161.9	77.1 ± 72.1
CD14	%	12.6 ± 7.1	25.6 ± 7.5	71.1 ± 12.2	39.0 ± 19.3	19.8 ± 15.1
	ΔMFI	14.7 ± 4.2	27.6 ± 15.5	55.3 ± 38.1	31.5 ± 29.0	60.7 ± 50.8
DC-SIGN	%	<1	87.6 ± 7.7	<2	84.7 ± 4.2	17.3 ± 15.4
	ΔMFI		460.3 ± 352.0		213.8 ± 160.1	40.2 ± 39.1
CD83	%	<1	<1	<1	<1	<1
CD11c	%	60.0 ± 14.2	94.1 ± 5.3	98.0 ± 1.6	93.7 ± 3.3	91.0 ± 8.5
	ΔMFI	132.1 ± 59.9	282.3 ± 37.2	202.3 ± 19.8	237.6 ± 17.8	97.4 ± 41.8
CD11b	%	59.4 ± 13.1	67.4 ± 29.0	48.8 ± 24.9	56.0 ± 5.4	59.6 ± 8.4
	ΔMFI	69.3 ± 23.0	52.9 ± 33.6	24.6 ± 14.3	47.9 ± 32.5	40.1 ± 35.3
CD123	%	35.5 ± 14.6	58.8 ± 12.3	63.5 ± 16.6	45.1 ± 7.9	29.4 ± 18.6
	ΔMFI	27.8 ± 15.2	35.9 ± 14.6	28.3 ± 12.6	33.9 ± 20.1	18.9 ± 15.3
HLA-ABC	%	79.6 ± 8.8	90.3 ± 8.4	91.8 ± 4.1	84.8 ± 9.3	99.2 ± 0.9
	ΔMFI	125.7 ± 61.6	92.4 ± 10.6	130.3 ± 62.1	111.2 ± 55.4	258.0 ± 47.9
HLA-DR	%	14.9 ± 12.0	90.1 ± 6.3	90.1 ± 4.1	82.1 ± 8.0	89.4 ± 7.7
	ΔMFI	189.5 ± 83.7	597.0 ± 204.3	267.3 ± 123.1	208.3 ± 82.9	509.8 ± 340.2
CD86	%	35.1 ± 9.1	93.4 ± 3.5	85.4 ± 7.3	90.1 ± 2.9	82.4 ± 14.1
	ΔMFI	60.2 ± 24.3	1767.4 ± 1122.3	158.5 ± 94.6	439 ± 131.0	125.3 ± 107.2
CD80	%	7.9 ± 7.8	81.2 ± 21.8	84.8 ± 10.7	81.8 ± 11.6	81.6 ± 19.3
	ΔMFI		621.2 ± 492.9	128.9 ± 80.4	295.8 ± 353.7	61.0 ± 13.2
CD40	%	4.6 ± 4.4	46.4 ± 16.9	43.3 ± 23.7	57.0 ± 1.6	53.9 ± 26.8
	ΔMFI		27.0 ± 11.4	16.6 ± 5.2	47.2 ± 32.5	21.0 ± 10.2

[a] Results are mean ± SD of four to five independent experiments; for step 3 cultures the percentage and ΔMFI were calculated for R1 gated cells (see Fig. 4).

nucleus containing two nucleoli and a moderate amount of cytoplasm without visible cytoplasmic granules (Fig. 2*B*, *inset*), thus resembling so-called agranular embryonic granuloblasts (30). During ontogeny, this transitory population of cells was found in the bone marrow at the initiation of bone marrow hemopoiesis (12–14 wk). However, embryonic granuloblasts have not been characterized functionally, and their relationship to classical myeloblasts is unknown.

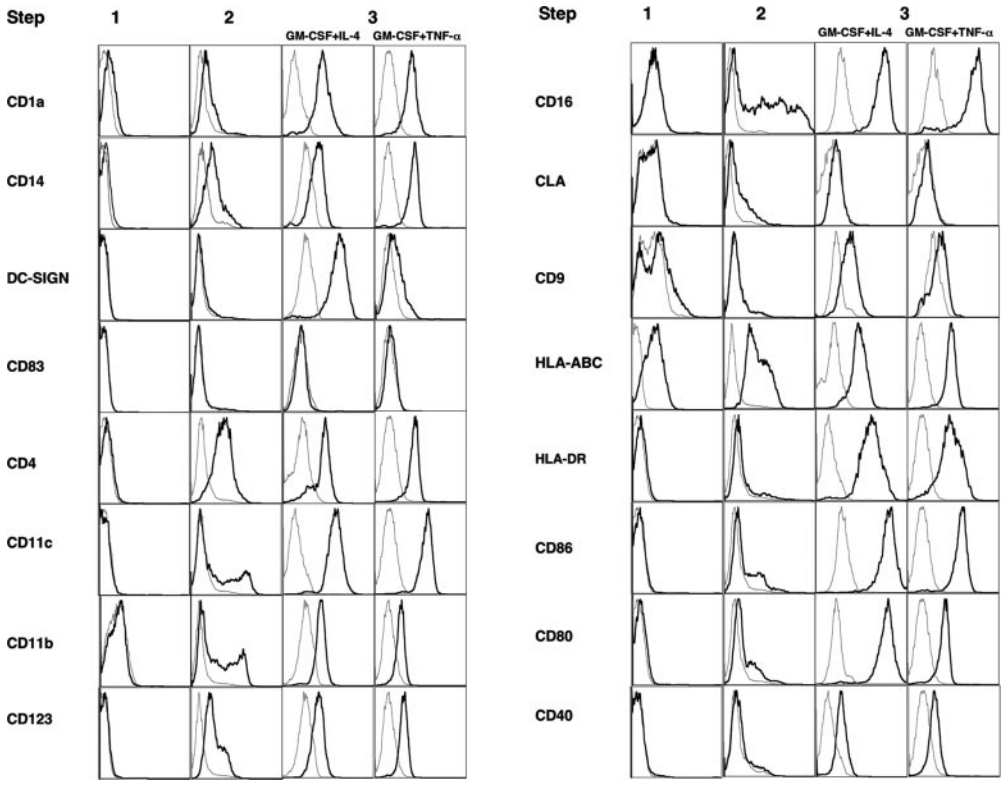

FIGURE 3. Phenotypic evolution of differentiated hES cells obtained in three-step culture. Representative experiment with H9 hES cells demonstrates expression of surface markers (black histogram in bold) relative to isotype control (gray histogram). ΔMFI and percentage of positive cells are presented in Table I.

Table II. *Relative yield of cells after each culture step*[a]

	Relative Cell Yield
Step 1	8.8 ± 4.4
Step 2	
GM-CSF	5.5 ± 3.7
GM-CSF + SCF	5.6 ± 5.7
GM-CSF + FLT3-L	4.6 ± 4.2
GM-CSF + SCF + FLT3-L	5.1 ± 5.0
Step 3	
GM-CSF + IL-4	3.3 ± 4.1
GM-CSF + TNF-α	2.3 ± 1.7
GM-CSF + IFN-α	2.3 ± 1.6
GM-CSF + IL-4 + TNF-α	1.9 ± 1.3

[a] Relative cell yield at each step was calculated as a number of cells obtained from one initially plated undifferentiated hES cell (total number hES cells plated on OP9/ total number of cells obtained after corresponding step); results were calculated as mean ± SD of 4 to 10 experiments.

Some of GM-CSF-expanded cells retained myeloid CFC potential, but neither erythroid nor multilineage CFC potential was detected (Fig. 2*C*). In addition, a relatively small population of cells at advanced stages of maturation expressed a moderate level of CD1a and CD14 as well as the HLA-DR, and CD80 and CD86 costimulator molecules (Table I, Fig. 3).

CLA expression on peripheral blood $CD34^+$ cells defines progenitors that further differentiate into Langerhans cells, while $CD34^+CLA^-$ cells give rise to interstitial DC-like cells (31). No significant CLA expression was detected in the total cell population obtained from OP9 cocultures (Fig. 3) or in isolated hES cell-derived $CD34^+$ cells (data not shown). However, CLA expression was found on a subset of myeloid cells generated with GM-CSF (Figs. 2*D* and 3).

GM-CSF appeared as the most important factor in expansion of myeloid cells, and the addition of SCF, FLT3L, or SCF^+FLT-3L to GM-CSF-supplemented cultures had little effect on total cell output (Table II) and myeloid CFC numbers (data not shown) during 10 days of culture in our system. Overall, these data demonstrate that culture of differentiated hES cells with GM-CSF allows us to obtain myeloid lineage cells at various stages of development, including myeloid progenitor and postprogenitor cells.

Differentiation of hES-cell derived myeloid cells into DCs

To induce differentiation of myeloid cells obtained in step 2 into DCs, we cultured them with various combinations of GM-CSF and IL-4, TNF-α, and IFN-α. In a typical experiment, after 7–10 days of culture with GM-CSF and IL-4, a majority of the cells appeared as aggregates. In addition, individual floating cells with well-defined dendrites can be seen (Fig. 4*A*). Morphologically, cells were large, exhibited an oval or kidney-shaped nucleus with a high nuclear cytoplasmic ratio, and had nonvacuolated, occasionally granular cytoplasm with very fine cytoplasmic processes (Fig. 4*C*). When cultured on the flat-bottom plates, cells formed well-defined dendrites (Fig. 4*B*). Flow cytometric scatter analysis of cells obtained in step 3 revealed two cell populations: R1, cells with high scatter profile and DC phenotype (Fig. 4*D*) and R2, cells with low scatter profile, which were lacking DC markers and were somewhat phenotypically similar to the myeloid progenitors generated in the second step (Fig. 4*D* and data not shown). DCs identified as R1 gated cells expressed CD1a, DC-SIGN, CD4, CD11c, CD16, MHC class I and class II molecules, CD80, and CD86 (Fig. 3, Table I). Additionally, these cells expressed low levels of CD9, CD11b, CD123, and CD40. CD14 expression was very weak, but detectable, and most of the CD14-positive cells coexpressed CD1a. However, the cells lacked CD83 expression. Neither CD208 (DC-LAMP) nor CD207 (Langerin) was detected by flow cytometry or immunocytochemistry in generated DCs (data not shown).

In addition to IL-4, differentiation of myeloid precursors into DCs was achieved by using other cytokines such as TNF-α and IFN-α or IL-4 and TNF-α in combination. However, most of the CD1a cells in cultures with TNF-α coexpressed relatively high levels of CD14 and lacked CD9 and DC-SIGN (Fig. 3, Table I). When TNF-α was added to cultures with GM-CSF and IL-4, CD14 expression was up-regulated while CD1a and DC-SIGN expression was down-regulated (Table I). As expected, the addition of IFN-α resulted in increased expression of MHC class I molecules; however, IFN-α decreased the number of $CD1a^+$ cells as well as CD14 expression. Similar to the monocyte-DC differentiation pathway (32), expression of DC-SIGN on hES cell-derived DCs was primarily dependent on IL-4 (Fig. 3, Table I). Based on cell yield and phenotypic and functional properties (Tables I and II, Fig. 3, and see Fig. 7), we concluded that a combination of GM-CSF and IL-4 provides the best conditions for generation of functional DCs from hES cells.

As shown by immunocytochemistry, hES cell-derived DCs were lightly CD68-positive and did not express CD83. Fascin, an actin-binding protein that has been shown to be a highly selective DC marker (33), was not detected. From this, we concluded that generated DCs were immature. To investigate whether generated DCs

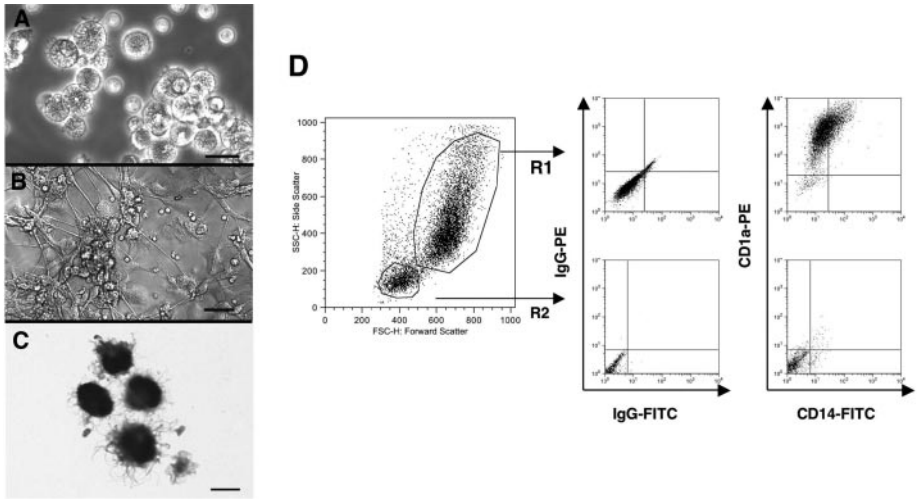

FIGURE 4. Morphology and light scatter properties of hES cell-derived DCs. *A*, Phase contrast micrograph of culture and (*C*) Wright-stained smears of differentiated H1 cells demonstrate numerous thin cytoplasmic processes ("veils"); (*A*) bar is 15 μm and (*C*) bar is 40 μm. *B*, When cultured on flat-bottom ultralow attachment plates, cells form long dendrites; bar is 25 μm. *D*, Light scatter properties and phenotype of cells obtained in step 3 after 9-day culture of hES cell-derived myeloid progenitors with GM-CSF and IL-4. Phenotypic analysis from representative experiments using the H1 cell line shows that R1-gated cells with a high scatter profile express CD1a and weakly CD14.

Table III. *Ag-presenting capacity of H1-derived DCs (hESDC), PBCD34$^+$DCs, and moDCs*[a]

DC	Peptide	CMV	
	Ag-specific proliferative response (cpm)[b]	Ag-specific proliferative response (cpm)[b]	Ag-specific IFN-γ production (pg/ml)[b]
hESDC	984 ± 332	3077 ± 1895	101.7 ± 22.4
PBCD34$^+$DC	3173 ± 502	NT	NT
moDC	2436 ± 434	NT	NT

[a] HLA-A02 H1-derived DCs (cells obtained in step 3 with GM-CSF + IL-4, PBCD34$^+$DCs, or moDCs from HLA-A02 donors incubated for 4 h with or without NLVPMVATV peptide and then added to the HLA-A0201-restricted allogeneic T cell line with specificity to CMV pp65. In addition, hESDCs incubated overnight with or without CMV virus.

[b] Ag-specific responses were calculated as follows: response in presence of virus/peptide − response in cultures without virus/peptide. Results expressed as a mean ± SD of triplicate. NT, not tested.

can be further matured, we treated cells generated in the third step with calcium ionophore A23187 (34). After this treatment, the intensity of intracytoplasmic CD68 staining substantially increased, and perinuclear condensation of CD68 was evident. In addition, cells began to express intracytoplasmic CD83 and fascin (Fig. 5).

Using serial analysis of gene expression of monocytes, monocyte-derived DCs, and macrophages, Hashimoto et al. (35) revealed several highly specific for DC genes, including *CCL17* and *CCL13* chemokines, *MMP-12*, *cathepsin C*, *HAI-2*, and *PFKP*, which were not expressed in macrophages and monocytes. We performed PCR analyses of cells obtained at different stages of differentiation and found that *CCL13*, *MMP-12* transcripts could already be detected in GM-CSF-expanded DC progenitors; however, expression of *CCL17* was significantly up-regulated in cells only after GM-CSF and IL-4 culture (Fig. 6). *PFKP*, *HAI-2*, and cathepsin C mRNAs were present at all stages of differentiation. The hES cell-derived DCs expressed the *PU1* transcription factor associated with development of myeloid DCs (36), but lacked *pre-Tα*, which is expressed in plasmacytoid DCs (37). Altogether, these data clearly demonstrate that cells with a typical myeloid DC morphology, phenotype, and gene expression profile can be generated from hES cells.

hES cell-derived DCs induce allogeneic T cell response and are able to process and present Ags

We next determined whether hES cell-derived DCs are functional. Using a self-quenched conjugate of OVA (DQ-OVA) that exhibits bright-green fluorescence upon proteolytic degradation, we found that hES cell-derived DCs were able to uptake and process OVA (Fig. 7). Cells obtained in cultures with GM-CSF and IL-4 were the most efficient in Ag processing, while cells differentiated with GM-CSF and TNF-α were less efficient.

The cells obtained in step 3 with different cytokine combinations were examined for their allostimulatory capacity in MLR. DCs obtained in cultures with GM-CSF and IL-4 or IFN-α induced a considerable proliferation of allogeneic adult lymphocytes (Fig. 8*A* and data not shown). A hallmark of DCs is their ability to stimulate naive cells (1, 2). As shown in Fig. 8*B*, hES cell-derived DCs were able to trigger proliferation of cord blood T cells, which are entirely naive. Immature DCs generated in cultures with GM-CSF and IL-4 were the most potent stimulatory cells, while the addition of TNF-α substantially diminished the cells' ability to stimulate naive T lymphocytes (data not shown). The capacity of hES cell-derived DCs to stimulate allogeneic adult or cord blood MLRs was similar to that observed with DCs generated from a peripheral blood monocytes (moDCs) but lower in comparison to PBCD34$^+$DCs (Fig. 8). As we found, PBCD34$^+$DCs, in contrast to hES cell-derived DCs or moDCs, contained a population of mature CD83$^+$ DCs (data not shown), which could explain the higher Ag-presenting capacity of PBCD34$^+$DCs.

To evaluate the capacity of DCs to present Ags through the MHC class I pathway, we pulsed HLA-A02 H1 cell line-derived DCs with inactivated CMV or NLVPMVATV peptide and evaluated their ability to stimulate HLA-A0201-restricted, allogeneic T cells with specificity to CMV pp65 NLVPMVATV peptide. As demonstrated in Table III, addition of H1-derived DCs pulsed with CMV or peptide to T cells induced a significant increase in proliferative response as compared with that seen with nonpulsed DCs. However, hES cell-derived DCs showed a lower peptide-presenting capacity when compared with moDCs or PBCD34$^+$DCs, possibly reflecting the more immature status of hES cell-derived DCs. Overall, these data clearly demonstrate that

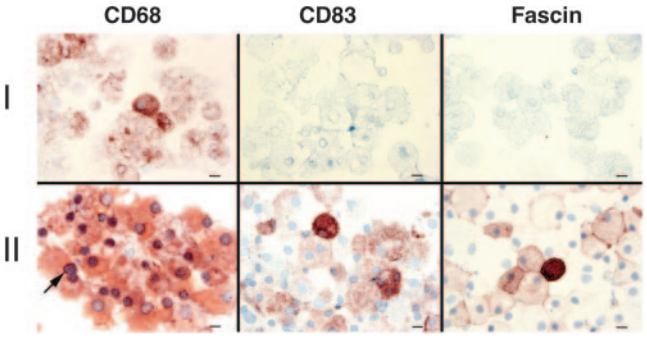

FIGURE 5. Immunocytochemical analysis of CD68, CD83, and fascin expression in immature H1-derived DCs obtained in culture with GM-CSF and IL-4 (*row I*) and following induction of their maturation with A23187 calcium ionophore (*row II*). Bar is 5 μm.

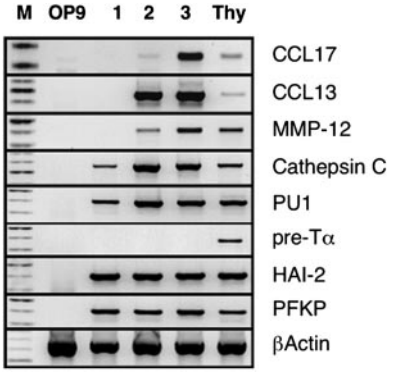

FIGURE 6. RT-PCR analysis of gene expression in H1 cells obtained after first (1), second (2), and third (3) steps of culture. Human thymus RNA (Thy) was used as positive control. Transcripts of studied genes were not amplified from OP9 cells alone. M indicates DNA markers.

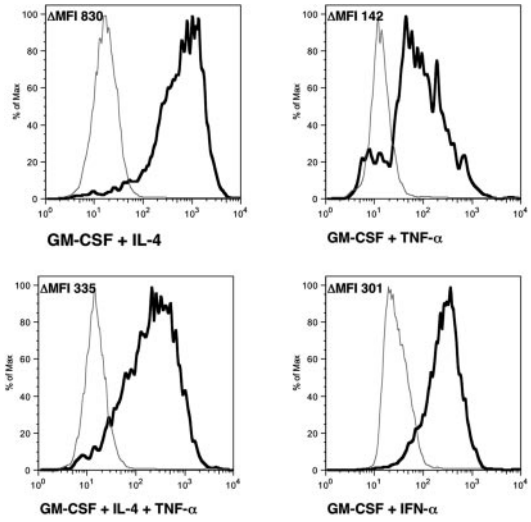

FIGURE 7. OVA uptake and processing by hES cell-derived DCs. Representative results from one of three typical experiments showing H1 cell-derived DCs; flow cytometric analysis of R1-gated DCs incubated 30 min with DQ-OVA at 4°C (gray histogram) and 37°C (black histogram in bold).

our culture system allows generation of cells with Ag-presenting properties characteristic of myeloid DCs.

Discussion

In this study, we have demonstrated generation of DCs from hES cells using selective expansion of myeloid cells obtained in hES cell/OP9 coculture followed by induction of their differentiation into DCs. The most critical step in our protocol for generating DCs was the efficiency of hemopoietic differentiation in hES cell/OP9 coculture. Cocultures with a low number of $CD34^+CD45^+$ hemopoietic precursors failed to expand myeloid lineage committed cells and, subsequently, differentiate to DCs. Using whole cell suspension, rather than isolated hemopoietic precursors from hES cell/OP9 coculture, and culturing the cells in nonadherent conditions were other important requirements for achieving substantial GM-CSF-mediated expansion of myeloid cells capable of differentiating into DCs. GM-CSF was the most critical factor for expansion of myeloid precursors, while SCF and FLT3-L, which have been shown to expand $CD34^+$ cell-derived dendritic precursors (38, 39), have little effect in our culture method. It is possible that SCF and FLT3-L are produced by cells generated in hES cell/OP9 coculture and, therefore, their addition was not required.

Myeloid cells expanded with GM-CSF contained myeloid CFCs, as well as a small population of more mature cells with DC phenotype, though the majority of the cells had a unique $CD4^+CD11b^+CD11c^+CD16^+MPO^+CD123^{low}$ phenotype. Distinct subpopulations of myeloid cells expressed M-CSFR, indicating monocyte-lineage commitment. However, these monocytic cells were immature, lacking HLA-DR expression. We also found that M-CSFR-positive cells coexpressed CD16 (data not shown), which was found on 15% of peripheral blood monocytes. $CD16^+$ monocytes expressing low levels of CD14 had increased potential to become migratory DCs (40). It is possible that myeloid progenitors in our system are enriched in $CD16^+$ DC precursors, which are less mature than $CD16^+CD14^{dim}$ peripheral blood monocytes and require additional maturation signaling to up-regulate MHC class II expression.

The hES cell-derived DCs obtained by our method had a $CD1a^+CD9^+CD68^+CD86^+CD207^-$ phenotype comparable with myeloid DCs differentiated from $CD34^+$ hemopoietic stem cells (3, 23) and expressed transcripts specific for myeloid DCs such CCL17, CCL13, MMP-12, and cathepsin C. However, a distinct phenotypic feature of these cells was coexpression of CD14. The level of CD14 expression was the lowest on cells differentiated by IL-4 but was substantially higher on cells differentiated by TNF-α. DCs that develop from human $CD34^+$ cord blood progenitors in the presence of GM-CSF and TNF-α differentiate into Langerhans cells and dermal/interstitial DCs through $CD1a^+CD14^-$ and $CD1a^-CD14^+$ intermediates, respectively (3). So far, in our cultures, CD1a expression has been always associated with at least a low level of CD14 expression, and we have not observed distinct $CD1a^+CD14^-$ and $CD1a^-CD14^+$ cell populations. This can be explained by different culture conditions used for differentiation or unique pathways of DC differentiation from hES cells.

Two-step OP9 coculture has been used successfully to generate mouse DCs from ES cells (19). Although our protocol has some commonalities with the described mouse system, there are differences between the two. The most important is that we were able to avoid a second OP9 coculture. We collected OP9-differentiated hES cells when an optimal amount of myeloid progenitors had been generated, and then expanded these progenitors with GM-CSF in feeder-free conditions. Another advantage is that our technique allowed us to obtain a discrete population of myeloid DC precursors, which is critical for further studies of DC development. It is important to emphasize that using the described technique, we were able to grow up to 4×10^7 DCs from 10^7 hES cells initially cocultured with OP9 in six 10-cm tissue culture dishes so, already, a sufficient number of cells for functional studies and genetic manipulation could be generated.

A recent study has shown that hemopoietic cells generated during embryoid body differentiation are able to trigger adult lymphocytes in MLR (41). However, the phenotype and Ag-presenting

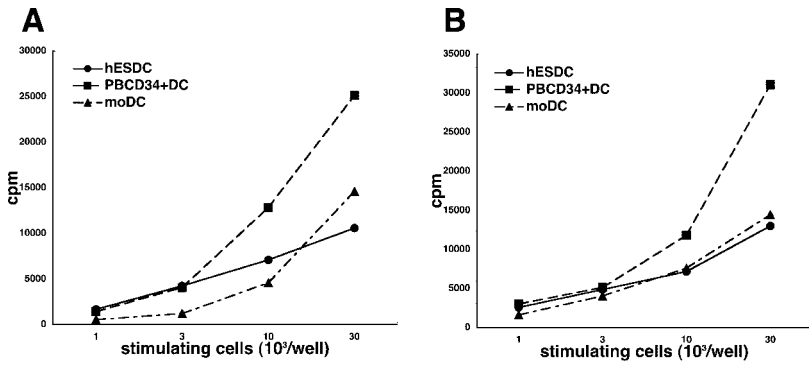

FIGURE 8. Comparison of the allogeneic stimulatory capacity of hES cell-derived DCs, $PBCD34^+$DCs and moDCs. Adult (*A*) and cord blood (*B*) mononuclear cells cultured with graded number of hESDCs obtained in step 3 with GM-CSF and IL-4, $PBCD34^+$DCs or moDCs. *A*, Data are mean of two to four separate experiments. The *x*-axis depicts graded doses of stimulating cells; the *y*-axis depicts [^{3}H]thymidine incorporation by responder cells.

properties of cells generated within embryoid bodies were not characterized. Our results provide first-time evidence that hES cells can be directly differentiated into cells with the morphology, phenotype, and functional properties of DCs.

Several studies have demonstrated that peptide-pulsed DCs transferred in vivo were able to efficiently induce antitumor immune responses in mice (2, 42), that encouraged the subsequent development of DC-based vaccines for cancer immunotherapy in humans (43, 44). Immature DC precursors isolated from peripheral blood, or DCs generated from PBMC and $CD34^+$ hemopoietic progenitors have been used in clinical trials of dendritic cell-based vaccines (7, 43, 45–48). However, these techniques are laborious, require repeated generation of new DCs for each vaccination, and are difficult to standardize (43). The hES cells can be expanded without limit and can differentiate into multiple types of cells (15, 49), and therefore, can be a universal and scalable source of cells for DC vaccines. In the clinical setting, hES cell-derived DCs would have several advantages over DCs from conventional sources. Large absolute numbers of DCs can be generated from the same donor ES cell line and used for multiple vaccinations. Derivation of DCs from hES cells can be less laborious and more amenable to standardization with the implementation of bioreactor technology. A low risk of pathogen contamination and risk-free donor collection are other important advantages of the clinical use of hES cell-derived DCs. Successful generation of isogenic hES cell lines in vitro that completely match the donor HLA isotype (50) demonstrates the feasibility of obtaining MHC-matched DCs from ES cells. However, poor efficiency and the ethical controversy of nuclear transfer technology will likely preclude its clinical application in the near future. Allogeneic DCs have been proposed as an alternative approach for cancer immunotherapy with an argument that the alloagression offers a generic tool to promote an effective T cell response to self MHC-restricted tumor peptides (51). Although recent clinical trials failed to demonstrate immunogenicity of allogeneic DCs loaded with tumor lysates (52), several investigations have shown the Ag presentation and induction of a potent tumor-specific immune response by allogeneic DCs fused with syngeneic or autologous cancer cells (53–57). Thus, generation of allogeneic hES cell-derived DC/autologous cancer cell hybrids could be seen as a feasible approach to the development of hES cell-based vaccines. Recently, significant antitumor protection has been achieved using OVA-expressing ES cell-derived DCs in semiallogeneic recipient mice (58), suggesting that ES cell-derived DCs sharing several common MHC alleles can be potentially used for immunization.

The differentiation system described here provides a good model to further evaluate the properties as well as developmental pathways of hES cell-derived DCs. Myeloid and lymphoid progenitors isolated from cord blood have been used successfully to identify developmental pathways of major DC subsets (13). We have recently shown that $CD34^+$ cells generated from hES cells in OP9 coculture are capable of giving rise to myeloid as well as B cells and $CD45^+CD56^+$ perforin-expressing NK cells (17). Further identification of hES cell-derived precursors for myeloid and lymphoid lineages will provide a unique opportunity to expand the studies on the origin of myeloid and plasmacytoid DCs in humans using genetic manipulation of hES cells to identify genes essential for the development of different DC lineages. The tolerogenic potential of hES cell-derived DC progenitors and DCs is another important question that should be addressed in the future. Generating tolerogenic DCs from hES cells may be important for reprogramming the immune system to tolerate grafted tissue obtained from the same hES cell line.

Acknowledgments

We thank Dr. T. Nakano for providing the OP9 bone marrow stromal cell line and Lynn Alford Schmidt for editorial assistance.

Disclosures

The authors have no financial conflict of interest.

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