Generation of immunogenic dendritic cells from human embryonic stem cells without serum and feeder cells

Aim: Dendritic cell (DC)-based vaccines have a potential utility for use in the treatment of malignancy. Human embryonic stem cells (hESCs) may provide a more cost-effective and reliable source of DCs for immunotherapy purposes, providing on-demand access for patients. **Method:** We developed a protocol to generate DCs from hESCs *in vitro* in the absence of serum and feeder cells. This protocol uses growth factors bone morphogenetic protein-4, granulocyte macrophage-colony stimulating factor (GM-CSF), stem cell factor and VEGF in serum-free media to generate hESC-derived monocytic cells. These cells are further differentiated to hESC-derived immature DCs with GM-CSF and IL-4, and matured to hESC-derived mature DCs with a maturation cocktail consisting of GM-CSF, TNF-α, IL-1β, IFN-γ and PGE2. **Results:** This study demonstrates the applicability of our defined differentiation process in generating functional hESC-derived DCs from multiple hESC lines. We show that hESC-derived immature DCs phagocytose, process, and present antigen upon maturation. hESC-derived mature DCs express the maturation marker CD83, produce Th1-directing cytokine IL-12p70, migrate in response to chemokine, and activate both viral and tumor antigen-specific T-cell responses. **Conclusion:** We developed a chemically defined system to generate unlimited numbers of DCs from hESCs. Our results demonstrate that hESC-derived DCs generated from this process are immunogenic and have the potential to be used for DC immunotherapy.

KEYWORDS: dendritic cells ■ human embryonic stem cells ■ immunotherapy ■ monocytic cells ■ vaccine

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Of all immune cells, dendritic cells (DCs) are the most potent for antigen presentation and naive T-cell activation. Therefore, there is significant interest in understanding their development, and in modulating their immune-stimulatory or -regulatory properties for immunotherapeutic purposes. DC immunotherapy harnesses the unique ability of DCs to optimally stimulate sustainable therapeutic antigen-specific immune responses. These DCs are generated from patient-specific hematopoetic stem cells or peripheral blood monocytes (PBMs). The challenges associated with these products are that autologous cell processing is laborious, and variability between donors renders production problematic. To circumvent these concerns, there is growing interest in developing alternative methods to generate DCs for therapeutic use.

The ability of human embryonic stem cells (hESCs) to self-renew indefinitely *in vitro* and their capacity to differentiate into all cell types of the body make them uniquely valuable for development of novel research tools and cell-based therapies [1,2]. The potential for differentiating DCs from hESCs has been established using either OP9 murine stromal cells or serum during the differentiation process [3–8]. However, all these methods pose challenges for clinical application owing to animal product contamination and serum variability.

Our studies utilize novel defined conditions in the absence of serum and feeder cells to generate hESC-derived DCs possessing immunogenic potency and phenotype. We show that hESC-derived DCs can present both viral and tumor antigens and activate antigen-specific T-cell responses. These studies are the first to identify critical growth factors directing hematopoietic precursor and DC differentiation from multiple hESC lines. The hESC-derived DCs generated from our process will be useful for many applications, including the exploitation of DCs as vaccines to enhance antigen-specific immune responses.

Materials & methods

■ hESCs & differentiation culture

hESC-derived DCs were generated from normal karyotyped H1 and H14 hESC lines derived from human blastocysts [1]. Undifferentiated hESCs were cultured and passaged as previously described [9,10]. In brief, hESCs were cultured on T75 flasks (Corning) coated with 1:30 diluted Matrigel™ (Becton Dickinson) and maintained in X-VIVO 10 medium (Lonza) supplemented

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with 80 ng/ml hbFGF and 0.5 ng/ml TGF-β (R&D Systems). To harvest hESC colonies for stage II differentiation, a confluent hESC culture was treated with collagenase to preferentially remove spontaneously differentiated accessory cells. The hESC colonies remaining on the plate were detached with a cell scraper. The undifferentiated hESC colonies were plated in six-well ultra low attachment (ULA) plates (Costar) at 3×10^6 cells/well in 4 ml of X-VIVO 15 medium (Lonza) supplemented with 1 mM Na-pyruvate, 1 × nonessential amino acids, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, stem cell factor (SCF) (20 ng/ml), VEGF (50 ng/ml), bone morphogenetic protein (BMP)-4 (50 ng/ml) and granulocyte macrophage-colony stimulating factor (GM-CSF) (50 ng/ml) to generate embryoid bodies (EB). The cells were fed every 2 days by removing 2 ml of media and replacing fresh medium supplemented with growth factors (1:1) or every 3 days at a 1:3 media change. During the differentiation process, BMP-4 was removed on day 5, VEGF was removed on day 10, and SCF was removed on day 15. After 17–25 days, round hematopoetic cells and monocytic cells were visible in culture.

To generate hESC-derived immature DCs (iDCs), supernatant and EB were collected from stage II culture. The EB were allowed to settle to the bottom of 50 ml conical tubes, collected, and replated in new ULA plates in X-VIVO 15 medium supplemented with GM-CSF (50 ng/ml). The EB culture of stage II was fed every 2–3 days, and continued to generate hESC-derived monocytic cells for an additional 25–40 days, making serial harvests of hESC-derived monocytic cells feasible. The supernatants containing progenitor cells and monocytic cells were then collected by centrifugation, resuspended in X-VIVO 15 medium with GM-CSF (50 ng/ml) and IL-4 (50 ng/ml), and plated onto the original ULA plates for 4–6 days. To generate hESC-derived mature DCs (mDCs), these hESC-derived iDCs were collected from the wells, centrifuged and resuspended in X-VIVO 15 media supplemented with the following growth factors: IFN-γ (20 ng/ml), IL-1-β (10 ng/ml), TNF-α (50 ng/ml), PGE_2 (1 µg/ml) and GM-CSF (50 ng/ml), and cultured for an additional 24–48 h to generate hESC-derived mDCs. We found that for every hESC plated for subsequent expansion and differentiation, our protocol generates three to five DCs. All growth factors were purchased from R&D Systems except PGE_2 (Sigma).

Statistical analysis

Statistical analysis of the growth factor requirements was performed as follows: a pairwise t-test was used to compare the original growth factor cocktail versus the reduced growth factor cocktails to determine whether the variances were equal. When variances were equal, a pooled t-test was used, whereas when variances were unequal, a Satterthwaite test was used.

Antibody, flow cytometry analysis & May–Grunwald staining

Cell samples were resuspended in 50 µl of fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline [PBS] + 0.1% BSA + 2 mM EDTA) and Fc receptor block (Miltenyi Biotec) was added for 10 min at 4°C before specific antibodies were added. After 20 min incubation at 4°C, the cells were washed twice in FACS buffer, and 0.1 µg/2 µl 7-aminoactinomycin (BD Bioscience) was added to each sample prior to data acquisition.

Sample data were collected using a FACSCalibur™ (Becton Dickinson), and analyzed using FlowJo software (Treestar). Dead cells were excluded from analysis by uptake of the 7-aminoactinomycin dye. Antibodies against the following targets were purchased from BD Biosciences: CD11b, CD11c, CD14, CD34, CD40, CD45, CD80, CD83, CD86, CD205, HLA-I and HLA-II. Intracellular staining with Oct-4 (Santa Cruz Biotechnology) was performed by first fixing the hESCs with BD Cytofix™ buffer and followed by BD Perm/Wash™ buffer, following the manufacturer's protocol. After washing with BD Perm/Wash buffer, antimouse Alexa-488 conjugated secondary antibody (Molecular Probes) was added to the sample at 1:500 dilution, incubated at 4°C for 30 min, and washed twice before data acquisition.

For May–Grunwald staining, the cells were washed and resuspended in 50 µl PBS, seeded onto glass slides by cytospin 3 (Shandon) at 1200 rpm for 5 min. The cells were then stained with May–Grunwald staining solution (Sigma) for 5 min at 25°C, washed three times with distilled water, and dried overnight before being mounted.

Cytokine profile

Supernatants from hESC-derived iDCs, mDCs, and PBM-derived mDCs were collected and concentrated by centrifugation using Amicon Ultra-15 10,000 NMWL centrifuge tubes (Millipore). IL-6, IL-10 and IL-12 were detected using BD Cytometric

Bead Array (BD Biosciences) following the manufacturer's instructions. Cytokine concentrations were determined using flow cytometric analysis program (FCAP) Array Software (BD Biosciences).

Migration assay

Serum-free lymphocyte media (AIM-V) was added to the upper and bottom chambers of 24-well transwell plates containing 8.0 µm pore size inserts (Corning), and incubated overnight at 37°C, 5% CO_2. After removal of the media from each well, 0.6 ml of AIM-V with or without 100 ng/ml of macrophage inflammatory protein (MIP)-3β (Pepro Tech) was added to the lower chamber. Mature hESC-derived DCs or PBM-derived DCs were harvested and washed twice in AIM-V medium. The cells were resuspended in AIM-V medium at 1.5×10^6 cell/ml, and 0.1 ml was added to the top chamber. The transwell plate was incubated for 2 h at 37°C, 5% CO_2. The number of cells that migrated to the bottom chamber was determined using hemocytometer counting.

Mixed leukocyte reaction assay

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors (Stanford Blood Bank) using a Ficoll-Paque™ (Amersham Pharmacia Biotech AB) gradient centrifugation method. Cells were washed and resuspended in complete RPMI 1640 (Invitrogen) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen) for use as responder cells. DCs that were used as stimulator cells were x-ray irradiated at 2000 rad, and various concentrations of stimulator cells were cocultured with 5×10^4 PBMC in a 96-well-bottom plate (Becton Dickinson). A mix lymphocyte reaction (MLR) assay was performed as previously described [11].

PBM-derived DCs

PBM-derived DCs were prepared by isolating monocytes from healthy donors. PBMCs were adhered to tissue culture flasks in AIM-V medium (Cellgro) for 2 h then washed with warm PBS to remove nonadherent cells. The remaining adherent cells, mostly monocytes, were incubated at 37°C and 5% CO_2 for 6 days with recombinant human (rh)IL-4 and rhGM-CSF at 1000 U/ml to generate iDCs. iDCs were then matured for 24 h in AIM-V medium supplemented with 800 U/ml rhGM-CSF, 10 ng/ml TNF-α and IL1-β, 50 ng/ml IL-6, and 1 µg/ml PGE_2 [12].

Generation of human telomerase reverse transcriptase antigen specific T-cell lines

The human telomerase reverse transcriptase (hTERT)-specific T-cell lines were generated as previously described [13]. Briefly, PBM-derived mDCs (HLA-A2$^+$) were pulsed with $hTERT_{540–548}$ peptide (100 µg/ml) (AnaSpec, Inc.) for 2 h at 37°C, 5% CO_2. Autologous CD8$^+$ T cells were isolated from PBMC by negative selection (Miltenyi Biotec), and resuspended in AIM-V medium containing 5% human AB serum (Valley Biomedical). DCs treated with $hTERT_{540–548}$ peptide were added to the T cells at a stimulator:responder ratio of 1:10. After 24 h culture at 37°C and 5% CO_2, rhIL-7 (10 ng/ml) (R&D systems) and IL-2 (10 U/ml) (R&D systems) were added to the cell culture. *In vitro* restimulations were performed every 7–10 days using irradiated (2000 rad) adherent PBMC treated with 10 µg/ml of $hTERT_{540–548}$ peptide. IL-12 (10 ng/ml) (R&D systems) was added to the culture the same day of restimulation; rhIL-7 (10 ng/ml) and IL-2 (10 U/ml) were added 24 h after restimulation. The culture medium was replenished every 3–4 days. The percentages of $hTERT_{540–548}$-specific CD8$^+$ T cells was determined by staining the cells with $hTERT_{540–548}$ pentamer labeled with allophycocyanin and anti-human CD8 fluorescein isothiocyanate-conjugated antibody (ProImmune).

Antigen processing & presentation assays

DQ™ ovalbumin (DQ-OVA, Invitrogen) was dissolved at 1 mg/ml in PBS and added to immature hESC-derived DCs at 100 µg/ml. The cells were incubated either at 37°C or at 4°C, washed twice with FACS buffer, and analyzed on the FACSCalibur.

IFN-γ ELISPOT assay was used to evaluate specific T-cell responses to cytomegalovirus (CMV), hTERT or mumps antigens. mDCs were treated with HLA-A2 binding peptides, $CMV_{495–503}$ peptide (10 µg/ml) or $hTERT_{540–548}$ peptide (100 µg/ml) (Anaspec), or untreated for 2 h in AIM-V medium at 37°C and 5% CO_2. For demonstration of antigen processing and presentation, mumps protein (Biodesign) was added to hESC-derived iDCs at 100 µg/ml for 2 h prior to maturation. After 24 h, untreated and mumps protein-treated hESC-derived mDCs were collected, washed twice with AIM-V medium, and plated on multiscreen polyvinylidene difluoride plates (Millipore) that were coated with anti-IFN-γ antibody according

to the manufacturer's protocol (Mabtech) at 1:10 stimulator: responder ratio. Characterized PBMCs (Cellular Technologies Limited) – cells that were previously tested for CMV or mumps antigen T-cell responses – were used as responder cells. PBMCs were thawed in a 37°C water bath, washed two times, resuspended in AIM-V medium, and plated at 1×10^5 cells/100 µl/well on ELISPOT plates. The hTERT-specific T-cell line was used when evaluating hTERT antigen presentation by DCs. The plate was cultured at 37°C and 5% CO_2 for 16–24 h, and developed following the manufacturer's instructions (Mabtech). Spots were enumerated using the CTL Analyzer and Immunospot software (Cellular Technology Limited).

Antigen-specific T-cell proliferation assay

For the $CMV_{495–503}$ antigen-specific assay, an HLA-A2$^+$ T-cell line recognizing $CMV_{495–503}$ peptide (ProImmune,) was thawed at 37°C and washed twice with RPMI 1640 medium containing 5% FBS and supplemented with 1 mM Na-pyruvate, nonessential amino acids, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 10 mM HEPES (Invitrogen). Either CMV or hTERT-specific T-cell lines were resuspended in pre-warmed PBS/0.1% BSA at 1.0×10^6/ml. Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) was added to the cells at a final concentration of 2 µM and the cells were incubated at 37°C for 10 min. CFSE was quenched by the addition of pre-chilled AIM-V medium containing 10% FBS, and washed twice. hESC-derived mDCs were untreated or treated with 10 µg/ml of $CMV_{495–503}$ peptide or 100 µg/ml of $hTERT_{540–548}$ peptide for 2 h at 37°C 5% CO_2 and washed twice in AIM-V medium before plating at 2×10^4/well in 96-well U bottom Falcon™ plate (Becton Dickinson). DCs were cocultured with CFSE-labeled T cells that were added at 2×10^5 cells/well. After 4 or 5 days, the cells were harvested and stained with pentamer reagent-recognizing $hTERT_{540–548}$ or $CMV_{495–503}$-specific T cells (ProImmune). The cells were washed twice with FACS buffer, and stained with 7AAD prior to analysis using the FACSCalibur. The analysis was performed using FlowJo software.

Results

Generation of hESC-derived DCs from hESCs

The process of hESC differentiation into DCs is divided into four stages. Stage I involves the expansion of hESCs, stage II encompasses the generation of hESC-derived monocytic cells, stage III generates hESC-derived iDCs, and stage IV matures hESC-derived iDCs to mDCs (Figure 1A and methods). As previously described, in stage I, hESC lines H1 and H14 were cultured in the absence of feeder cells and serum [9,10]. These serum-free cultured hESC colonies have distinct borders surrounded by accessory cells, which were largely spindle shaped and stromal, as seen in Figure 1B. These accessory cells are cells that spontaneously differentiated from hESCs. Flow cytometric analysis showed that more than 85% of hESCs expressed Oct-4 (Figure 1C), a marker of the undifferentiated state.

In stage II, we found that the growth factors BMP-4, VEGF, SCF and GM-CSF efficiently drive hESC differentiation to myeloid cells. In order to identify the critical growth factors required for stage II differentiation, we performed an experiment to deconvolute the required growth factors. The removal of BMP-4 from the stage II culture resulted in a 20-fold reduction in CD34$^+$ cell generation as compared with control cultures (Figure 2A). This decrease in CD34$^+$ cell number translated into a statistically significant reduction in generating CD45$^+$ and CD11c$^+$ cells (Figures 2B & 2C). The removal of GM-CSF from the culture also resulted in a significant decrease in generating CD45$^+$ and CD11c$^+$ cells (Figures 2B & 2C). T-test analysis demonstrated that these differences were statistically significant with a p-value less than 0.05. Although the removal of either VEGF or SCF from the culture did not result in a significant difference in generating the numbers of CD45$^+$ or CD11c$^+$ cells (Figures 2B & 2C), their presence ensured consistency in generating hESC-derived monocytic cells.

We phenotyped cells generated from stage II differentiation for hematopoietic and myeloid surface markers. As early as 5 days after initiation of stage II culture, 4% of the cells expressed surface CD34 protein (Figure 3A). These data are consistent with previous studies [14–16]. We observed CD45$^+$ cells as early as day 15 of stage II culture and, by day 20, the culture contained at least 60% CD45$^+$ cells and 25–35% CD14$^+$ cells (Figure 3A & Table 1). The hESC-derived monocytic cells generated from this process expressed CD11c, CD11b, HLA-I, HLA-II and CD86, but not CD83 (Figure 3B). To better depict the phenotype of hESC-derived monocytic cells in flow cytometry analysis, these cells were gated on the larger cell population by forward and side scatter. The hESC-derived monocytic cells possessed ruffled membranes with a high cytoplasm to nucleus ratio (Figures 3C–3E).

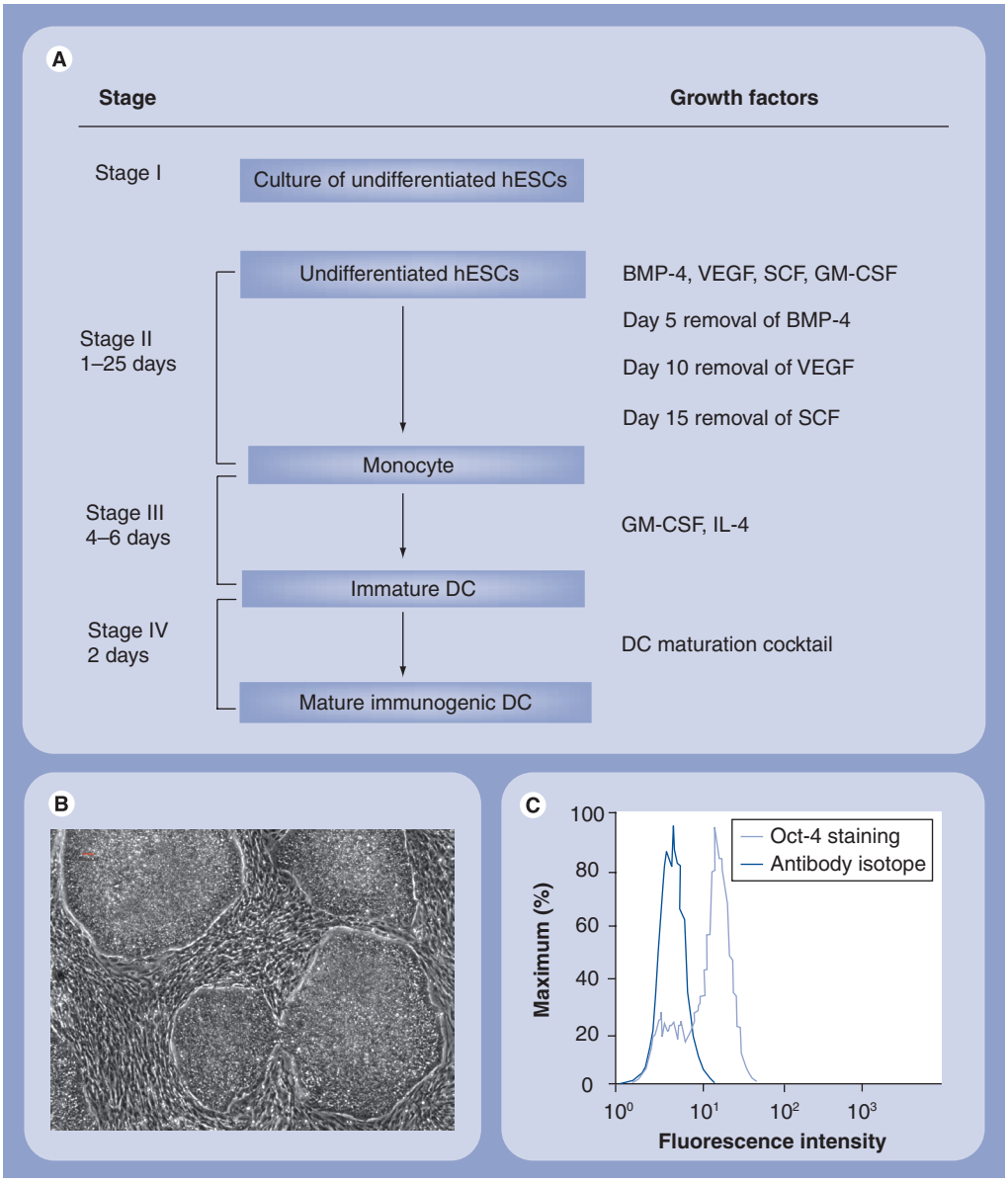

Figure 1. Schematics of a defined differentiation process. (A) Protocol schematic: the differentiation protocol is divided into four stages. Stage I is the expansion of hESCs. Stage II differentiates hESCs to hESC-derived monocytic cells. Stage III involves the generation of hESC-derived immature DCs. Stage IV mature DCs with a maturation cocktail containing IFN-γ, IL-1β, TNF-α, PEG2 and GM-CSF. **(B)** Transmitted light image of hESC colonies grown on X-VIVO 10 medium supplemented with TGF-β and basic FGF. **(C)** Characterization of undifferentiated hESCs by flow cytometry. The H1 hESCs expressed undifferentiated hESC marker Oct-4.
BMP: Bone morphogenetic protein; DC: Dendritic cell; GM-CSF: Granulocyte monocyte colony stimulating factor; hESC: Human embryonic stem cell; SCF: Stem cell factor.

Stage III of the defined differentiation process involves converting hESC-derived monocytic cells to iDCs. While hESC-derived monocytic cells generated from stage II were harvested, EBs from stage I culture continued to generate hESC-derived monocytic cells for an additional 25–40 days, making serial harvests feasible. hESC-derived monocytic cells treated with GM-CSF and IL-4 for 4–6 days differentiated to hESC-derived iDCs. hESC-derived iDCs possessed the following cell surface markers: $CD14^{lo/neg}$, MHC class I^{+}, MHC class II^{+}, $CD205^{neg}$, and $CD83^{lo}$ (Figure 3F). For the flow cytometry analysis, these cells were gated on the larger cell population by forward and side scatter.

Stage IV of the defined differentiation process involves the maturation of hESC-derived iDCs to mDCs. Treatment of hESC-derived

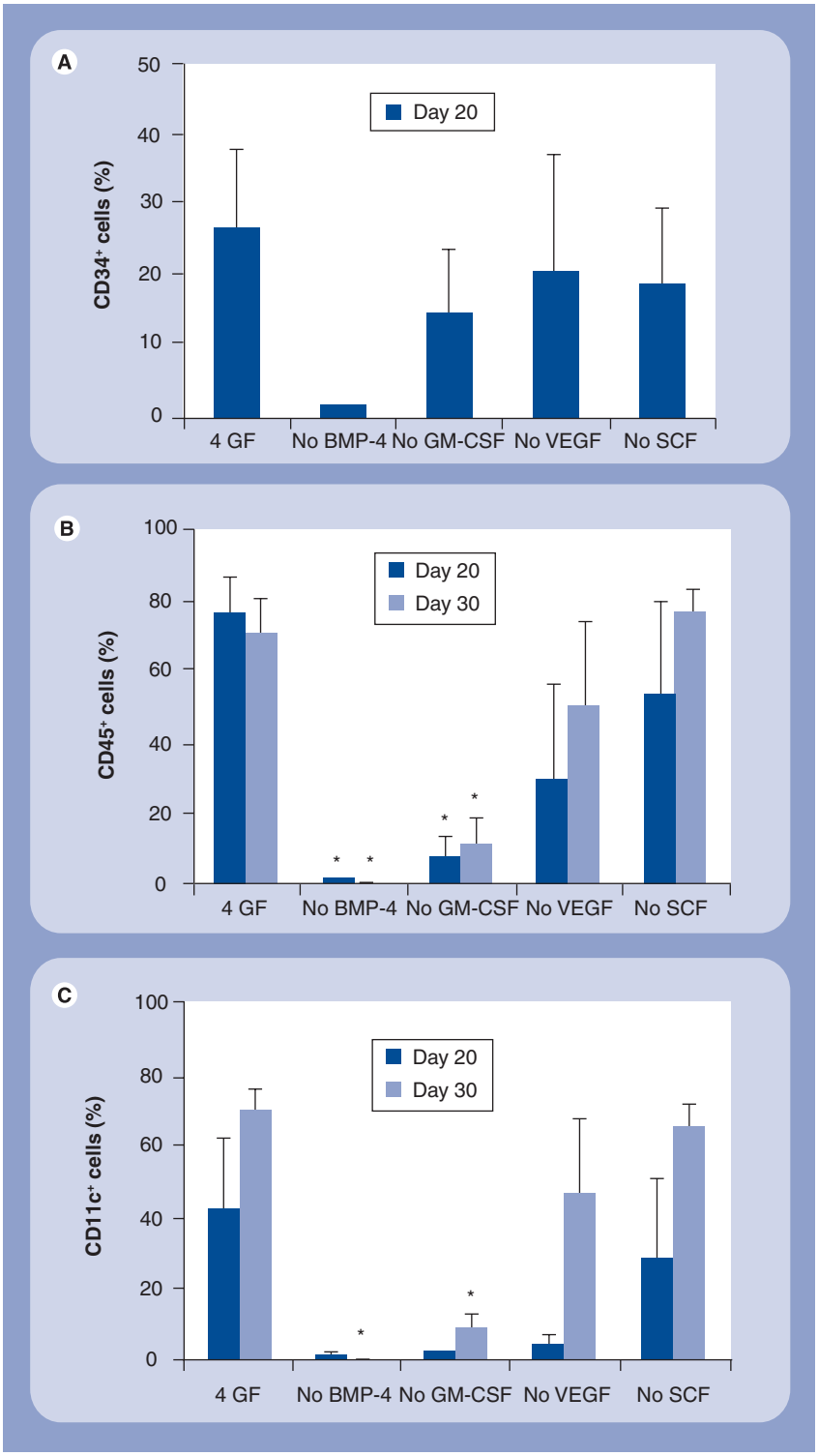

Figure 2. Identification of essential growth factors for stage II differentiation. Comparison of four growth factor cocktails with three growth factors cocktails in generating cells expressing the following surface markers: **(A)** $CD34^+$, **(B)** $CD45^+$ and **(C)** $CD11c^+$. Cells of the various culture conditions were collected at day 20 and day 30. Data represent the average of three independent experiments. Error bars indicate the standard error of the mean.
*The difference is statistically significant with a p-value less than 0.05 as demonstrated by t-tests.
BMP: Bone morphogenetic protein; GF: Growth factor; GM-CSF: Granulocyte-monocyte colony stimulating factor; SCF: Stem cell factor.

iDCs with a maturation cocktail consisting of GM-CSF, IFN-γ, IL1-β, TNF-α, and PGE_2 for 24–48 h generated mDCs that comprised 60–80% of the total cell population in the terminal culture (Table 1). The hESC-derived mDCs expressed MHC class I^+, MHC class II^+, $CD86^+$, $CD83^+$, $CD205^+$, $CD11b^+$, $CD11c^{hi}$, and $CD40^+$ (Figure 4A). These cells were gated on the forward and side scatter for DCs. The hESC-derived mDCs had a similar phenotype to that of PBM-derived mDCs (Figure 4B), exhibited veiled structures (Figure 4C) and formed homotypic clusters (Figure 4D). These are all characteristics of mDCs. Importantly, this protocol produced hESC-derived DCs from H14 and H1 hESC lines at similar efficiencies (Table 1), demonstrating the applicability of our protocol across hESC lines.

hESC-derived DCs produce cytokines, migrate & stimulate allogeneic T-cell responses

We evaluated the ability of hESC-derived DCs to produce pro-inflammatory cytokines known to regulate the function of other immune cells, in particular T cells. Since we found that hESC-derived mDCs produced significantly greater levels of IL-6 (300-fold) than hESC-derived iDCs (Figure 5A), IL-6 was not added to the maturation cocktail even though PBM-derived DCs were matured with a cocktail containing IL-6. We found that hESC-derived mDCs produced comparable amounts of IL-12p70 to PBM-derived DCs (Figure 5B). hESC-derived iDCs produced 2.5-fold greater levels of the anti-inflammatory cytokine IL-10 than mDCs. PBM-derived DCs produced tenfold greater levels of IL-10 than hESC-derived mDCs (figure 5C). Other cytokines that hESC-derived DCs produced upon maturation include: IL-8, Rantes, growth-regulated oncogene-α, monocyte chemotactic protein-2, IL-7 and thymus and activation-regulated chemokine (Supplementary Table 1) (see online www.futuremedicine.com/toc/rme/4/4). A key function of DCs *in vivo* is their ability to take up antigen in the periphery and migrate to secondary lymphoid organs to activate antigen-specific T cells. This is of particular importance in the DC vaccine setting, where DCs are expected to migrate from the injection site to the lymphoid organs. The migratory capacity of hESC-derived mDCs was assessed in an *in vitro* chemotactic assay. We found that 50% of hESC-derived mDCs migrated in response to MIP-3β chemokine in transwells, while mDCs in the control transwells without chemokine

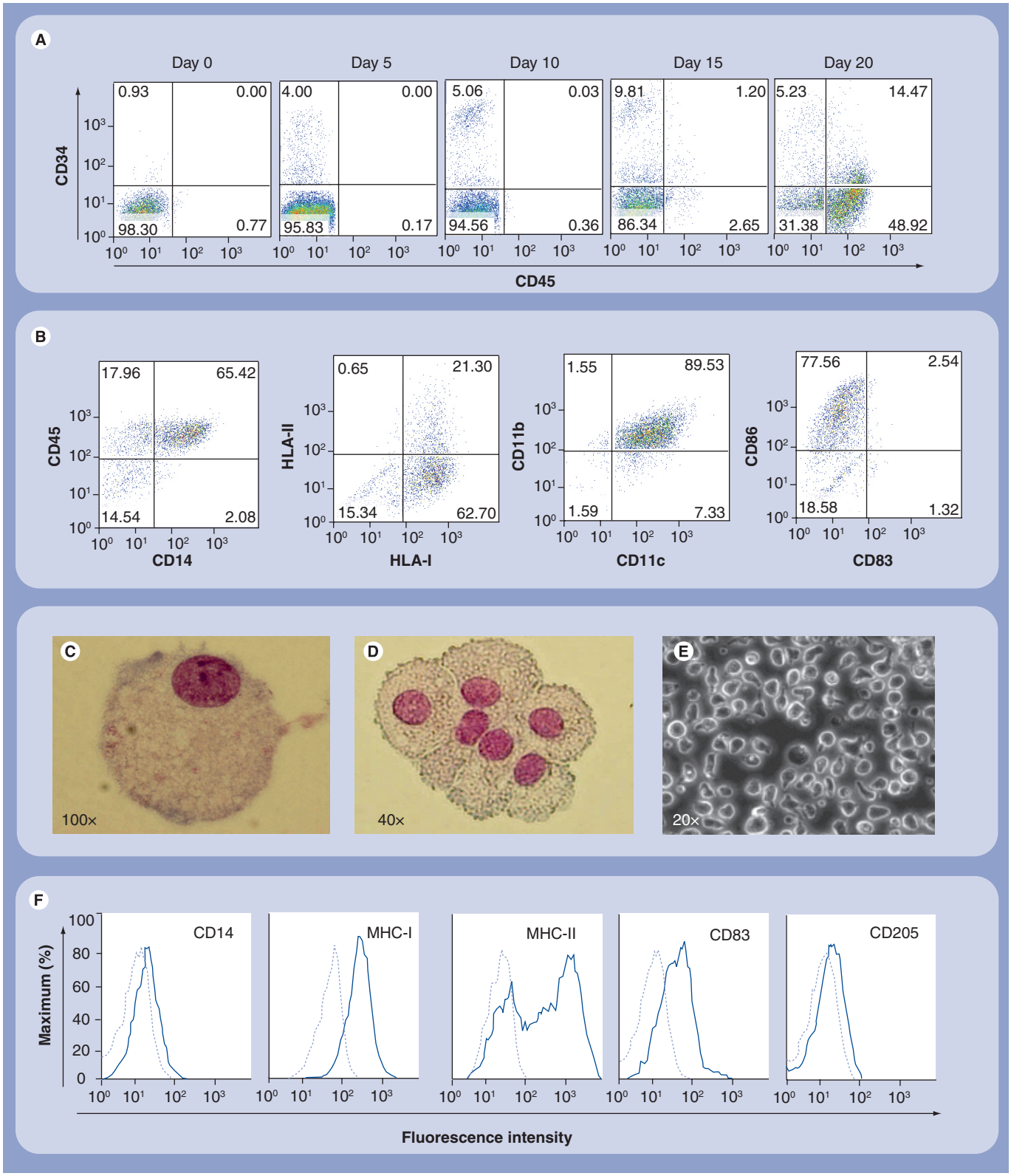

Figure 3. Characterization of hESC-derived cells in stage II and III of the differentiation culture. (A) Time course expression of $CD34^+$ and $CD45^+$ population. **(B)** Phenotype of hESC-derived myeloid cells before entering stage III. hESC-derived monocytic cells expressed HLA-I, HLA-II, CD11c and CD11b. **(C & D)** May–Grunwald staining of hESC-derived monocytic cells. Images were taken with an upright Zeiss microscope with **(C)** 100× oil objective and **(D)** 40× Neofluar objective. **(E)** Transmitted light image of hESC-derived monocytic cells. **(F)** Phenotype of hESC-derived immature dendritic cells generated from stage III. These data are representative of five differentiation experiments. Dotted line depicts isotype control and solid line depicts sample staining.
hESC: Human embryonic stem cell.

Table 1. Characterization of human embryonic stem cell-derived dendritic cell differentiation.

hESC line	H1	H14
Growth factors		
BMP-4	+	+
GM-CSF	+	+
VEGF	+	+
SCF	+	+
Stage II		
Day 20		
CD45	71 ± 15	73 ± 11
CD11c	37 ± 14	34 ± 2
CD14	25 ± 17	33 ± 2
Day 30		
CD45	87 ± 6	92 ± 2
CD11c	73 ± 9	75 ± 2
CD14	27 ± 8	37 ± 16
Stage III		
iDC		
CD86	72 ± 6	75 ± 5
CD83	45 ± 7	67 ± 6
MHC II	26 ± 12	32 ± 6
Stage IV		
mDC		
CD86	75 ± 5	81 ± 2
CD83	71 ± 8	75 ± 1
MHC II	34 ± 15	53 ± 5
CCR7	57 ± 10	70 ± 4

Values represent % positive cell surface expression of total population ± standard error of the mean.
hESC line H1 data represent the average of four independent differentiations.
hESC line H14 data represent the average of two independent differentiations.
hESC-derived DC yield and function between H1 and H14 line hESC-derived DCs were similar.
BMP: Bone morphogenetic protein; hESC: Human embryonic stem cell; iDC: Immature dendritic cell; mDC: Mature dendritic cell; SCF: Stem cell factor.

displayed less than 5% migration (Figure 5D). Similar migration responses were observed from PBM-derived DCs.

One of the hallmarks of mDCs is their ability to stimulate strong allogeneic T-cell responses. In an MLR assay, PBMCs were cocultured with hESC-derived mDCs, PBM-derived mDCs, or undifferentiated hESCs. The results suggest that hESC-derived mDCs, similar to PBM-derived DCs, stimulated significant allogeneic T-cell proliferation (Figure 5E). Although it may appear that hESC-derived mDCs generated lower allogeneic responses than PBM-derived DCs in this particular experiment, results from other experiments indicated that hESC-derived mDCs could generate stronger allogeneic responses than PBM-derived DCs (data not shown). This is likely related to the degree of HLA matching between donor and responder cells.

hESC-derived DCs phagocytose, process & present viral antigens

An important task iDCs perform in the periphery is phagocytosis and processing of antigens. We characterized hESC-derived iDCs for these functions by DQ-OVA protein uptake and by a semi-allogeneic assay designed to measure antigen-specific T-cell activation. DQ-OVA protein was labeled with a pH insensitive BODIPY-FL dye. Owing to autoquenching, intact DQ-OVA protein is nonfluorescent, but exhibits bright green fluorescence upon denaturation and proteolysis. The result indicated that hESC-derived iDCs phagocytosed and processed DQ-OVA protein, displaying approximately a one log shift in fluorescence intensity compared with the control (Figure 6A).

In order to determine whether our hESC-derived DCs can process and present antigen to antigen-specific T cells, hESC-derived iDCs were treated with mumps protein, matured, and then cocultured with semiallogenic PBMC. We observed a five- to 18-fold increase in the number of IFN-γ spots in the ELISPOT assay when semiallogeneic PBMCs were cocultured with either the hESC line H14-derived-DCs (Figure 6B) or the hESC line H1-derived-DCs (Figure 6C) treated with mumps protein as compared with untreated DCs. Semiallogeneic PBMCs that were previously characterized as nonresponders to mumps antigen did not produce IFN-γ responses when cultured with hESC-derived DCs treated with mumps protein demonstrating antigen-specific T-cell activation (data not shown).

hESC-derived DCs stimulate viral-specific CD8$^+$ T-cell responses

We further tested the efficacy of our hESC-derived mDCs to stimulate viral antigen-specific CD8$^+$ T-cell responses *in vitro*. We first investigated the ability of hESC-derived DCs to stimulate antigen-specific CD8$^+$ T-cell activation by measuring IFN-γ production in a semiallogeneic ELISPOT assay. HLA-A2$^+$ hESC-derived mDCs and PBM-derived mDCs were treated with $CMV_{495–503}$ peptide and cocultured with HLA-A2$^+$ semiallogeneic PBMC. hESC-derived DCs treated with $CMV_{495–503}$ peptide stimulated several hundred-fold increase in the number of T cells producing IFN-γ, as compared with untreated DCs. In addition, hESC-derived mDCs stimulated $CMV_{495–503}$-specific T cells to produce IFN-γ at a similar level to PBM-derived mDCs (Figure 7A).

We next examined the ability of hESC-derived mDCs to stimulate $CMV_{495–503}$-specific $CD8^+$ T-cell expansion *in vitro*. T cells were labeled with CFSE and cocultured with hESC-derived mDCs or PBM-derived mDCs that were treated with $CMV_{495–503}$ peptide. T-cell proliferation was determined by positive $CMV_{495–503}$ pentamer-specific staining and CFSE dilution, indicated by a decrease in fluorescence intensity. Both hESC-derived mDCs and PBM-derived mDCs stimulated CMV-specific $CD8^+$ T cells that underwent five rounds of cell division, with more than 95% of the cells completing at least a single cell division, demonstrating that hESC-derived mDCs can stimulate antigen-specific T-cell proliferation (Figure 7B).

hESC-derived DCs stimulate tumor antigen-specific $CD8^+$ T-cell responses

The induction of T-cell responses directed against foreign viral antigens can be achieved more readily than that of tumor antigens, which are generally derived from self antigens. hESC-derived mDCs were studied for their ability to stimulate tumor antigen-specific T-cell responses directed against the hTERT antigen, which is widely expressed in tumor cells. We generated $hTERT_{540}$-specific $CD8^+$ T-cell lines from $HLA\text{-}A2^+$ healthy donors and demonstrated that hESC-derived mDCs treated with $hTERT_{540}$ peptide stimulated more than a 14-fold increase in IFN-γ production by hTERT-specific T cells as compared with untreated hESC-derived mDCs (Figure 7C). hESC-derived DCs appeared to stimulate significantly greater hTERT antigen-specific T-cell responses as compared with PBM-derived DCs (Figure 7C).

We also assessed the ability of hESC-derived mDCs to stimulate hTERT antigen-specific T-cell proliferation. The hTERT-specific T-cell line was labeled with CFSE and cocultured with untreated or $hTERT_{540}$ peptide-treated hESC-derived mDCs. hESC-derived mDCs stimulated hTERT $CD8^+$ T cells that underwent six rounds of cell division, with 86% of the cells completing at least one cell division (Figure 7D).

Discussion

DC-based vaccines offer an attractive route to therapeutic manipulation of the immune system, such as the enhancement of immune responses in cancer patients. Issues inherent to autologous DC products pose technical and manufacturing challenges such as variable yield and costs. The observed variability of DC products reflects individual differences among patients, and the potential that the DC maturation and function in patients with advanced cancer are compromised.

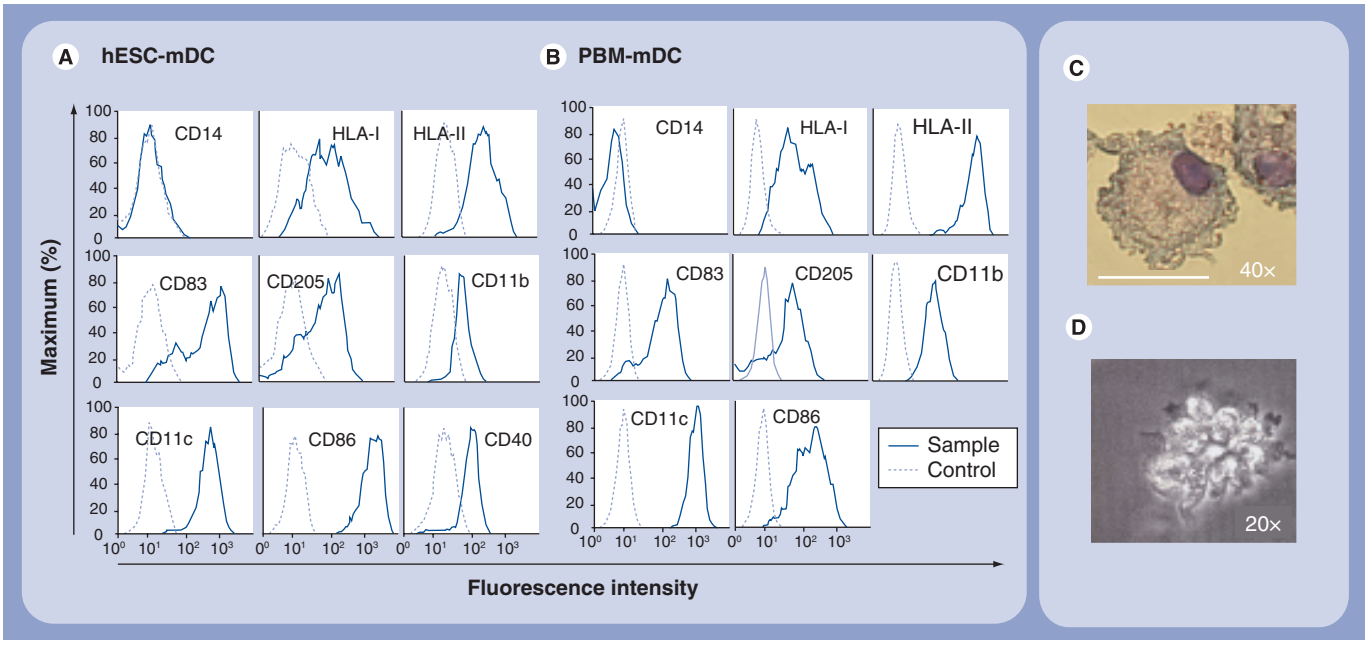

Figure 4. Characterization of hESC-derived mDCs in stage IV of the differentiation culture. (A & B) Flow cytometric analysis of surface protein expression by DCs. **(A)** hESC-derived mDCs. **(B)** PBM-derived mDCs. These data are representative of n = 6 differentiation experiments. **(C)** May–Grunwald staining of hESC-derived mDCs. The white bar denotes 30 μm. Images were taken with an upright Zeiss microscope with 40× Neofluar objective. **(D)** Transmitted light image of a hESC-derived mDC cluster.
hESC: Human embryonic stem cell; mDC: Mature dendritic cell; PBM: Peripheral blood monocyte.

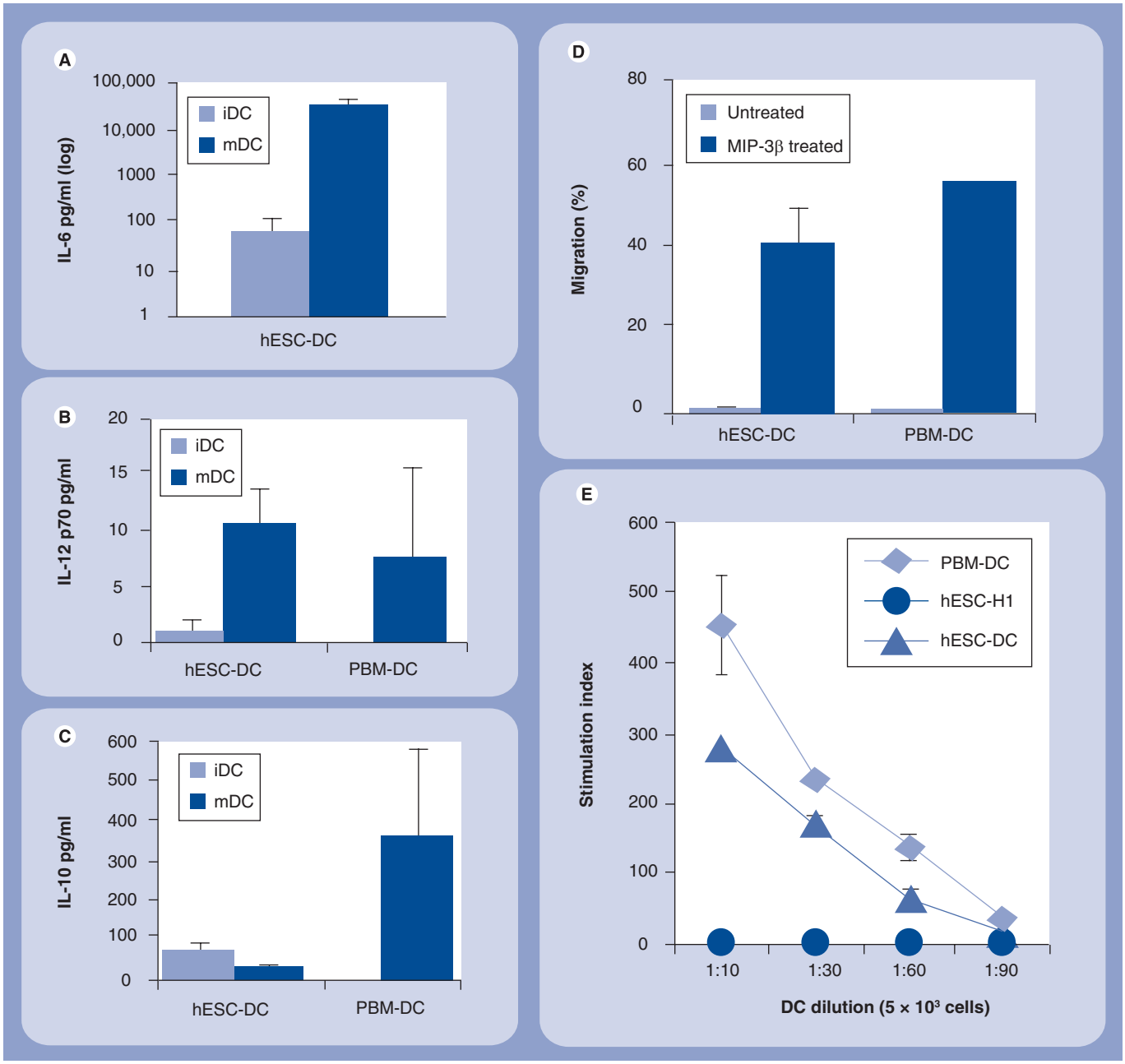

Figure 5. Function of hESC-derived mDCs. hESC-derived mDCs secrete pro-inflammatory cytokines **(A)** IL-6 and **(B)** IL-12p70, and anti-inflammatory cytokine **(C)** IL-10. Supernatant from hESC-derived iDCs was collected prior to the addition of maturation cocktail, and supernatant from hESC-derived mDCs and PBM-derived mDCs were collected 48 h after the addition of maturation cocktail. These data are the average of three experiments. **(D)** hESC-derived mDCs possess migratory function. In an *in vitro* chemotaxis assay, hESC-derived mDCs migrated towards MIP-3β in a transwell vessel. These data are the average of three experiments. **(E)** hESC-derived mDCs stimulated allogenic lymphocyte proliferation. Peripheral blood mononuclear cells were cocultured with hESC, hESC-derived DCs and PBM-derived DCs for 5 days and pulsed with ^{3}H thymidine for 12–16 h before harvest. The error bars indicate standard deviation of triplicate wells. These data are representative of three experiments.
DC: Dendritic cell; hESC: Human embryonic stem cell; iDC: Immature dendritic cell; mDC: Mature dendritic cell; MIP: Macrophage inflammatory protein; PBM: Peripheral blood monocyte.

We have demonstrated a protocol to generate DCs from hESCs in a defined stepwise culture system. A combination of the growth factors BMP-4, GM-CSF, SCF and VEGF efficiently generated a significant percentage of hematopoietic and myeloid cells, as indicated by the expression of CD34, CD45, CD11c and CD14 (Table 1), and we identified BMP-4 and GM-CSF as essential growth factors for the generation of CD45^{+} and CD11c^{+} cells. The robustness of this protocol is demonstrated by its capability of generating hESC-derived DCs from more than one hESC line.

Important functional criteria for DC use in the vaccine settings are their ability to migrate, produce pro-inflammatory cytokines, and stimulate antigen-specific T-cell activation and expansion. We demonstrated that hESC-derived DCs generated using the described serum-free conditions can migrate in response to MIP-3β, and stimulate T-cell responses directed against both viral and tumor antigens. Furthermore, unlike other studies [3,4,7], we were able to observe clear antigen-specific T-cell responses in our semiallogeneic assays using antigen-specific pentamers and distinguish these from allogeneic T-cell responses (Supplementary Figure 1). In addition, we demonstrated that hESC-derived DCs have the ability to prime and stimulate T cells that can recognize antigen-specific targets as measured by granzyme B secretion (Supplementary Figure 2). The antigen model platforms exploited in our studies support the feasibility of using hESC-derived DCs to generate immune responses against malignant and infectious diseases.

Since pluripotent hematopoetic stem cells can generate a wide range of hematopoietic cells, we investigated the potential generation of other cell types in our differentiation cultures. The presence of B cells, T cells, natural killer cells, erythrocytes and platelets were not detected in our differentiation cultures (data not shown). In addition, myelomonocytic cells have the potential to differentiate into either monocytes or granulocytes. We examined the distribution of granulocytes in our differentiation cultures and found that approximately 5–20% of the total population expressed $CD15^+$, HLA-II^- and $CD86^-$, and May–Grunwald staining confirmed the granulocyte phenotype (data not shown). We also examined for the presence of undifferentiated hESCs in the final hESC-derived DC culture by flow cytometric analysis, and did not detect Oct-4 and Tra-1–60 (data not shown).

The ability to use semiallogeneic DCs for immunotherapy will greatly reduce the number of hESC lines required to treat a given population. A previously published report describes antitumor protection achieved using semiallogeneic OVA-expressing murine ESC-derived DCs [17], indicating that semiallogeneic cell therapy may be efficacious. Although a certain degree of allogeneic-mediated responses may facilitate immunization through adjuvant-mediated effects, current DC cancer vaccination schedules require the incorporation of successive treatments to maximize efficacy. Since the priming of nonspecific allogeneic T-cell responses could potentially reduce the potency of a hESC-derived DC vaccine, the number of vaccinations required to effectively generate immunity will require

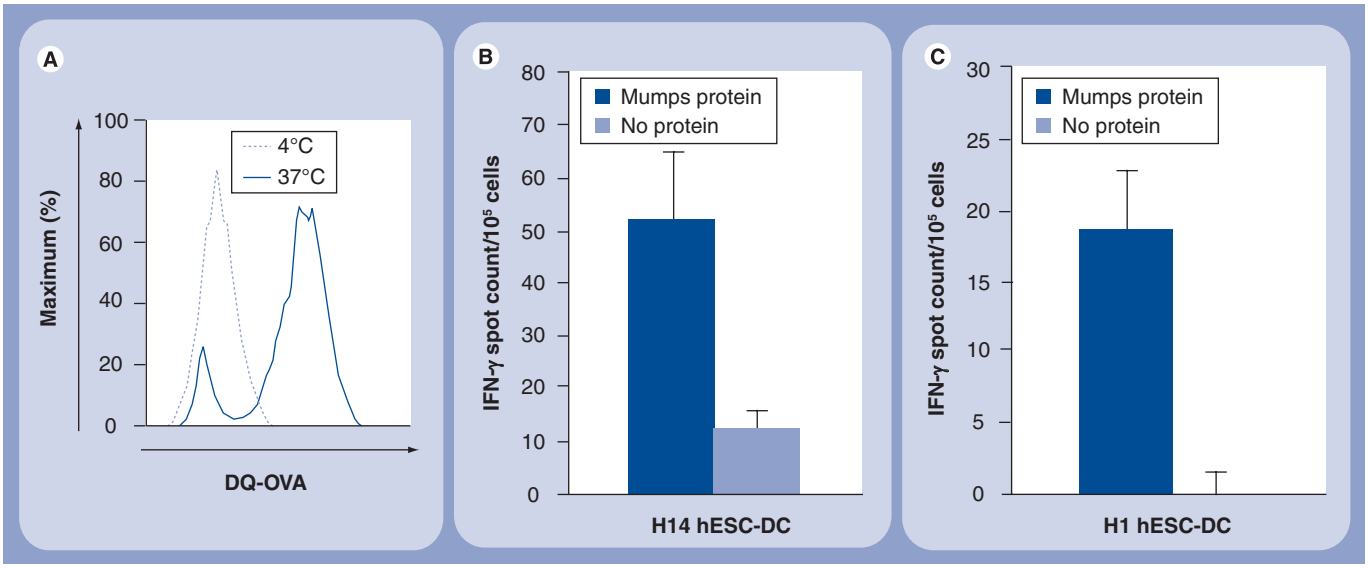

Figure 6. hESC-derived DCs processed and presented viral antigen to blood T cells. (A) hESC-derived immature DCs (iDCs) phagocytosed and proteolysed DQ-OVA protein. The hESC-derived iDCs were incubated with DQ-OVA for 30 min either at 4°C or at 37°C. **(B & C)** hESC-derived DCs phagocytosed, processed and presented mumps antigen, and stimulated antigen-specific T-cell responses. The hESC-derived iDCs were untreated or treated with mumps protein and matured for 24 h before coculture with peripheral blood mononuclear cells. **(B)** Blood peripheral blood mononuclear cell and hESC line H14-derived DCs were matched on MHC class I and class II alleles: *HLA-B7*, *HLA-Cw7*, *HLA-DR1* and *HLA-DQ5*. **(C)** Blood peripheral blood mononuclear cell and hESC line H1-derived DCs were matched on MHC class I and class II alleles: *HLA-A2*, *HLA-B35*, *HLA-DR1* and *HLA-DQ5*. This assay was performed in triplicates and error bars indicate standard deviations. Data are representative of three experiments.
DC: Dendritic cell; DQ-OVA: DQ™ ovalbumin; hESC: Human embryonic stem cell.

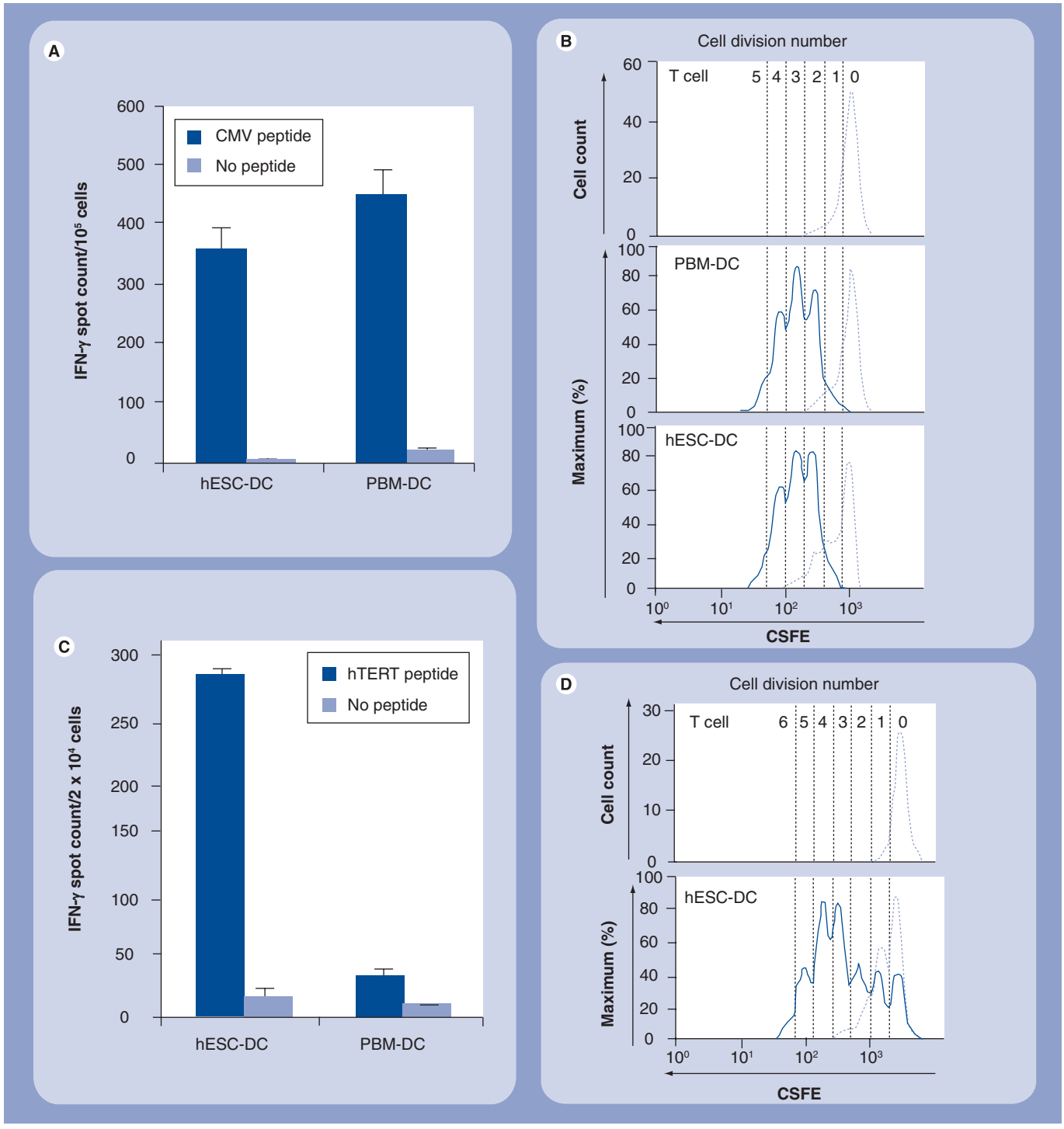

Figure 7. hESC-derived mDCs stimulated viral and tumor antigen-specific T-cell responses. (A) hESC-derived mature DCs (mDCs) presented $CMV_{495–503}$ peptide on HLA-A2 and stimulated antigen-specific T cells to produce IFN-γ. HLA-A2$^+$ PBMCs were cocultured with HLA-A2$^+$ hESC-derived mDCs or PBM-derived mDCs treated or untreated with $CMV_{495–503}$ peptide (n = 3). **(B)** hESC-derived mDCs stimulated CMV-specific CD8$^+$ T-cell proliferation. CFSE labeled T cells were cocultured with hESC-derived mDCs or PBM-derived mDCs treated or untreated with $CMV_{495–503}$ peptide for 4–5 days (n = 2). **(C)** hESC-derived mDCs presented $hTERT_{540–548}$ peptide antigen on HLA-A2 and stimulated antigen-specific T cells to produce IFN-γ. The hTERT-specific T-cell line was cocultured with hESC-derived mDCs treated or untreated with $hTERT_{540–548}$ peptide. The hESC-derived DC data are representative of five experiments and the PBM-derived DC data are representative of two experiments. **(D)** hESC-derived mDCs stimulated hTERT-specific CD8$^+$ T-cell proliferation. CFSE-labeled T cells were cocultured with hESC-derived mDCs treated or untreated with $hTERT_{540–548}$ peptide for 4–5 days (n = 2). Antigen-specific proliferation was shown as positive pentamer-specific staining and reduction in CFSE fluorescence intensity. The dashed lines delineate cell divisions. CFSE: Carboxyfluorescein succinimidyl ester; CMV: Cytomegalovirus; DC: Dendritic cell; hESC: Human embryonic stem cell; hTERT: Human telomerase reverse transcriptase; PBM: Peripheral blood monocyte.

consideration. However, there have been intensive studies using semiallogeneic DC vaccines by somatic cell hybrid methods. The publication of successful preclinical and clinical studies that validate this immunotherapeutic approach in cancer and viral infection demonstrate the feasibility of semiallogeneic cell therapy [18–22].

Conclusion

The advantage of semiallogeneic nonpatient-specific products is that they offer the opportunity for an 'off-the-shelf' vaccine suitable for treating all patients. DCs generated from hESC lines are a unique source of immunogenic cells that permit consistency and reproducibility between production lots. Our success in generating functional hESC-derived DCs in a serum-free and feeder-free system eliminates concerns over animal product contaminations and serum product variations. These improvements facilitate the realization of developing vaccine treatments available for a wide spectrum of patients.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Development of a defined human embryonic stem cell (hESC)-derived dendritic cell (DC) differentiation system without the need for feeder cells and serum allows the generation of unlimited DC for clinical applications.
- Allogeneic DC generated from hESC as vaccine will have more production and functional consistency then current autologous DC-based vaccines.
- Our protocol generated hESC-derived DC that possess equivalent phenotype and functions as peripheral blood monocyte-derived DC.
- The use of semi-allogeneic or allogenic DC for immunotherapy can significantly increase the availability of this type of treatment for patients.

Bibliography

1 Thomson JA, Itskovitz-Eldor J, Shapiro SS *et al.*: Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147 (1998).

2 Murry CE, Keller G: Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 132, 661–680 (2008).

3 Slukvin II, Vodyanik MA, Thomson JA, Gumenyuk ME, Choi KD: Directed differentiation of human embryonic stem cells into functional dendritic cells through the myeloid pathway. *J. Immunol.* 176, 2924–2932 (2006).

4 Senju S, Suemori H, Zembutsu H *et al.*: Genetically manipulated human embryonic stem cell-derived dendritic cells with immune regulatory function. *Stem Cells* 25, 2720–2729 (2007).

5 Zhan X, Dravid G, Ye Z *et al.*: Functional antigen-presenting leucocytes derived from human embryonic stem cells *in vitro*. *Lancet* 364, 163–171 (2004).

6 Bandi S, Akkina R: Human embryonic stem cell (hES) derived dendritic cells are functionally normal and are susceptible to HIV-1 infection. *AIDS Res. Ther.* 5, 1 (2008).

7 Su Z, Frye C, Bae KM, Kelley V, Vieweg J: Differentiation of human embryonic stem cells into immunostimulatory dendritic cells under feeder-free culture conditions. *Clin. Cancer Res.* 14, 6207–6217 (2008).

8 Karlsson KR, Cowley S, Martinez FO *et al.*: Homogeneous monocytes and macrophages from human embryonic stem cells following coculture-free differentiation in M-CSF and IL-3. *Exp. Hematol.* 36, 1167–1175 (2008).

9 Xu C, Inokuma MS, Denham J *et al.*: Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* 19, 971–974 (2001).

10 Li Y, Powell S, Brunette E, Lebkowski J, Mandalam R: Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. *Biotechnol. Bioeng.* 91, 688–698 (2005).

11 Okamura RM, Lebkowski J, Au M *et al.*: Immunological properties of human embryonic stem cell-derived oligodendrocyte progenitor cells. *J. Neuroimmunol.* 192, 134–144 (2007).

12 Jonuleit H, Kuhn U, Muller G *et al.*: Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* 27, 3135–3142 (1997).

13 Minev B, Hipp J, Firat H *et al.*: Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. *Proc. Natl Acad. Sci. USA* 97, 4796–4801 (2000).

14 Kennedy M, D'Souza SL, Lynch-Kattman M, Schwantz S, Keller G: Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood* 109, 2679–2687 (2007).

15 Lugus JJ, Chung YS, Mills JC *et al.*: GATA2 functions at multiple steps in hemangioblast development and differentiation. *Development* 134, 393–405 (2007).

16 Eichmann A, Corbel C, Nataf V *et al.*: Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc. Natl Acad. Sci. USA* 94, 5141–5146 (1997).

17 Fukuma D, Matsuyoshi H, Hirata S *et al.*: Cancer prevention with semi-allogeneic ES cell-derived dendritic cells. *Biochem. Biophys. Res. Commun*. 335, 5–13 (2005).

18 Avigan DE, Vasir B, George DJ *et al.*: Phase I/II study of vaccination with electrofused allogeneic dendritic cells/autologous tumor-derived cells in patients with stage IV renal cell carcinoma. *J. Immunother*. 30, 749–761 (2007).

19 Gong J, Nikrui N, Chen D *et al.*: Fusions of human ovarian carcinoma cells with autologous or allogeneic dendritic cells induce antitumor immunity. *J. Immunol*. 165, 1705–1711 (2000).

20 Newton DA, Acierno PM, Brescia FJ, Brown EA, Gattoni-Celli S: Semi-allogeneic vaccines for patients with cancer and AIDS. *J. Immunother*. 25, 334–341 (2002).

21 Fabre JW: The allogeneic response and tumor immunity. *Nat. Med*. 7, 649–652 (2001).

22 Yasuda T, Kamigaki T, Kawasaki K *et al.*: Superior anti-tumor protection and therapeutic efficacy of vaccination with allogeneic and semiallogeneic dendritic cell/tumor cell fusion hybrids for murine colon adenocarcinoma. *Cancer Immunol. Immunother*. 56, 1025–1036 (2007).