

## **Differentiation of Human Embryonic Stem Cells into Immunostimulatory Dendritic Cells under Feeder-Free Culture Conditions**

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**Abstract Purpose:** The objective of this study was to develop a scalable and broadly applicable active immunotherapy approach against cancer, circumventing the limitations typically encountered with autologous vaccination strategies. We hypothesized that human embryonic stem cells (hESC) can serve as a virtually unlimited source for generating dendritic cells (DC) with potent antigen-presenting function. Here, we investigated the developmental processes and requirements for generating large numbers of mature, antigen-presenting DC from pluripotent hESC.

**Experimental Design:** A feeder cell-free culture system was developed to differentiate hESC into mature DC sequentially through hematopoietic and myeloid precursor stages.

**Results:** Using this method, we were able to yield large numbers of mature immunostimulatory DC from hESC to enable clinical investigation. Upon activation, the hESC-derived DC secreted interleukin-12p70, migrated in response to MIP-3 $\beta$ , and exhibited allostimulatory capacity. Most importantly, antigen-loaded, hESC-derived DC were capable of stimulating potent antigen-specific CD8<sup>+</sup> T-cell responses in an HLA class I – matched semiallogeneic assay system. Moreover, HLA class II – mismatched hESC-derived DC induced a potent Th1-type cytokine response without expanding FOXP3<sup>+</sup> regulatory T cells *in vitro*.

**Conclusions:** These data suggest the development of a novel active immunotherapy platform to stimulate potent T-cell immunity in patients with intractable diseases, such as cancer or viral infection.

Active immunotherapy with antigen-presenting cells (APC), such as dendritic cells (DC), has become the focus of many academic-based and industry-based research and development programs, because they are highly effective in overcoming immune tolerance by priming potent T-cell immunity in models for cancer or viral disease (1). The primary drawback of using autologous DC vaccines in clinical trials is that this mode of vaccination is a customized form of cell therapy, necessitating APC generation from each individual patient. Preparing human DC from autologous hematopoietic progenitor cells remains a complex and laborious task, thereby limiting the availability of cells for multiple vaccinations (2). This issue becomes highly relevant, because recent studies now

suggest that the stimulation of a clinically effective immune response may require repeated vaccinations and continuous boosting to maintain the vaccine-induced T-cell response (3). Secondly, the time interval between the initial peripheral blood mononuclear cell (PBMC) harvest and the ultimate availability of the autologous DC vaccine significantly delays, or even precludes, immunization of patients with rapidly progressing tumors or with acute viral disease. Finally, DC generated from peripheral blood progenitors of advanced cancer patients often exhibit impaired antigen-presenting function due to prior exposure to immunosuppressive tumor-derived factors (4). Recent murine studies have shown that pluripotent embryonic stem cells can be differentiated into APC with either immunostimulatory (5–8) or tolerogenic (9) function. *In vitro* differentiation of embryonic stem cells can be directed along multiple lineage pathways and may offer prospects for reprogramming the immune system to either stimulate T-cell immunity against tumor-associated or viral antigens or dampen immune responses in autoimmune or transplant settings. Fairchild and coworkers have shown the feasibility of generating immunoregulatory DC by directed differentiation of murine embryonic stem cells stably transfected with the green fluorescent protein (GFP) reporter gene (10). More recently, other groups have explored the possibility of differentiating human embryonic stem cells (hESC) through the myeloid pathway into immunostimulatory APC (11, 12). Despite this recent progress, the developmental pathways and conditions

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### Translational Relevance

In this study, we show, for the first time, the feasibility of generating functionally intact dendritic cells (DC) from an established human embryonic stem cell (hESC) line through the myeloid differentiation pathway using a clinically compatible feeder cell-free culture system. The hESC-derived DC could be loaded with HLA-A0201 – restricted peptides or with mRNA-encoded antigens and exhibit potent T-cell stimulatory function upon maturation. The *ex vivo* – generated hESC-derived DC showed migratory, cytokine secretion and allostimulatory properties comparable with monocyte-derived DC that we and others have tested in clinical trials. Using hESC-derived DC as stimulators, we consistently detected primary, antigen-specific CTL responses capable of recognizing and killing HLA class I – matched human target cells, whereas avoiding activation of FOXP3-expressing regulatory T cells. These data suggest that hESC may function as a universal and scalable source for DC-based cancer immunotherapy, providing immediate availability of antigen-presenting cells for cancer patients. Importantly, the allogeneic responses mediated by the semiallogeneic hESC-derived DC via HLA class II could provide potent Th1-type cytokine help for the generation of CTL responses to peptides presented by shared HLA class I molecules. The data derived from these experiments serve as the scientific basis for upcoming clinical trials with semiallogeneic hESC-derived DC immunotherapy in patients with malignant diseases.

for propagating large numbers of mature antigen-presenting DC from hESC, under conditions acceptable for use in active immunotherapy trials, have not yet been established. Moreover, little is known regarding the migratory, secretory, and functional capacity of hESC-derived DC to stimulate antigen-specific CTL responses *in vitro*.

In this study, we show, for the first time, the feasibility of generating functionally intact DC from an established hESC line through the myeloid differentiation pathway using a clinically compatible feeder cell-free culture system. The hESC-derived DC generated under these conditions could be loaded with HLA-A0201 – restricted peptides or with mRNA-encoded antigens and exhibit potent T-cell stimulatory function upon maturation. The *ex vivo* – generated hESC-derived DC showed migratory cytokine secretion and allostimulatory properties comparable with monocyte-derived DC that we and others have tested in clinical trials (3, 13). Using hESC-derived DC as stimulators, we consistently detected primary, antigen-specific CTL responses capable of recognizing and killing HLA class I – matched human target cells while avoiding activation of FOXP3-expressing regulatory T cells. Moreover, terminally differentiated hESC-derived DC completely abrogated their ability to form teratoma in immune-deficient vertebrate animals (data not shown).

These data suggest that hESC may function as a universal and scalable source for DC-based cancer immunotherapy, providing immediate availability of APC for patients with all stages and types of disease. Importantly, the allogeneic

responses mediated by the semiallogeneic hESC-derived DC (deliberately matched to the HLA class I repertoire of the recipient and expressed allogeneic HLA class II determinants) via HLA class II could provide potent Th1-type cytokine help for the generation of CTL responses to peptides presented by shared HLA class I molecules. The data derived from these experiments serve as the scientific basis for upcoming clinical trials with semiallogeneic hESC-derived DC immunotherapy in patients with malignant and viral diseases.

### Materials and Methods

**hESC culture.** The HLA-A2<sup>+</sup> H9 hESC line (passage 18) was acquired from Wicell Research Institute (14) and passed weekly on human fibronectin (Chemicon) – coated plates in standard hESC culture medium, as described previously (15). H9 hESC used in all differentiation experiments exhibited normal growth characteristics, phenotype, and karyotype *in vitro*. For hESC characterization, antibody panels consisting of cell surface and intranuclear hESC markers were used, including CD9, E-cadherin, Podocalyxin, Nanog, Oct-3/4, SOX2, SSEA-1, and SSEA-4 (R&D Systems).

**Reverse transcription-PCR analysis of differentiated hESC.** Total RNA was extracted from hESC harvested on culture days 0, 8, 12, and 21 during embryoid body (EB) formation using the RNeasy Mini kit (Qiagen). Reverse transcription was done using a Transcriptor First Strand cDNA Synthesis kit (Roche). Primer sets used for these analyses were described previously (16). Glyceraldehyde-3-phosphate dehydrogenase transcripts were determined in all experiments and used to normalize the amount of cDNA in each sample.

**Hematopoietic differentiation of hESC.** Spontaneous EB formation was tested to allow hESC differentiation. Briefly, hESC colonies were suspended in  $\alpha$ -MEM (Invitrogen) supplemented with 20% human antibody serum (Valley Biomedical) and cultured in ultralow attachment flasks (Corning Costar) for 12 to 14 d. After differentiation, EBs were disassociated with TrypLE Select (Invitrogen) into single-cell suspensions and used for further analysis. In addition to the spontaneous EB formation, 50 ng/mL of bone morphogenetic protein 4 (BMP-4; R&D Systems) were added in some experiments to improve hematopoietic differentiation, as previously described (17). For comparative experiments, murine OP9 and human HS-5 cells were obtained from American Type Culture Collection and expanded, according to previously published protocols (11). The cultures were incubated for up to 12 d at 37°C and 5% CO<sub>2</sub>, with fresh medium changes every other day.

**Myeloid expansion and generation of mature hESC-derived DC.** To achieve myeloid expansion, single-cell suspensions after EB formation were harvested and subsequently cultured in  $\alpha$ -MEM supplemented with 10% human antibody serum (Valley Biomedical), 100  $\mu$ M monothioglycerol, 100  $\mu$ g/L stem cell factor, 50  $\mu$ g/L FLT3 ligand, 800 units/mL granulocyte macrophage colony-stimulating factor, 20  $\mu$ g/L interleukin-3 (IL-3), and 20  $\mu$ g/L thrombopoietin (PeproTech) for 10 d. Differentiation into hESC-derived DC was facilitated by culturing the resulting monocytic precursors in 800 units/mL granulocyte macrophage colony-stimulating factor, 800 units/mL IL-4, and 10 ng/mL tumor necrosis factor- $\alpha$  (R&D Systems). At 2 d before cell harvest, immature DC were loaded with antigen and subsequently matured with a cytokine cocktail consisting of 10 ng/mL tumor necrosis factor- $\alpha$ , 10 ng/mL IL-1 $\beta$ , and 150 ng/mL IL-6, cytokine cocktail plus 1  $\mu$ g/mL prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; Sigma), cytokine cocktail plus 3,000 units/mL IFN- $\alpha$ 2b (Schering-Plough), and cytokine cocktail plus IFN- $\alpha$ 2b supplemented with 20  $\mu$ g/mL poly I:C (Sigma). Monocyte-derived DC, generated as described previously (3), were used as controls in all experiments.

**Flow cytometric analysis.** Three-color fluorescence-activated cell sorting analyses were done on hESC-derived DC using a Becton Dickinson Biosciences FACSCalibur. After labeling with CD3, CD19,

CD56, CD14, CD1a, CD45, CD43, CD33, CD34, CD31, CD11c, CD11b, CD40, CD86, CD80, CD54, CD83, HLA class I and HLA class II, CD209, and isotype control monoclonal antibodies (all BD PharMingen), cells were tested for cell surface expression by flow cytometry using CellQuest software.

**IL-12p70 production and chemotaxis assays.** Unstimulated or stimulated hESC-derived DC and monocyte-derived DC were cultured, washed, and plated in 96-well plates at densities of  $2 \times 10^5$  cells per well. Supernatants were then analyzed for IL-12p70 secretion after 24 h of culture (Endogen). Chemotaxis of DC was measured and assessed by their migration through a polycarbonate filter of 8- $\mu$ m pore size in 6-well transwell chambers (Corning).

**mRNA electroporation of DC.** Transfection of immature hESC-derived DC with GFP or prostate-specific antigen (PSA) mRNA was done by electroporation, as previously described (3).

**T-cell proliferation analysis.** For proliferation assays, T cells were purified with Pan T-Cell Isolation Kit II (Miltenyi Biotec) and then cocultured with increasing numbers of DC. Triplicate wells of T cells alone were used as a background control. After 5 d of culture, 1  $\mu$ Ci (0.037 MBq) [methyl- $^3$ H]thymidine (NEN) was added to each well, and incubation was continued for an additional 16 h. Cells were collected onto glass fiber filters (Wallac) with a cell harvester, and uptake of thymidine was determined using a liquid scintillation counter.

**Antigen-specific CD8 T-cell analysis.** To analyze the antigen-presenting function of hESC-derived DC, cells were first pulsed with HLA-A0201-restricted BMLF1 peptide 280-288 (GLCTLVAML) and incubated overnight using various maturation stimuli (see Figs. 4 and 5). Cells were then harvested and used as targets in standard ELISPOT assays. As effector cells, polyclonal EBV-specific CTL lines (18) that were established from the PBMC of HLA-A0201<sup>+</sup> and HLA-A0201<sup>-</sup> donors were used. For peptide-specific CTL induction, CD8<sup>+</sup> T cells (96-98% pure) from HLA-A0201<sup>+</sup> donors were isolated using magnetic bead separation techniques (Miltenyi Biotec) and stimulated with HLA-A0201<sup>+</sup> MART-1 peptide (ELAGIGILTV)-loaded semiallogeneic hESC-derived DC at a stimulator-to-responder ratio of 1:10 in the presence of rhIL-2 (20 units/mL) and rhIL-7 (10 ng/mL; PeproTech). On day 7, the frequency of MART-1-specific CD8<sup>+</sup> T cells was tested using IFN- $\gamma$  ELISPOT analysis.

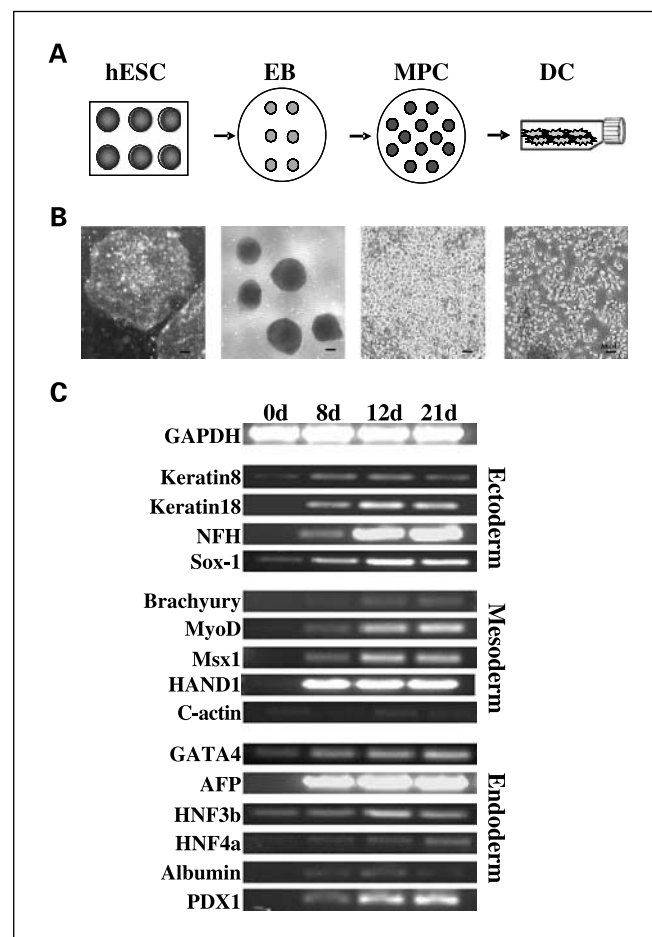
Furthermore, standard  $^{51}$ chromium release assays were done on CTL derived from the CD8<sup>+</sup> T cells of an HLA-A2<sup>+</sup> donor and stimulated with RNA-transfected DC. T cells were restimulated once with RNA-transfected DC, and IL-2 (20 units/mL) was added after 5 d and every other day thereafter. After 12 d of culture, effector cells were harvested and tested in cytolytic assays, as previously described (3). Results from triplicate wells were averaged, and the percentage of specific lysis was calculated.

**Cytokine analysis and FOXP3 staining of CD4 T cells.** Naive CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from human PBMC using magnetic bead separation techniques. Briefly, untouched CD4<sup>+</sup> T cells were isolated with CD4<sup>+</sup> T-Cell Isolation Kit II and then the CD4<sup>+</sup>CD25<sup>+</sup> cells were depleted with CD25 Microbeads II (Miltenyi Biotec). The naive CD4<sup>+</sup>CD25<sup>-</sup> T cells were then cocultured with either autologous monocyte-derived DC or allogeneic hESC-derived DC. After 6 d, T cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 1  $\mu$ g/mL ionomycin (Sigma) and analyzed after 6 h for cytokine secretion. The cytokines and the antibody pairs used for IFN- $\gamma$ , IL-4 ELISAs were purchased from R&D Systems. FOXP3 expression by CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells was assessed by fluorescence-activated cell sorting analysis using the antihuman FOXP3 staining kit (e-Biosciences).

## Results

**Pluripotent hESC can be maintained and differentiated into hematopoietic precursors.** The objective of these initial experiments was to develop clinically compatible and feeder cell-free

culture conditions that would allow the propagation of large numbers of hESC without interfering with their capability of differentiating into DC. To mimic the physiologic differentiation of hESC into DC via the myeloid pathway, we sought to develop a three-step culture protocol to generate functionally intact DC from established hESC lines through hematopoietic precursor and myeloid precursor stages, as schematically shown in Fig. 1A and B. For hESC culture, we adapted a culture method, in which H9 hESC were passed on human fibronectin-coated plates using serum-free media supplemented with high concentrations of basic fibroblast growth factor (19, 20). Using these defined culture conditions, we were able to successfully maintain hESC in an undifferentiated state from July 2006 to the present. hESC propagated in defined, high basic fibroblast growth factor-supplemented medium exhibited identical proliferative capacity and normal karyotype when compared with H9 hESC cocultured with the current gold



**Fig. 1.** Differentiation of hESC into DC. **A**, schematic diagram of the protocol for generating hESC-derived DC. Steps include culture and maintenance of hESC under feeder-free conditions; hematopoietic differentiation via EB formation (*EB*); expansion of myeloid precursor cells (*MPC*) and differentiation into mature DC. **B**, morphology of cells derived by targeted differentiation. Images were captured with an inverted microscope using 4 $\times$  magnification and a digital camera. **C**, reverse transcription – PCR (RT-PCR) analysis of differentiated hESC. hESC differentiated by EB formation revealed time-dependent acquisition of lineage-specific markers, including keratin 5, 15, 18, Sox1, NFH (ectoderm); Brachyury, Msx1, MyoD, HAND1, cardiac actin (mesoderm); GATA4, AFP, HNF-4a, HNF-3b, albumin, and PDX1 (endoderm).



standard, namely mouse embryonic fibroblast feeder layers (data not shown).

To test the pluripotency of hESC maintained under these defined conditions, we first did reverse transcription-PCR analysis on hESC spontaneously differentiated via EB formation (16). It is well established that *in vitro*-cultured hESC spontaneously aggregate into cell clusters, termed EB, which trigger the differentiation of hESC into cells of endodermal, ectodermal, and mesodermal origin (21). As shown in Fig. 1C, hESC, differentiated via EB formation, acquired increased gene expression characteristics of endodermal, ectodermal, and mesodermal development, including *Keratin 5*, *15*, *18*, *Sox1*, *NFH* (ectoderm); *Brachyury*, *Msx1*, *MyoD*, *HAND1*, *cardiac actin* (mesoderm); *GATA4*, *AFP*, *HNF-4a*, *HNF-3b*, *albumin*, and *PDX1* (endoderm). As shown in Fig. 1C, gene expression increased over time and peaked after 12 to 21 days of EB-mediated differentiation. These data provided a first line of evidence that the pluripotency of hESC is maintained after defined feeder-free cell culture and EB-mediated differentiation.

We next determined whether EB-mediated culture is capable of differentiating hESC into CD34<sup>+</sup> hematopoietic precursors (HSC), the first differentiation step as depicted in Fig. 1A and B. As shown in Fig. 2A, CD34<sup>+</sup> hematopoietic precursors could be generated in a reproducible manner from hESC after EB-mediated differentiation in the presence of 20% human antibody serum. CD34<sup>+</sup> cell yields averaged  $7.5 \pm 2.3\%$  (10 consecutive experiments) of the originally undifferentiated hESC population after a 12-day culture period. Whereas CD34 expression on hESC typically peaked ~12 days after EB-mediated differentiation (Fig. 2A), hESC surface markers (SSEA-4, CD9, and E-cadherin) proportionally decreased during the differentiation process (data not shown).

To further improve the yield of CD34<sup>+</sup> hematopoietic precursors, we tested EB-mediated differentiation in the presence of BMP-4, a member of the transforming growth factor- $\beta$  superfamily, which has been shown to promote hematopoietic differentiation of hESC (17). In parallel, we did comparative experiments to test hESC differentiation using murine (OP9; ref. 22) and human bone marrow (HS-5) feeder cells (23). As shown in Fig. 2B, we were able to significantly improve the yield of CD34<sup>+</sup> hematopoietic precursors by adding BMP-4 protein to the culture and, thereby, almost double (average yield,  $15.3 \pm 2.1\%$ ;  $n = 10$ ) the CD34<sup>+</sup> cell numbers typically obtained after EB culture alone (average yield,  $7.2 \pm 1.2\%$ ;  $n = 10$ ) or after culture with murine OP9 bone marrow feeder cells (average yield,  $7.8 \pm 1.9\%$ ;  $n = 10$ ). As shown in Fig. 2B, human bone marrow HS-5 feeder cells were also capable of triggering differentiation of H9 hESC into hematopoietic precursors. However, these culture conditions are not yet optimized, as evidenced by the fact that the yield of CD34<sup>+</sup> hematopoietic precursors was consistently lower (average yield,  $1.5 \pm 1.2\%$ ;  $n = 10$ ) under these conditions when compared with cells derived from EB-mediated differentiation or by murine OP9 coculture. Cumulatively, the experiments in Figs. 1 and 2 show that hESC can successfully be cultured and differentiated into CD34<sup>+</sup> hematopoietic precursors using feeder-free culture conditions that eventually may yield a stem cell-based product acceptable for clinical use.

**Generation of mature APC from hESC-derived hematopoietic precursors.** To facilitate the second differentiation step from

the CD34<sup>+</sup> hematopoietic precursor stage toward cells of myeloid lineage, the differentiated hESC cells were harvested 12 days after EB formation and single-cell suspensions containing the hematopoietic precursors were subsequently incubated with recombinant human stem cell factor, Flt-3 ligand, thrombopoietin, IL-3, and granulocyte macrophage colony-stimulating factor to yield cells of myeloid lineage. Three days after culture, semiadherent, rapidly proliferating cells became visible (myeloid expansion). After 12 days of culture and after removal of dead cells, expansion of a homogenous cell population (>95% purity) expressing the leukocyte common antigen CD45 was obtained (Fig. 2C, *left*). Typical cell yields after myeloid expansion averaged an approximate 3-fold increase of the starting CD34<sup>+</sup> cell population, as determined in 10 consecutive experiments. Furthermore, the majority of the CD45<sup>+</sup> cells exhibited low or intermediate expression of the myeloid marker CD33 (Fig. 2C, *middle*) and stained positive for CD31 (Fig. 2C, *right*), providing further evidence for the myeloid lineage commitment of hESC-derived HSC.

To facilitate the final differentiation step from the myeloid precursor to the DC stage (Fig. 1A and B), myeloid precursors were cultured for 10 to 12 days in media supplemented with the cytokines IL-4 and granulocyte macrophage colony-stimulating factor (24). As shown in Fig. 3A (*left*), most cells formed typical clusters of aggregated "veiled cells" characteristic for monocyte-derived DC. The hESC-derived DC exhibited well-defined dendritic processes (Fig. 3A, *middle*), as well as large eccentric nuclei (Fig. 3A, *right*). Moreover, after differentiation, this large cell population revealed expression of the APC cell surface markers CD11c, CD54, CD11b, CD40, CD45, CD86, HLA class I, and HLA class II molecules. Additionally, these cells exhibited low levels of CD80, CD209, CD43, and CD31 on their cell surface, whereas no significant expression of CD14, CD3, CD19, CD56, CD33, CD34, and CD1a was observed. Similar to their monocyte-derived counterparts, hESC-derived DC up-regulated the activation marker CD83 upon maturation using the cytokines IL-1 $\beta$ , IL-6, tumor necrosis factor- $\alpha$ , and PGE<sub>2</sub> (Fig. 3B; ref. 25). As shown in Fig. 3C, under these experimental conditions, we were able to generate  $\sim 2 \times 10^7$  mature hESC-derived DC from a starting population of  $1 \times 10^7$  hESC with  $\geq 80\%$  purity (calculated as a percentage of CD11c positive cells in total cell population).

**hESC-derived DC are capable of secreting IL-12p70, migrating in response to MIP-3 $\beta$ , and stimulating potent antigen-specific T-cell responses.** Next, we investigated whether the hESC-derived DC are functionally intact, as judged by their immunostimulatory and migratory function, as well as by their ability to secrete the Th-1 cytokine IL-12p70. We did allogeneic mixed lymphocyte reaction assays to assess their allostimulatory capacity *in vitro*. As shown in Fig. 4A, hESC-derived DC were able to trigger the proliferation of allogeneic, HLA-mismatched T cells. Expectedly, mature hESC-derived DC used in the mixed lymphocyte reaction were more potent in stimulating T-cell proliferation than their immature counterparts. Importantly, hESC-derived DC were equally effective in stimulating allo-responses when compared with DC generated from peripheral blood monocytes (Fig. 4A). To determine DC migratory behavior and the ability of secreting IL-12p70 upon activation *in vitro*, immature hESC-derived DC were matured using various cytokine combinations, including the above-mentioned

cytokine cocktail, cytokine cocktail plus PGE<sub>2</sub>, cytokine cocktail plus IFN- $\alpha$ , and cytokine cocktail plus IFN- $\alpha$  with poly I:C (Fig. 4B). Similar to monocyte-derived DC, ~40% of the hESC-derived DC migrated in response to MIP-3 $\beta$  but only after activation by PGE<sub>2</sub> (26). As shown in Fig. 4B, DC migration was less efficient with the other activation protocols tested in parallel in these experiments. Expectedly, hESC-derived DC secreted higher amounts of IL-12p70 when cytokine cocktail/IFN- $\alpha$ /poly I:C was used for maturation (Fig. 4C).

In summary, these data show that hESC-derived DC possess virtually identical migratory, allostimulatory and Th-1 polarizing properties as monocyte-derived DC, thus providing further evidence regarding their intactness and potent APC function *in vitro* (27).

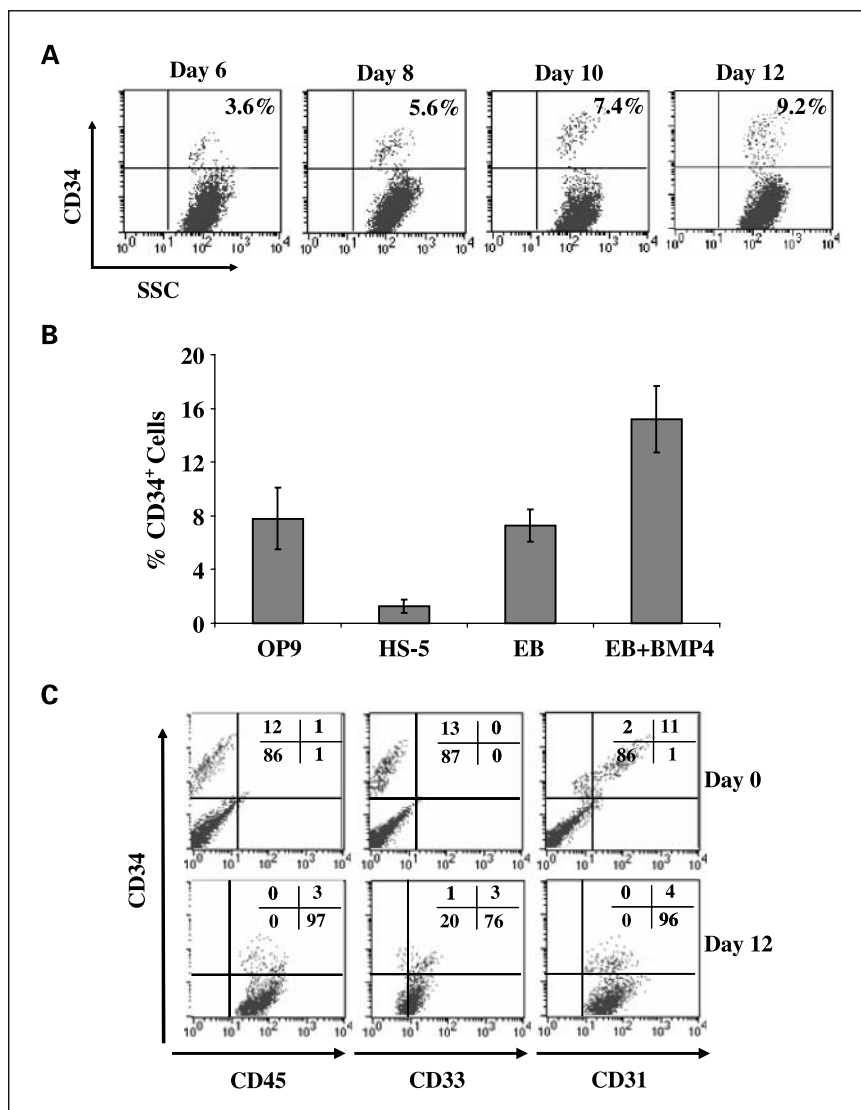
**Peptide loaded hESC-derived DC can induce antigen-specific cytotoxic T-cell responses.** To assess the capability of mature hESC-derived DC to stimulate antigen-specific T-cell responses, we first used an EBV-specific recall antigen test system to determine whether or not EBV peptide-specific T cells can recognize antigens presented by EBV antigen-bearing hESC-derived DC in an HLA-restricted manner. EBV-specific CTL were

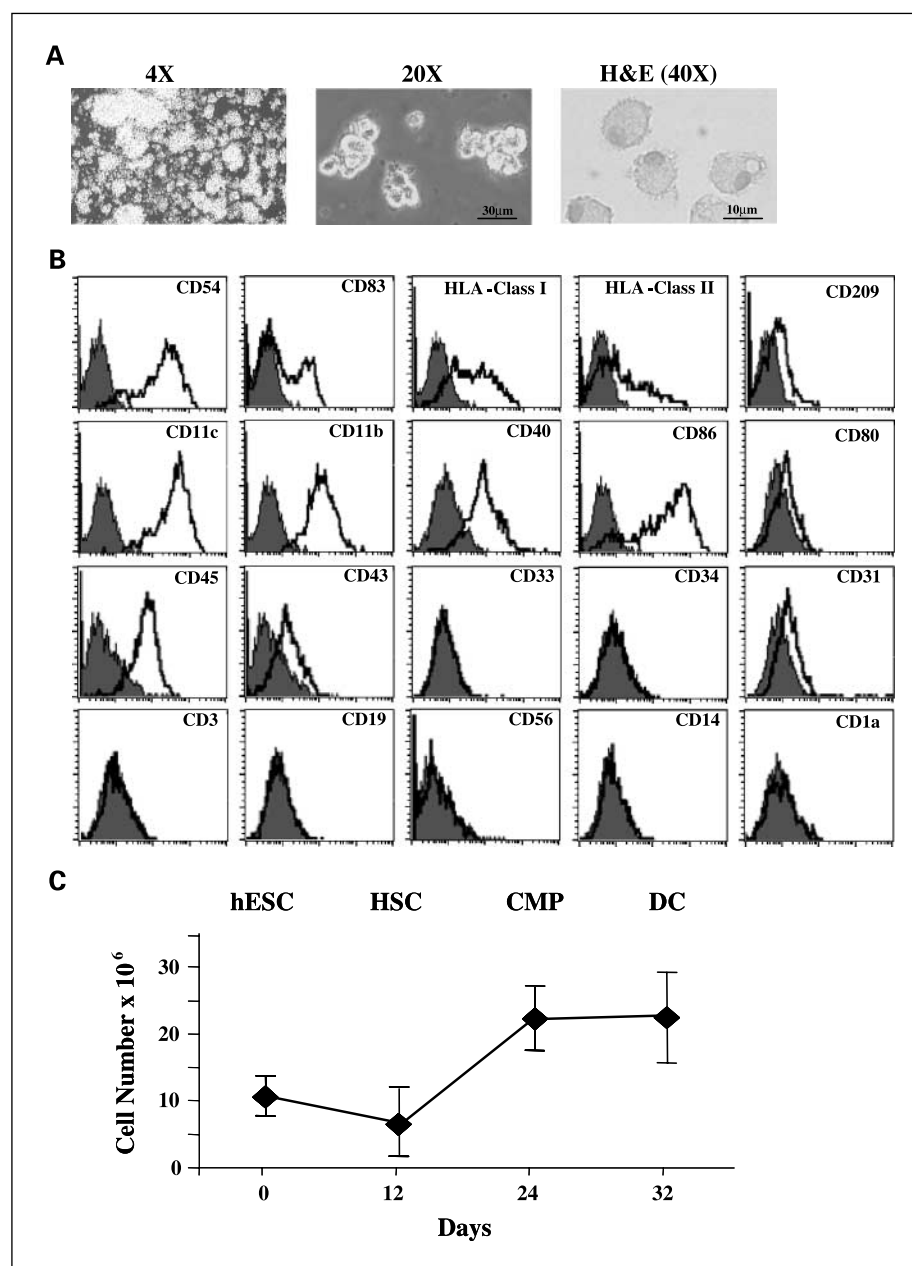
stimulated from the T cells of HLA-A2<sup>+</sup> and HLA-A2<sup>-</sup> donors, using irradiated autologous BLCL (Fig. 5A, *left*). These established EBV-specific CTL were then used in IFN- $\gamma$  ELISPOT assays to test whether they could recognize HLA-A2<sup>+</sup> hESC-derived DC pulsed with HLA-A2 restricted EBV BMLF1<sub>280-288</sub> peptide.

As shown in Fig. 5A (*right*), EBV peptide-specific CTL responses were detected by using both mature and immature peptide-pulsed hESC-derived DC cells as stimulators, whereas, expectedly, no significant differences in CTL lysis were observed when immature and mature hESC-derived DC were used as targets. These experiments show that hESC-derived DC can efficiently present peptide antigens on the cell surface in the context with MHC class I molecules that can be recognized by CTL.

To further test whether hESC-derived DC are capable of priming a tumor (self) antigen-specific CD8<sup>+</sup> T-cell response, we used an *in vitro* melanoma tumor system, as shown in Fig. 5B–D. HLA-A2<sup>+</sup> CTL were generated using CD8<sup>+</sup> cells isolated from the PBMC of HLA-A2<sup>+</sup> donors that were stimulated with HLA-A2-matched MART-1 (ELAGIGITV)

**Fig. 2.** Hematopoietic differentiation of feeder-free maintained hESC. **A**, sequential phenotypic analysis of hematopoietic differentiation of hESC via EB culture. Representative phenotypes of single-cell suspensions of total live cells harvested at 6, 8, 10, and 12 d after the beginning of EB culture. Differentiated cells were obtained at the indicated times and labeled with CD34 PE antibody. The percentages of CD34<sup>+</sup> cells are shown in the corresponding quadrant. **B**, hematopoietic precursor cell yields obtained after various differentiation conditions. Percentage of CD34<sup>+</sup> hESC after hESC/OP9 coculture (OP9), hESC/HS-5 coculture (HS-5), hESC/EB formation, and hESC/EB formation in the presence of BMP-4 (EB + BMP-4) are shown after 12 d of differentiation. **C**, myeloid expansion of CD34<sup>+</sup> hESC-derived HSC. HSC were differentiated and expanded in cytokine-conditioned medium for 12 d. Cells were obtained before (day 0) and after (day 12) expansion and costained with CD34, CD45, CD33, or CD31. The percentages are shown in the corresponding quadrant.





**Fig. 3.** Generation of mature hESC-derived DC from hESC-derived hematopoietic precursor cells. **A**, morphologic analysis of hESC-derived DC. hESC-derived DC were generated from myeloid precursors by culture in media supplemented with granulocyte macrophage colony-stimulating factor, IL-4, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Phase contrast images of cultured hESC-derived DC were acquired (*left*, 4 $\times$  magnification) and (*middle*, 20 $\times$  magnification). In addition, H&E staining (*right*, 40 $\times$  magnification) was done on cytopins of cells obtained after DC culture. **B**, phenotypic analysis of hESC-derived DC. A panel of cell surface – based DC and lineage markers was used to analyze cell surface expression on hESC-derived DC. The results are presented as fluorescence-activated cell sorting histograms as shown in the image. Histogram overlay (*filled line*) represents isotype control for nonspecific IgG staining. **C**, cell yields (average  $\pm$  SD calculated from 10 consecutive experiments) and culture duration at each differentiation step (hESC, EB, MP, DC) using a starting population of  $1 \times 10^7$  undifferentiated hESC.

peptide-pulsed hESC-derived DC *in vitro*. T-cell cultures were then harvested and analyzed in standard IFN- $\gamma$  ELISPOT assays using Malme-3M (HLA-A2+, A\*0201 allele, MART-1<sup>+</sup>, melanoma), Malme-3 (HLA-A2+, A\*0201 allele, MART-1<sup>-</sup>, normal skin fibroblasts isolated from the same patients as Malme-3M) and T2 cells pulsed with MART-1 peptide as targets. K562 cells, CEF peptide pool, and unstimulated T cells served as controls (28). In addition, unloaded hESC-derived DC and MART-1 peptide-pulsed hESC-derived DC were also tested in the same experiment for identifying the nonspecific allogeneic T-cell response. As shown in Fig. 5B, the peptide-specific CTL not only recognized the semiallogeneic, HLA class I–matched melanoma cell line Malme-3M but also recognized, to a greater extent, MART-1 peptide–pulsed T2 cells, whereas Malme-3 normal cells and K562 target cells did not trigger IFN- $\gamma$  release

by activated T cells. As expected, semiallogeneic, but antigen-naïve hESC-derived, DC were also recognized by CTL as a result of the bystander allo-response (Fig. 5C). However, when hESC-derived DC were loaded with MART-1 peptide, a dramatic increase in spot forming colonies was observed statistically significantly exceeding those stimulated by the bystander allo-response.

Cumulatively, we show that hESC-derived DC are capable of stimulating potent antigen-specific T-cell responses *in vitro* against target cells bearing either viral or tumor antigens in an HLA class I–restricted manner.

***hESC-derived DC can be transfected with mRNA-encoded antigens and can stimulate antigen-specific CD8<sup>+</sup> T-cell responses.*** These results shown in Fig. 5 prompted further experiments to investigate whether hESC-derived DC possess normal antigen

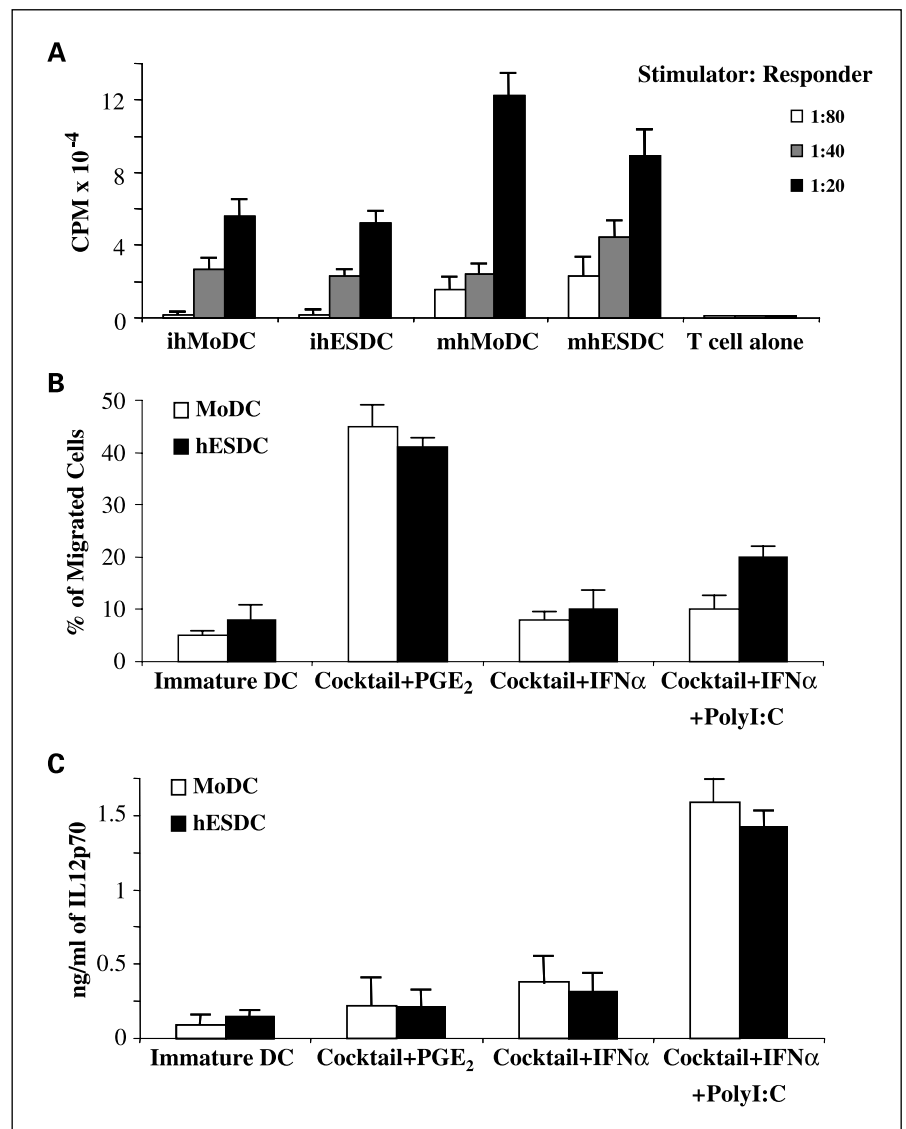
processing capability and function. Furthermore, the antigen-presenting function of the semiallogeneic hESC-derived DC was compared with autologous monocyte-derived DC *in vitro*. We have previously shown that monocyte-derived DC can successfully be transfected with mRNA-encoded tumor antigens and effectively be used for active cancer immunotherapy in patients with genitourinary malignancies (3). Using these well-established techniques, mature hESC-derived DC and monocyte-derived DC were transfected with GFP or PSA mRNA ( $2\mu\text{g mRNA}/1 \times 10^6$  cells) by electroporation (experimental settings are shown in Fig. 6). Transfection of hESC-derived and monocyte-derived DC with GFP mRNA resulted in equally efficient expression of GFP protein by transfected cells. Specifically, >50% of cells expressed GFP 12 hours after transfection with the transient expression lasting >3 days (Fig. 6A). Notably, these transfection techniques routinely yielded hESC-derived DC with >90% viability 24 hours after transfection (data not shown). Next, we tested the T-cell stimulatory capacity of mRNA-transfected hESC-derived DC in HLA-A2 matched cytolytic T-cell assays and found that PSA-specific and

GFP-specific CTL stimulated by mRNA transfected hESC-derived DC were able to kill their cognate target cells in an antigen-specific and HLA-restricted manner. As shown in Fig. 6B, semiallogeneic hESC-derived DC consistently stimulated more potent CTL responses against PSA or GFP than autologous monocyte-derived DC, most likely due to the concomitant induction of an allogeneic CD8<sup>+</sup> T-cell response.

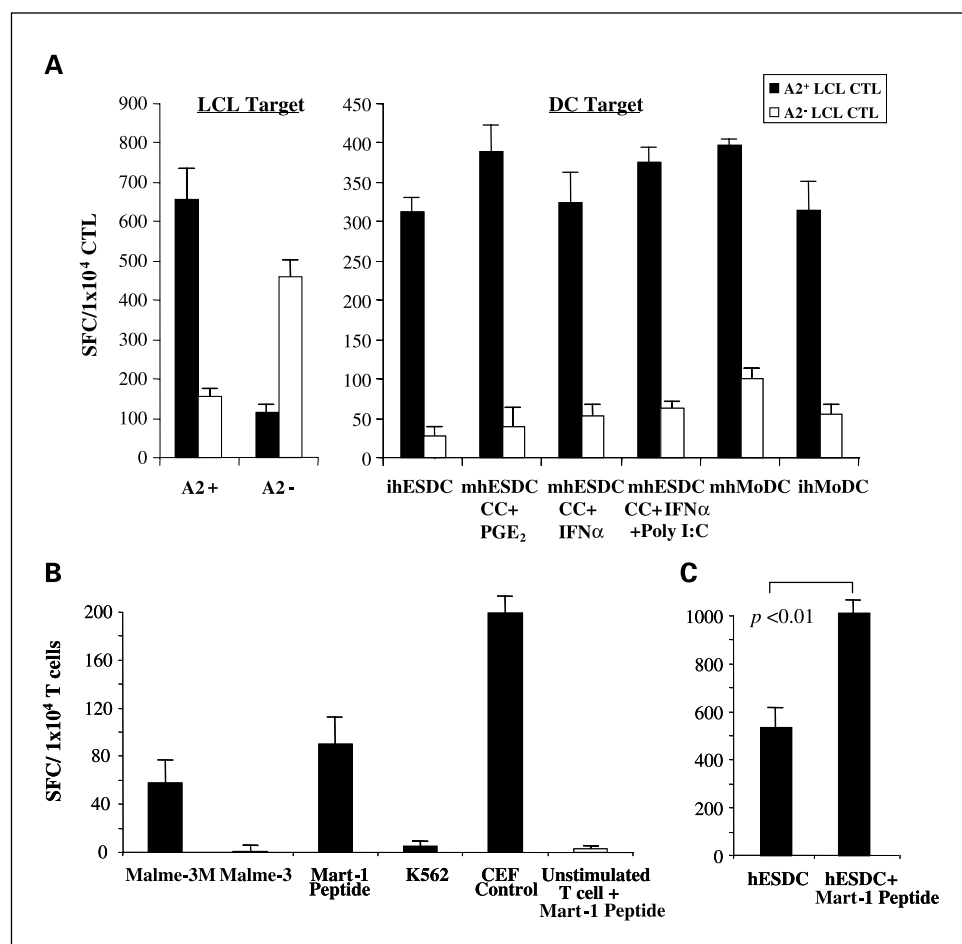
In summary, these data show that hESC-derived DC have comparable antigen processing and presenting function to monocyte-derived DC. Moreover, allogeneic CD8<sup>+</sup> T-cell responses, induced by semiallogeneic hESC-derived DC, may augment HLA-class I–restricted antigen-specific CTL responses, as recently shown (29).

**Semiallogeneic hESC-derived DC stimulate an allogeneic Th-1 type CD4 T-cell response without expanding regulatory T cells.** Recent studies have suggested that stimulation with antigen-loaded DC cannot only trigger potent T-cell responses but also a concomitant regulatory T-cell response (30). Here, we hypothesized that using HLA class I matched, but HLA class II mismatched, hESC-derived DC for stimulation, potent

**Fig. 4.** Allogeneic stimulatory capacity, migration efficiency, and IL-12p70 production of hESC-derived DC. **A**, allogeneic PBMC were incubated with immature and mature hESC-derived or monocyte-derived DC at the indicated stimulator-to-responder ratios. Proliferation was assessed by measuring incorporation of tritiated thymidine. **B**, cultured hESC-derived and monocyte-derived DC were matured using the proinflammatory cytokines tumor necrosis factor- $\alpha$ , IL-1 $\beta$ , and IL-6 (Cocktail) plus PGE<sub>2</sub>, cocktail plus IFN $\alpha$ , or cocktail plus IFN $\alpha$  and poly I:C and analyzed for their chemotactic response to MIP-3 $\beta$ . **C**, in addition, IL-12p70 ELISA assays were done using culture supernatants collected from the DC generated under the above maturation conditions.







**Fig. 5.** Antigen-presenting function of hESC-derived DC. **A**, IFN- $\gamma$  ELISPOT assays of EBV-specific CTL stimulated by hESC-derived DC. hESC-derived DC were exposed to different maturation stimuli, as indicated in image, and pulsed with the EBV-specific and HLA-A2-restricted BMLF<sub>280-288</sub> EBV peptide. After antigen loading, the DC were cocultured with HLA-A2<sup>+</sup> and HLA-A2<sup>-</sup> (control) BLCL-CTL lines. The number of IFN- $\gamma$  spot-forming colonies (SFC) per 10<sup>4</sup> T cells was measured after 24 h. Monocyte-derived immature and mature DC (Cocktail + plus PGE<sub>2</sub>) were included as controls (right). The specificity of HLA-A2<sup>+</sup> and HLA-A2<sup>-</sup> BLCL-CTL lines were determined using BLCL as targets (left). **B**, generation of functional, MART-1-specific CTL after stimulation with MART-1 peptide-loaded mature hESC-derived DC. CD8<sup>+</sup> T cells were isolated from a HLA-A2<sup>+</sup> donor and stimulated once with MART-1 peptide pulsed hESC-derived DC for 7 d. T-cell cultures were then harvested and analyzed in standard IFN- $\gamma$  ELISPOT assays using Malme-3M tumor cells (HLA-A2<sup>+</sup>, MART-1<sup>+</sup>), Malme-3 normal cells (HLA-A2<sup>+</sup>, MART-1<sup>-</sup>), and T2 cells pulsed with MART-1 peptide as targets (MART-1 peptide). K562 cells, CEF peptide pools (positive control for activating CD8 memory cells), and unstimulated T cells served as controls. **C**, antigen-naive hESC-derived DC and MART-1 peptide-pulsed hESC-derived DC were tested in the same experiment.

antigen-specific T-cell responses can be induced while bypassing activation of antigen-specific regulatory T cells. At the same time, we sought to determine the cytokine profile of stem cell-derived DC-induced allogeneic CD4<sup>+</sup> T cells that could provide potent Th-1 type cytokine help to augment the antigen-specific CD8<sup>+</sup> CTL response against tumor antigens presented by shared HLA class I molecules. To address these issues, we determined secretion of IFN- $\gamma$  (Th-1) and IL-4 (Th-2) from naive (nonpolarized) CD4<sup>+</sup>/CD25<sup>-</sup> T cells stimulated from autologous, monocyte-derived DC or from hES-derived DC using cytokine ELISA. Moreover, we serially determined the frequencies of FOXP3-expressing CD4<sup>+</sup> T cells stimulated over time by the HLA class II matched (autologous) or mismatched hES-derived DC.

As shown in Fig. 6C, naive CD4<sup>+</sup> T cells stimulated by allogeneic hESC-derived DC, but not by autologous monocyte-derived DC, exhibited a strong Th1-type response, as evidenced by secretion of high amounts of IFN- $\gamma$ , but not IL-4 or IL-5 (latter not shown). Moreover, mature hESC-derived DC only insignificantly activated and expanded FOXP3<sup>+</sup> regulatory CD4<sup>+</sup> T cells over the culture period. In contrast, significant regulatory T-cell expansion was noted after 6 days of stimulation using autologous monocyte-derived DC (Fig. 6D).

These data suggest that semiallogeneic HLA class II-mismatched hESC-derived DC may avoid the shortcomings of autologous vaccination by not only triggering a Th-1 type

cytokine environment but also by circumventing the expansion of immunosuppressive regulatory T cells.

## Discussion

Recently, several groups have shown the feasibility of using hESC for generating functional hematopoietic cells (17, 19, 22, 31–35). Other studies suggest the possibility that hESC can successfully be differentiated into APC (11, 36). However, limited data is available regarding the exact phenotype, cell yield, and the functional properties of the stem cell-derived APC that would justify their use in clinical immunotherapy protocols. Most importantly, the ability to use stem cell-derived products in clinical applications has been currently compromised by mouse feeder culture methods that are incompatible with human application. Here we show that using feeder-free culture conditions, hESC can successfully be differentiated into functional mature DC in the presence of human antibody serum and are capable of stimulating potent antitumor and antiviral T-cell responses *in vitro*, which may prove highly relevant for the clinical investigation of hESC-derived cellular products (37, 38). Although, the phenotype of this novel DC type is different from the monocyte-derived DC, which may depend on the differentiation pathways (11, 12), the hESC-derived DC showed potent capacity in the functional analysis. Similar to monocyte-derived DC, hESC-derived DC

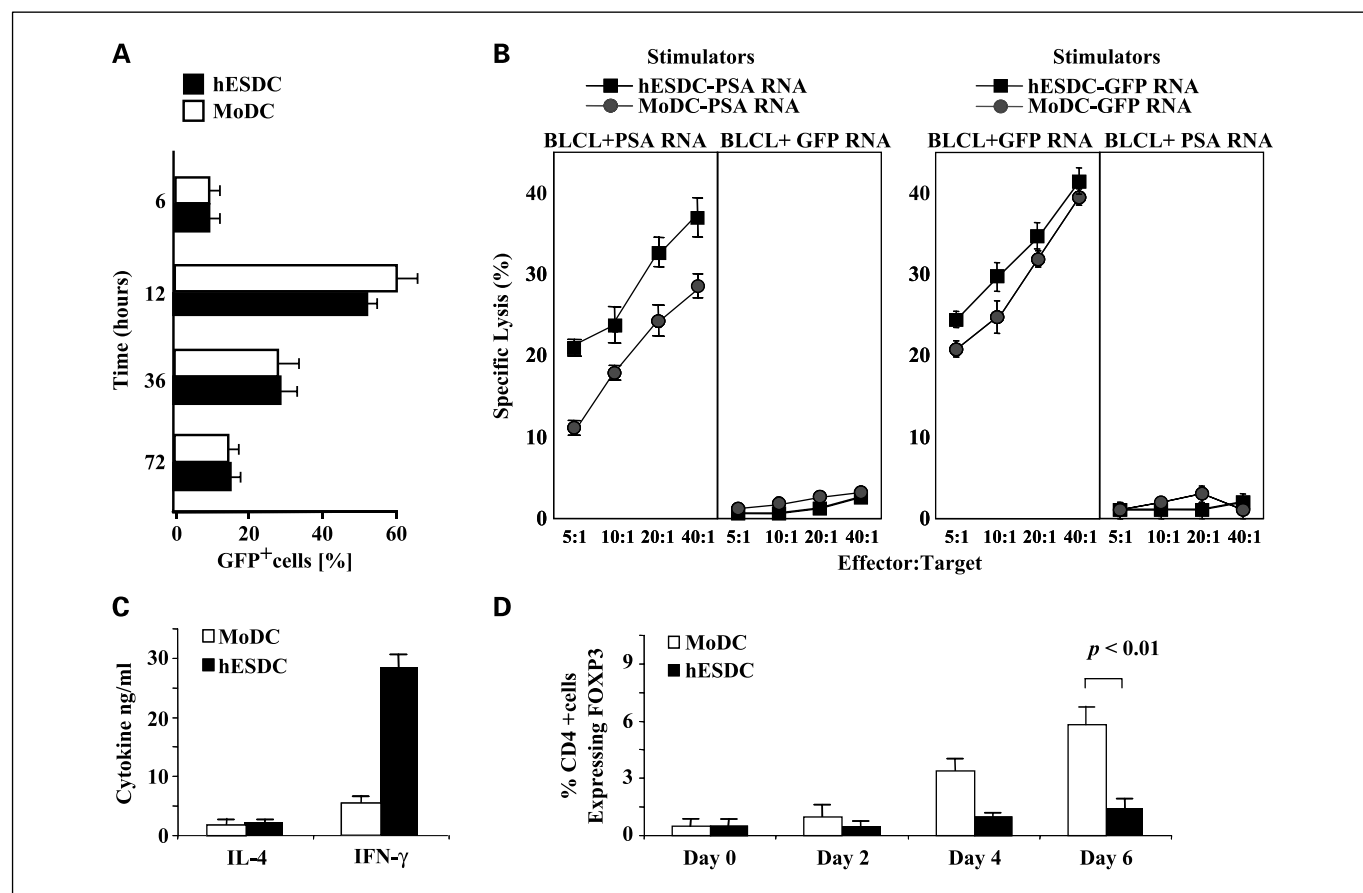


spontaneously secreted IL-12p70, migrated toward chemokine gradients, and stimulated potent T-cell responses *in vitro*. Moreover, hESC-derived DC seem to overcome obstacles typically encountered during autologous DC vaccination by inducing an antigen-specific immune response against tumor antigens without expanding and activating regulatory T cells (Fig. 6C and D).

The results of the experiments described in this report may have significant implications for the therapeutic application of hESC-derived DC in clinical disease settings. First, a major advantage of using semiallogeneic, stem cell-derived DC vaccines for active immunotherapy is the ability to generate large numbers of well-defined antigen-bearing cells that are amenable to a degree of standardization. It has been shown that undifferentiated hESCs can be expanded up to  $2\text{--}3 \times 10^8$  cells *in vitro* (39, 40). Using the methods described above, we were able to double cell yields when differentiating hESCs into DC. These conditions allow for the generation of up to 50 to 100 million viable, antigen-loaded DC, making clinical trials feasible. The “off the shelf” availability of stem cell-derived DC vaccines not only allows for repeated vaccinations but also

enables immediate treatment of patients with rapidly progressing tumors or with infectious diseases. Secondly, generation of DC from hESC lines for human immunotherapy circumvents the possibility that tumor-derived factors elicit DC dysfunction, as is often the case when autologous DC are used for tumor immunotherapy (41–43).

One relevant requirement for the successful application of allogeneic cellular vaccines is that the vaccine should specifically be matched to the HLA class I repertoire of the recipient patient (44). Without such matching, antigen-loaded hESC-derived DC cannot process antigen along the class I pathway and may only induce an unspecific T-cell response. In our study, we investigated the antigen-presenting function of hESC-derived DC in an HLA-A2-matched semiallogeneic system. In these experiments, we were not only able to show the generation of allogeneic responses but also a vaccine-mediated potent antigen-specific CD8<sup>+</sup> T-cell response. Therefore, hESC-derived DC, deliberately matched to the HLA class I repertoire of the recipient, have the capacity to stimulate potent CTL responses that are further augmented by T-cell cross-reactivity mediated by allogeneic responses (see ref. 44; Figs. 5 and 6).



**Fig. 6.** CD8 and CD4 T-cell responses induced by semiallogeneic mRNA transfected, hESC-derived DC. **A**, hESC-derived DC and monocyte-derived DC were transfected by electroporation at 300 V/150  $\mu$ F with 2  $\mu$ g GFP mRNA/ $1 \times 10^6$  cells. The degree of GFP expression was longitudinally monitored by fluorescence-activated cell sorting analysis 6, 12, 36, and 72 h after transfection. **B**, PBMC from a healthy HLA-A0201<sup>+</sup> donor were stimulated twice with PSA mRNA-transfected or GFP mRNA-transfected hESC-derived and monocyte-derived DC to generate antigen-specific CTL. These CTL were then tested in cytolytic assays using the after autologous HLA-A2<sup>+</sup> BLCL targets transfected with PSA mRNA and GFP mRNA using electroporation. **C**, Th-1 polarization of naive CD4<sup>+</sup> T-cell response induced by hESC-derived DC. IFN- $\gamma$  and IL-4 secretion were measured by cytokine ELISA after coculture of isolated naive CD4<sup>+</sup>CD25<sup>+</sup> T cells with cytokine-matured autologous monocyte-derived DC or allogeneic hESC-derived DC. **D**, low induction of FOXP3<sup>+</sup> CD4<sup>+</sup> T cells from naive CD4<sup>+</sup>CD25<sup>+</sup> T cells by hESC-derived DC. CD4<sup>+</sup>CD25<sup>+</sup> T cells were sorted with magnetic beads and then cocultured with autologous monocyte-derived DC or allogeneic hESC-derived DC without additional cytokines for 6 d. The percentage of FOXP3<sup>+</sup> cells were monitored at indicated culture days. Data represents the summary of three experiments.

Although our data suggest the effective induction of tumor-specific CTL response by semiallogeneic hESC-derived DC, it is possible that the allo-specific CTL induced by repeated vaccination may attack the injected APC and therefore eliminate them *in vivo*. Thus, it will be critical that the injected hES-derived DC could activate tumor antigen-specific CTL restricted to the shared MHC class I molecules before they are eliminated by allo-reactive CTL. However, in direct relevance to our semiallogeneic hESC-derived DC platform, this important issue was previously addressed by Fukuma et al., demonstrating that semiallogeneic murine embryonic stem-derived DC vaccines can induce an antigen-specific immune response even in the presence of allo-specific T cells (8). Moreover, clinical studies have not shown any evidence of rejection or tolerance using allogeneic vaccine. Specifically, in one recent study, up to 12 boost injections were necessary to induce an antigen-specific immune response using an allogeneic tumor cell vaccine platform (45). Conversely, the adjuvant effect of allo-reaction may enhance the HLA-A2-matched CTL response in the tumor-bearing host. Based on the data presented, it is conceivable that the direct CD4<sup>+</sup> T-cell response to defined allogeneic HLA class II antigens on the DC used in the vaccines will provide a potent Th1-type cytokine environment for the generation of CD8<sup>+</sup> CTL responses to tumor peptides presented by shared HLA class I molecules even in the absence of classic adjuvants. This result is critical because the provision of Th-1 type cytokines in tumor cell-based vaccines and peptide-based vaccines is likely to be weak or absent. The allogeneic response induced by semiallogeneic hESC-derived DC has the unique

potential to create a microenvironment rich in cytokines and T-cell costimulatory ligands for the promotion of polyclonal T-cell responses that may overcome any previously generated T-cell anergy. Moreover, the lack of activating antigen-specific regulatory T cells may increase the duration and magnitude of vaccine-induced antigen-specific immune response.

Studies have shown that the embryonic stem cell-derived DC platform could be further improved by stably transfecting hESC with recombinatorial molecules encoding either antigenic proteins, HLA, or costimulatory molecules to enhance their immunostimulatory function (5, 10, 46). In one recent study, preliminary evidence of using genetically modified murine stem cell-derived DC to induce protective immunity in a murine melanoma model (5) was provided. These studies strongly suggested the great potential of this novel APC platform for future improvement. In summary, the use of stem cell-derived DC vaccines has considerable advantages and, at the same time, provides a mechanism to eliminate or reduce the drawbacks associated with other immune-based technologies.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Differentiation of Human Embryonic Stem Cells into Immunostimulatory Dendritic Cells under Feeder-Free Culture Conditions

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