METHOD OF FORMING DENDRITIC CELLS FROM EMBRYONIC STEM CELLS

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**Abstract**

This invention relates to the culture of dendritic cells from human embryonic stem (ES) cells. Human ES cells are first cultured into hematopoietic cells by co-culture with stromal cells, and lineage are then cultured with GM-CSF to create a culture of myeloid precursor cells. Culture of the myeloid precursor cells induces functional dendritic cells to be generated. The dendritic cells have a unique phenotype, as indicated by their combination of cell surface markers.

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ABSTRACT

This invention relates to the culture of dendritic cells from human embryonic stem (ES) cells. Human ES cells are first cultured into hematopoietic cells by co-culture with stromal cells. The cells now differentiated into the hematopoietic lineage are then cultured with GM-CSF to create a culture of myeloid precursor cells. Culture of the myeloid precursor cells with the cytokines GM-CSF and IL-4 causes functional dendritic cells to be generated. The dendritic cells have a unique phenotype, as indicated by their combination of cell surface markers.

1 Claim, 9 Drawing Sheets
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OTHER PUBLICATIONS


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* cited by examiner
Step 1

hESC

OP9

hESC+OP9 9-9-10 days

Dissociation

Expansion of myeloid precursors
a-MEM-10% FCS
GM-CSF (8-10 days)

Percoll separation

Step 2

DC generation
SFEM
GM-CSF+IL-4
or other cytokine combinations
(7-9 days)

Figure 1A
Figure 1B

hESC

OP9

9 days

No cytokines

Cytokine addition had no effect on differentiation

CD34+
CD43+
Lin-

Multipotent lymphohematopoietic progenitor

GM-CSF

(Addition of other cytokines: Flt-3L, SCF, IL-3 or their combinations had no effect on differentiation or expansion)

CD45+
CD4+
CD123a
CD11c+
CD11b+
CD15–
CD16–
MPO–
M-CSFR–

GM-CSF + TNF-α or GM-CSF+IFN-α

Regulatory DCs

CD1a
CD8–
CD80a
CD86a

GM-CSF

7-9 days

Immature DC

GM-CSF + IL4

CD1a+
DC-SIGN+
CD4+
CD9
CD11c+
CD40
CD80+
CD86+
HLA-ABC+
HLA-DR+
CD207+
CD208+

Mature DC

Calcium ionophore (LPS, IL1β, IL5, PGE2, TNF-α are not efficient in induction of maturation)

CD80+

Fascin+
Figure 2C
Figure 2D
Figure 3

D

SSC-H: Side Scatter

FSC-H: Forward Scatter

R1

R2

IgG-PE

CD1a-PE

IgG-FITC

CD14-FITC
METHOD OF FORMING DENDRITIC CELLS FROM EMBRYONIC STEM CELLS

BACKGROUND OF THE INVENTION

Embryonic stem cells are pluripotent cells capable of both proliferation in cell culture as well as differentiation towards a variety of lineage restricted cell populations that exhibit multipotent properties (Odorico, et al., Proc. Natl. Acad. Sci. U.S.A. 2001). Human embryonic stem (ES) cells are thus capable of commitment and differentiation to a variety of lineage-restricted paths resulting in very specific cell types that perform unique functions.

Generally, ES cells are highly homogeneous, exhibit the capacity for self-renewal, and have the ability to differentiate into any functional cell in the body. This self-renewal property can lead under appropriate conditions to a long-term proliferating capability with the potential for unlimited expansion in cell culture. Furthermore, it is understood that if human ES cells are allowed to differentiate in an undirected fashion, a heterogeneous population of cells is obtained expressing markers for a plurality of different tissue types (WO 01/51616; Shamblott, et al., 2001). These features make these cells a unique homogeneous starting population for the production of cells having therapeutic utility.

There have been efforts by researchers in the field to develop methods to culture a variety of progeny cell types from human ES cells. For example, U.S. Pat. No. 6,280,718 describes a method for culturing human ES cells into hematopoietic cells by culturing the human ES cell with stromal cells. Some methods of creating progeny cell types from human ES cells involve the creation of embryoid bodies, which are three dimensional structures which can be formed by ES cells in culture and which foster the diverse differentiation of ES cells into various differentiated progeny lineages. Other methods for creating progeny lineages depend on the culturing of human ES cells with particular media, agents or types of cells to expose the ES cells to factors which encourage differentiation in a particular direction. All these methods have a common objective, which is to provide a source for particular cell types for scientific research and experimentation and, for some cell types, for ultimate transplantation into human bodies for therapeutic purposes.

Dendritic cells are immune cells that perform a critical function in the mammalian immune system. Dendritic cells (sometimes here DCs) are powerful antigen-presenting cells which are present at low frequency in tissues of the body in contact with the environment such as skin, and linings of the nose, lungs, stomach and intestines. Dendritic cells have the ability to uptake antigens and induce primary T cell responses to initiate generalized immune system responses to pathogens.

The availability of human immature dendritic cells would be useful for the study of antigen processing and presentation, as well as for understanding the mechanisms of the induction of immunity and tolerance. Functional analysis of human dendritic cell subsets has significantly facilitated by the development of in vitro systems for the differentiation of dendritic cells from CD34+ hematopoietic stem cells and monocytes. However, these existing protocols, obtaining large numbers of human dendritic cell progenitors is a laborious process and is associated with potential risks for donors. Other aspects of dendritic cell biology, such as dendritic cell ontogeny, have not been studied in humans due to the difficulties in obtaining tissues during early development.

The advent of human ES cells represents an opportunity to overcome these limitations.

Functional dendritic cells have been generated from mouse ES cells using embryoid bodies and by co-culture with mouse macrophage colony-stimulating factor deficient bone-marrow stromal cell line, OP9. We have previously demonstrated that OP9 cells can be used to induce hematopoietic cells from human ES cells. The full potential of these hematopoietic cells to produce progeny of the various lineages was unexplored previously.

BRIEF SUMMARY OF THE INVENTION

In one embodiment, the present invention is a method of culturing human embryonic stem cells into dendritic cells, the method comprising the steps of co-culturing human embryonic stem cells with stromal cells that do not express macrophage colony-stimulating factor, wherein the stem cells are induced to differentiate into multipotent lympho-hematopoietic progenitor cells and wherein the culture is not in the presence of cytokines; cultivating the progenitor cells with granulocyte/macrophage colony stimulating factor (GM-CSF) to cause the expansion of myeloid precursors; and recovering cells which have the phenotype of immature dendritic cells. Preferably the step of recovering cells with the phenotype of dendritic cells includes culturing the myeloid precursor cells with at least one cytokine selected from the
group consisting of IL-4, TNF-α, INF-α, and GM-CSF. Preferably, the stromal cells are OP9 cells and the culturing of step (b) is under non-adherent conditions.

In another embodiment, the present invention includes the step of culturing the myeloid precursor cells with GM-CSF and TNFα or GM-CSF and INF-α and recovering regulatory accessory cells, wherein the regulatory accessory cells are characterized by the markers CD1a<sup>low</sup>, CD9<sup>−</sup>, CD80<sup>high</sup> and CD86<sup>low</sup>.

In another embodiment, the present invention includes the step of culturing the myeloid precursor cells with GM-CSF and TNFα or GM-CSF and INF-α and recovering regulatory accessory cells, wherein the regulatory accessory cells are characterized by the markers CD1a<sup>low</sup>, CD9<sup>−</sup>, CD80<sup>high</sup> and CD86<sup>low</sup>.

The present invention is also a culture of human dendritic cells, in which a majority of the cells in the culture have a phenotype of CD1a<sup>+</sup>, DC-SIGN<sup>+</sup>, CD4<sup>+</sup>, CD9<sup>low</sup>, CD86<sup>+</sup>, CD86<sup>a</sup>, IL-1α, IL-4, and are negative for CD207 and CD208. Preferably, at least 10% of the cells in the culture have the phenotype.

In another embodiment, the invention is a culture of myeloid precursor cells in which a majority of the cells have a phenotype of myeloid precursors and in which an excess of 90% of the cells are CD34<sup>+</sup>, CD4<sup>+</sup>, CD123<sup>low</sup>, negative for HLA-DR and include subpopulations of cells expressing MPO, M-CSFR, CD11b, CD11c, CD15 and CD16.

In another embodiment, the present invention is a method of making of cellular vaccine, comprising differentiating human embryonic stem cells into population of dendritic cells, characterized by the markers CD1a, CD80, CD86, DC-SIGN, HLA-DR<sup>high</sup>, obtaining and preparing single cell suspension of tumor cells from a patient, and fusing the embryonic stem cell-derived dendritic cells with the tumor cells so that a cellular vaccine is created.

Other embodiments of the present invention will be apparent to one of skill in the art after review of the specification, claims and drawings.

**BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS**

FIGS. 1A and B are schematic illustrations of the overall method of the present invention.

FIG. 2 illustrates the morphology and phenotypical features of myeloid precursor cells generated in step 2 of FIG. 1.

FIG. 2A is a phase-contrast micrograph of differentiated human ES cells growing in the presence of GM-CSF. FIG. 2B is a Wright-stained cytospin of cells obtained from that culture. FIG. 2C charts the colony forming cell (CFC) potential of the expanded cells (counts are mean of five experiments). FIG. 2D are graphs of data from representative experiments demonstrating expression of surface and intracellular myeloid markers on GM-CSF expanded human ES cells.

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

**DETAILED DESCRIPTION OF THE INVENTION**

We report here that dendritic cells can be created in large numbers from human ES cells. The co-culture system with a macrophage colony-stimulating factor (M-CSF) deficient stromal cell line, such as the murine line OP9, fosters the differentiation of human ES cells into hematopoietic cells. These hematopoietic cells have the capacity to generate dendritic cells, a capacity which is exploited by GM-CSF culture of the hematopoietic cells. The dendritic cells derived from human ES cells are morphologically, phenotypically and functionally comparable to interstitial human dendritic cells naturally produced in vivo.

Slukvin, et al., *J. Immunology*, 2006, 176:2924-2932, is an academic article by the inventors describing the present invention. It is incorporated by reference as if fully set forth below.

The overall method is schematically illustrated in FIGS. 1A and B, in which the process is broken down into three overall steps and is demonstrated in its preferable form in the Examples below and in Slukvin, et al., supra. By "multipotent lymphohematopoietic progenitor cells," "myeloid dendritic cell (DC) precursor cells," "immature DC," "mature DC," and "regulatory DC," we mean the cell populations derived in FIG. 1B.

In Step 1, human ES cells are co-cultured with stromal cells, preferably M-CSF deficient stromal cells, to induce differentiation of the cells into multipotent lymphohematopoietic progenitor cells. Preferably, the cells are OP9 cells.

In Step 2, the disassociated ES-derived cells from that culture are then cultured so that myeloid cell expansion occurs. Preferably, this is done by culture of the cells with granulocyte/macrophage colony stimulating factor (GM-CSF) preferably as described below in the Examples. Also preferably, this step is performed in non-adherent conditions. Preferable non-adherent conditions require the tissue culture flask to be coated with poly 2-hydroxyethyl methacrylate (HEMA, Sigma) as described below. One could also prevent cell adherence by other means, such as cell shaking, using substances known to have non-adherent properties to cover the plastic container or using commercially available non-adherent tissue flakes.

The result of this expansion step is a culture rich in myeloid precursors, and that culture is then used in Step 3 to make dendritic cells by culture in serum free medium with GM-CSF and IL-4, or other combination of cytokines (as described below) which condition development to dendritic cells.

An optional separation procedure, which can be done with PERCOLL separation, is shown between steps 2 and 3 and is used to remove both clumps of cells and dead cells from the culture prior to inducing dendritic cell formation.

In the examples described below, dendritic cells are generated from human ES cells by selective expansion of myeloid precursors obtained by the co-culture of human ES cells with M-CSF deficient stromal cells without cytokine addition. The hematopoietic cells resulting from the coculture step are competent to be induced to differentiate into myeloid precursor cells and then into immature dendritic cells.

A critical step in our protocol for generating DCs was the efficiency of hematopoietic differentiation in human ES cell/OP9 co-culture. Co-cultures with a low number of CD34<sup>+</sup> CD43<sup>+</sup>Lin<sup>−</sup> multipotent lymphohematopoietic progenitors
myeloid lineage and expressed CD4, CD45, myeloid precursor cells to undergo this expansion was GM-CSF, in contrast to other factors, such as SCF and FLT3-L, which in our hands had little effect on the expansion of myeloid dendritic cell precursors.

The myeloid precursors derived from human ES cells and expanded with GM-CSF, contained myeloid colony-forming cells (CFCs), as well as small populations of more mature cells with the dendritic cell phenotype. However, the majority of the cells morphologically resembled blasts of the myelomonocytic lineage and expressed CD4, CD45, CD123low and low levels of CD14. These cells were HLA-DR-negative. We found that these cells included subpopulations of cells expressing MPO, M-CSFR, CD11b, CD11c, CD15, and CD16.

The human ES cell-derived immature dendritic cells obtained by our method had a phenotype of CD1a+, DC-SIGN+, CD44+, CD9low, CD11c+, CD40low, CD80+, CD86+, HLA-ABC+, HLA-DR+, CD207low and CD208-, a phenotype comparable with interstitial dendritic cells differentiated from the cord blood or bone marrow CD34+ hematopoietic stem cells. Preferably, at these immature dendritic cells comprise at least 70% of the cultured cells at this point. However, a distinct phenotypic feature of the human ES-derived dendritic cells was co-expression of CD14. The level of CD14 expression was the lowest in cells differentiated using IL-4, but was substantially higher on cells differentiated using TNF-α. Dendritic cells that develop from human CD34+ hematopoietic stem cells in the presence of GM-CSF and TNF-α differentiate into Langerhans cells and dermal and interstitial dendritic cells through intermediates that have phenotypes that are CD1a+CD14- and CD1a-CD14+ respectively. So far, in our cultures, CD1a expression has always been associated with at least low level expression of CD14, and we have not seen distinct CD1a+CD14- or CD1a-CD14+ populations in our cell cultures. Thus the culture conditions used for differentiation of the human ES cells into dendritic cells appears to use unique pathways that may not exact replicates of the corresponding pathways of differentiation from CD34+ hematopoietic stem cells in vivo.

The Examples below describe another embodiment of the present invention, a population of cells, wherein at least 70% are mature DCs.

Additionally, another embodiment of the invention is a population of regulatory DC cells. Myeloid DC precursors cultured with GM-CSF and TNF-α or GM-CSF and IFN-α develop into CD1a+CD4+, CD9+, CD40low, CD80low, CD86low accessory cells with low stimulatory activity. These cells can represent regulatory DCs.

The co-culture system used here with the M-CSF deficient stromal cells (OP9 cells) differs from the system based on OP9 cells used with murine ES cells. The method described here does not use a second co-culture with the OP9 cells, unlike the mouse system. We collected the human ES cell derivatives from the co-culture when the maximal amount of myeloid progenitors were generated and then expanded those progenitors with GM-CSF in feeder-free non-adherent conditions. This technique resulted in the discrete population of dendritic cell precursors which is useful for further studies of dendritic cell development.

It has been shown recently that during embryoid body differentiation, cells expressing HLA-DR and capable of triggering proliferation of adult lymphocytes were generated. Zhan et al., 2004, Lancet Jul 10; 364(9429):163-71. However, the antigen-presenting properties and the phenotype of the cells generated in this system were not demonstrated. It is possible that cells obtained as described in this report were macrophages. Our results provide, for the first time, evidence that human ES cells can be directly differentiated into cells with morphology, phenotype and functional properties of antigen-presenting dendritic cells. Furthermore, this process is already relatively efficient. We have been able to grow as many as 4x10⁷ dendritic cells at a time from 10⁷ initially plated human ES cells.

While dendritic cells have evident scientific interest and application, they also have potential use in human medicine. Several studies have demonstrated that peptide-pulsed dendritic cells transferred in vivo were able to induce efficiently anti-tumor immune response in mice. These studies have encouraged subsequent development of dendritic cell-based vaccines for cancer immunotherapy in humans. In these techniques, immature dendritic cell precursors isolated from peripheral blood or dendritic cells generated from peripheral blood mononuclear cells and CD34+ hematopoietic progenitors are used in clinical trials of dendritic cell based vaccines. However, these techniques are laborious, require repeated generation of new dendritic cells for each vaccination and are difficult to standardize. Embryonic stem cells can be expanded without limit and can differentiate into multiple types of cells, and therefore can be universal and scalable source of cells for dendritic cell vaccines. Potentially, dendritic cells with major HLA haplotype combinations can be obtained from human ES cells to match donor MHC haplotype. In the clinical setting, human ES cell-derived dendritic cells would have several advantages over dendritic cells from conventional sources. Large absolute numbers of dendritic cells could be generated from the same donor cell line, and the same line of dendritic cells could be used for multiple vaccinations. Derivation of dendritic cells from human ES cells can be less laborious and more amenable for standardization with implementation of bioreactor technology. Low risk of pathogen contamination and risk free donor collection are another important advantages of clinical use of human ES cell-derived dendritic cells.

In another embodiment, the present invention is a method of making a cellular vaccine, comprising differentiating the human embryonic stem cells into a population of dendritic cells, characterized in that they are CD1a+, CD80+, CD86+, DC-SIGN+, HLA-DRhigh, obtaining and preparing single cell suspension of tumor cells from a patient, and fusing the embryonic stem cell-derived dendritic cells with cancer cells. Gong et al., J. Immunology, 2000, 165:1705-1711 and Parkhurst et al., 2003, J. Immunology, 170:5317-5325 (both incorporated by reference) describe general techniques for cellular fusion.

In another embodiment, the invention is a method of forming a dendritic cell vaccine for treating of cancer, comprising dendritic cells differentiating from human embryonic stem cells, where dendritic cells have been fused with allogeneic cancer cells. One of skill in the art would understand and appreciate the various methods of creating tumor vaccines.

In another embodiment, the present invention is a method of making a dendritic cell vaccine for treating cancer, comprising differentiating human embryonic stem cells into CD45−CD4−CD123low myeloid precursors which include subpopulations of cells expressing CD11b, CD11c, and CD16, genetically altering the myeloid precursors to express immunogenic tumor proteins/peptides, and differentiating the genetically modified myeloid precursors into immunogenic dendritic cells. For example, one may wish to transfec cells with tumor genes that will be the target of an immune response. For example, one may wish to transfec cells with melanoma-antigen-3 (MAGE-3), prostate acid phosphatase (PAP) or prostate specific membrane antigen (PSMA).

In another embodiment, the present invention is a method of making dendritic cells with tolerogenic properties which can be used for treatment of rejection of human embryonic stem cell-derived tissues obtained from the same cell line. By "tolerogenic properties," we mean that the cell suppresses rejection of a transplant by the host immune system. The cells will down-regulate a detrimental immune response of the host towards a transplanted tissue. For this purpose hES cell-derived myeloid precursors will be induced to differentiate into regulatory DCs by culture with GM-CSF and TNF-α or GM-CSF and IFN-α.

EXEMPLARY

Experimental Protocol and Results

Expansion of Human ES Cell-Derived Myeloid Progenitors with GM-CSF.

Recently we developed an in vitro culture system for hematopoietic differentiation from human ES cells, using cells of mouse M-CSF deficient bone marrow stromal cell line OP9 as feeder cells, a step used to start the protocols described here. Human ES cells were co-cultured with OP9 cells so that they would differentiate into CD34+ cells which are highly enriched in colony-forming cells and contain erythroid, myeloid, as well as lymphoid, progenitors and include a population of CD34+ CD43− Lin- multipotent hematopoietic progenitors. This step does not require cytokine addition. The maximal expansion of myeloid colony-forming cells (CFCs) in the OP9 co-culture system was observed on days 9 to 10 of differentiation. To induce selective expansion of myeloid progenitors, we harvested the resulting cells from days 9 or 10 of human ES cell/OP9 co-culture and cultured the cells in non-adherent conditions in presence of GM-CSF. At the beginning of culture, aggregates of large cells were formed. Approximately 3 days after initiation of GM-CSF culture, individual cells appeared and rapidly expanded. After 9-10 days of culture with GM-CSF, and following the removal of clumps and dead cells by PERCOLL separation, we obtained a population of cells of which 90% of the cells were CD45 positive. More than 90% of these CD45+ cells contained intracellular MPO (myeloperoxidase, a marker of myeloid cells) but not Tdt (terminal deoxynucleotidyl transferase, a marker of lymphoid cells) and expressed a marker of myeloid progenitors, CD33. In addition, these human ES cell-derived myeloid cells were CD34 positive, and weakly expressed IL-3 receptor a-chain CD123. More than 50% of these cells expressed CD16, CD15, CD11b, and CD11c (Table 2). Morphologically, the GM-CSF-expanded cells had multi-lobed or round nuclei and a moderate amount grayish, occasionally vacuolated, cytoplasm without visible granules (FIG. 2B), resembling bone marrow myelomonocytic precursors. Some of the GM-CSF-expanded cells retained myeloid CFC potential, but no erythroid or multi-lineage CFC potential was detected (FIG. 2C). In addition, a relatively small population of cells at advanced stages of maturation that expressed a moderate level of CD14, low level of CD1a as well as the ILA-DR, and CD80 and CD86 co-stimulator molecules were present (Table 2).

Cutaneous lymphocyte-associated antigen (CLA) expression on peripheral blood CD34+ cells defines progenitors which further differentiate into Langerhans’ cells, while CD34+CLA- cell give rise to interstitial DC-like cells. No significant CLA expression was detected in the total cell population obtained from OP9 co-cultures or isolated human ES cell-derived CD34+ cells. However, CLA expression was found on a small subset of myeloid progenitors generated with GM-CSF.

GM-CSF appeared to be the most important factor in expansion of myeloid precursors. Separately, the addition of SCF, FLT3L, or SCF with FLT3L to GM-CSF-supplemented cultures had little effect on total cell output and myeloid CFCs numbers during 10 days of culture (Table 1). These data demonstrate that culture of differentiated human ES cells generated in OP9 system with GM-CSF predominantly expand into a unique population of CD45+CD34+CD123low myeloid precursors which include subpopulation of cells expressing MPO, M-CSFR, CD11b, CD11c, CD15 and CD16. Differentiation of Human ES-Cell Derived Myeloid Precursors into Dendritic Cells.

To induce differentiation of myeloid precursors into dendritic cells, we cultured the culture of precursor cells with GM-CSF and various combinations of IL-4, TNF-α, and IFN-α. In typical experiment, after 7-10 days of culture with GM-CSF and IL-4, most of the cells appeared as clumps. In addition, individual floating cells with well-defined dendrites appeared in the cultures. Morphologically, these cells were large, had high nuclear cytoplasmic ratio, and had oval or kidney-shaped nuclei and nonvacuolated, occasionally granular cytoplasm with very fine cytoplasmic processes (FIGS. 3A and C). Based on flow cytometric analysis of size and granularity, two cell populations were observed (FIG. 3D): R1, cells with high scatter profile and dendritic cell phenotype; and R2, cells with a low scatter profile, which lacked dendritic cell markers and which were more phenotypically similar to myeloid progenitors generated in the second step. Dendritic cells identified as R1 gated cells expressed CD1a, DC-SIGN, CD4, CD11 c, HLA-ABC and HLA-DR, CD80, and CD86. Additionally, these cells expressed a low level of CD9, CD11b, CD123, and CD40. CD14 expression was very weak, but detectable, and most of the CD14-positive cells co-expressed CD1a. However all cells were lacking CD83 expression.

In addition to IL-4, differentiation of myeloid precursors into dendritic cells was achieved by using other cytokines such as TNF-α and IFN-α or their combinations. However, most of the cells in cultures with TNF-α co-expressed low level of CD1a, high levels of CD14 and were lacking expression of CD9. In addition, in cultures with TNF-α, cells down-regulated expression of costimulatory molecules. As expected, addition of IFN-α to these cell cultures resulted in increased expression of MHC class I molecules. However, IFN-α culture resulted in a decreased number of CD14+ cells, as well decreased CD14 expression. Similar to the monocyte-DC differentiation pathway, expression of DC-SIGN on human ES cell-derived dendritic cells was primarily dependent on IL-4. Based on cell yield, phenotypic, and functional
properties (Table 1 and 2), we concluded that a combination of
GM-CSF and IL-4 provides the best conditions for genera-
tion of functional dendritic cells from human ES cells.

By immunochemistry, human ES cell-derived dend-
ritic cells were positive for CD68, but not strongly so, and
expressed a very low level of intracytoplasmic but not mem-
branous CD83. Fascin, an actin-binding protein that has been
shown to be a highly selective marker of mature dendritic
cells, was not detected. From this, we concluded that the
dendritic cells generated by the process described so far were
immature. To investigate whether these immature dendritic
cells could be further matured, we treated cells generated
from above protocols with calcium ionophore A23187.

This treatment resulted in the up-regulation of CD83, CD86
and HLA-DR expression. The intensity of intracytoplasmic
CD68 staining substantially increased and perinuclear con-
centration of CD68 was evident in the cells so produced.

In addition, some cells became fascin-positive. LPS, TNF-α,
IL-1β, PGE2, and IL-6 were not efficient in induction of
maturation of hES cell-derived DCs. Taken together, these
data demonstrate that cells with typical dendritic cell mor-
phology and phenotype can be generated from human ES
cells.

The dendritic cells induce allo genetic T Cell response and
are capable of antigen processing and presentation.

We next investigated to determine whether our human ES
cell-derived dendritic cells were fully functional as dendritic
cells. As determined by DQ ovalbumin assay, human ES
cell-derived dendritic cells were capable of taking up and
processing antigen. Cells obtained in cultures treated with
GM-CSF and IL-4 were the most efficient in antigen process-
ing, while the dendritic cells differentiated with GM-CSF and
TNF-α were less efficient.

A hallmark of the functionality of dendritic cells is their
ability to stimulate naïve cells. By our tests, human ES cell-
derived dendritic cells were able to trigger cord blood T cells,
which are entirely naïve. Immature dendritic cells, generat-
ed in cultures with GM-CSF and IL-4 were added, were the
most powerful stimulatory cells, while addition of TNF-α to the
cell culture significantly diminished ability of the cells to
stimulate naïve T lymphocytes. In addition, the dendritic cells
were able to stimulate adult donor T-cells.

To evaluate the capacity of dendritic cells to present anti-
gens through the MHC class I pathway, we pulsed HLA-A02
H1 cell line-derived dendritic cells with inactivated CMV
virus and evaluated the ability of the cells to stimulate HLA-
A0201 restricted CMV-specific T cell clone HLA with specif-
icity to CMV pp65 NLVPVMTAV peptide. While the addi-
tion of dendritic cells to T-cells induced allogeneic response,
a significant increase in response by the T cells was obtained
when cells were stimulated with CMV pulsed H1-derived
dendritic cells (Table 3). Altogether, these data demon-
strate that our culture system allows generation of cells with
phenotype, morphology and unique antigen-presenting proper-
ties characteristic of dendritic cells.

Methods and Materials

Cell Lines, Cytokines and Monoclonal Antibodies
(mAbs).

Human ES cell lines H1 (passages 32-51) and H9 (pass-
ages 40-44) were maintained in an undifferentiated state by
weekly passage on mouse embryonic fibroblasts. A mouse
bone marrow stromal cell line OP9 was obtained from Dr.
Tori Nakano (Research Institute for Microbial Diseases,
Osaka University, Japan). This cell line was maintained on
gelatinized 10 cm dishes (BD Bioscience, Bedford, Mass.) in
OP9 growth medium consisting of αMEM (INVITROGEN,
Carlsbad, Calif.), supplemented with 20% defined fetal
bovine serum (FBS; HyClone Laboratories, Logan, Utah).
Sterile, recombinant, endotoxin and pyrogen-free SCF,
FLT3-L, TNF-α, IL-4 were obtained from PeproTech (Rocky
Hill, N.J.), GM-CSF from Berlex Laboratories (Richmond,
Calif.) and IFN-α from Schering Corporation (Kenilworth,
N.J.). The following mouse anti-human mAbs without detect-
able cross-reactivity with murine cells have been used for
flow cytometric analysis: CD1a-PE, CD34-PE, CD11b-FITC,
CD16-PE, CD33-FITC, CD80-PE, CD86-PE, HLA-DR-PE,
myeloperoxidase (MPO)-FITC, terminal deoxynucleotidyl
transferase (TdT) FITC (CalTag, Burlingame, Calif.); CD9-
PE, CD14-FITC, CD40-PE, CD43-FITC, CD45-PE, CD209
(DC-SIGN)-FITC, CLA-FITC (BD Pharmingen); CD41e-
PE, CD34-PerCP-Cy5.5 (Becton Dickinson Immunocyto-
metry Systems [BDIS], San Diego, Calif.); CD83-FITC, CD208
(DC-LAMP; Beckman Coulter, Miami, Fla.); CD123-FITC
(Miltenyi Biotec, Auburn, Calif.); HLA-ABC-FITC
(Sigma, St. Louis, Mo.); CD207 (Vector Laboratories).

Hematopoietic Differentiation of Human ES Cells in Co-
Culture with OP9 Cells.

The induction of human ES cells differentiation into
hematopoietic cells was done as previously described, Vodya-
by reference. Briefly, undifferentiated human ES cells were
harvested by treatment with 1 mg/ml collagenase IV (INVITI-
ROGEN) and added to OP9 cultures at approximate density
of 1.5×10^5/20 ml per 10 cm dish in aMEM supplemented with
10% FBS (HyClone) and 100 µM Methyl β-D-thiogalacto-
pyranoside (MTG) (Sigma, St. Louis, Mo.). Human ES cell/
OP9 co-cultures were incubated for 9-10 days with a half
medium change on days 4, 6, and 8 without added cytokines.
The human ES cells then differentiated into hematopoietic
cells.

Generation of Human ES Cell-Derived Dendritic Cells.

A schematic diagram of the protocol used for generation of
dendritic cells from human ES cells is depicted in FIG. 1. On
day 9-10 of human ES cell/OP9 co-culture, differentiated
derivatives of human ES cells were harvested by treatment
with collagenase IV (INVITROGEN; 1 mg/ml in αMEM) for
20 min at 37° C., followed by treatment with 0.05%
Trypsin-0.5 mM EDTA (INVITROGEN) for 15 min at 37° C.
After trypsin inactivation by FBS, these cells were re-sus-
pended in α-MEM supplemented with 10% FBS (HyClone) and
100 ng/ml GM-CSF, and transferred into tissue culture
flasks (BD Bioscience) coated with poly 2-hydroxyethyl
methacrylate (HEMA, Sigma) to prevent cell adherence. The
cells were then cultured for 8-10 days with a half medium
change every fourth day to expand dendritic cells. To
evaluate the effect of SCF on FLT3-L, on the expansion of
these human ES cell-derived dendritic cell precursors, we
cultured the cells in the presence of (1) 100 ng/ml GM-CSF
+20 ng/ml SCF; (2) 100 ng/ml GM-CSF+50 ng/ml FLT3-L;
or (3) 100 ng/ml GM-CSF+20 ng/ml SCF+50 ng/ml FLT3-L.
Subsequently, the cells were spun over 20% PERCOLL
(Sigma) to remove dead cells and cell aggregates. As a third
step, PERCOLL-isolated cells were cultured for 7-9 days in
HEMA-coated flasks in StemSpan® serum-free expansion
medium (SFEM; Stem Cell Technologies, Vancouver,
Canada) supplemented with lipid mixture 1 (Sigma) and 100
ng/ml GM-CSF, with the addition of the following cytokines:
(1) 100 ng/ml IL-4, (2) 20 ng/ml TNF-α, (3) 10^4 U/ml IFN-α,
and (4) 100 ng/ml IL-4+20 ng/ml TNF-α Cells were cultured
for 7-9 days with a half medium change every fourth day. To
further mature dendritic cells, we cultured the cells obtained
Cells were prepared in PBS-FBS (PBS containing 0.05% sodium azide, 1 mM EDTA, and 2% FBS), supplemented with 2% normal mouse serum (Sigma), and labeled with a combination of mAbs. Samples were analyzed using a FACScan (Becton Dickinson) with CellQuest (BD) acquisition software. List mode files were analyzed by FlowJo software (Tree Star, Inc., Ashland, Oreg.). Control staining with appropriate isotype-matched control mAbs (BD Pharmingen) was included to establish thresholds for positive staining and background linear scale mean fluorescence intensity (MFI) values. The percentage (%) of positive cells was calculated as % of positive cells stained with specific mAb-MFI of cells stained with corresponding isotype control. MFI was calculated as MFI of cells stained with specific mAb-MFI of cells stained with corresponding isotype control. Linear scaled MFI was used as an indicator of relative antigen density on given cells.

Clonal Progenitor Cell Assay

Hematopoietic clonogenic assays were performed in 35 mm low adherent plastic dishes (StemCell Technologies) using a 1 ml/dish of MethoCult™ GM with H4435 semisolid medium (StemCell Technologies) consisting of 1% methylcellulose, 30% FBS, 1% BSA, 50 ng/ml SCF, 20 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), 20 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml granulocyte colony stimulating factor (G-CSF), and 3 units/ml erythropoietin. All clonogenic progenitor assays were performed in duplicates and CFCS were scored after 14-21 days of incubation according to their colony morphology as erythroid (E-CFC), granulocyte-macrophage (GM-CFC), granulocyte (G-CFC), and macrophage (M-CFC). The frequency of CFCS was calculated per 10^5 total cells.

Allogeneic Mixed Lymphocyte Reaction (MLR)

Adult mononuclear cells were isolated from peripheral blood samples obtained from healthy laboratory volunteers by density gradient centrifugation on Histopaque-1077. Mononuclear cord blood cells were also purchased from Cambrex Bio Science (Walkersville, Md.). The mononuclear cells were depleted of monocytes by plastic adherence and used as responder cells. Graded numbers (1x10^3 to 3x10^5/well) of irradiated (35 Gy) stimulatory cells were co-cultured with 1x10^5 responder cells for 6 days in 96-well flat bottom plates (Corning) in RPMI 1640 containing 5% human AB serum (Sigma). [3H]thymidine (Sigma) was added (1 µCi/well) during the last 16 hours of incubation. Cells were harvested onto glass fiber filters and incorporation of [3H]thy­midine was measured by scintillation counting.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is understood that certain adaptations of the invention are a matter of routine optimization for those skilled in the art, and can be implemented without departing from the spirit of the invention, or the scope of the appended claims.

### TABLE 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Relative Cell Yield%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>8.8 ± 4.4</td>
</tr>
<tr>
<td>Step 2</td>
<td>5.5 ± 3.7</td>
</tr>
<tr>
<td>Step 3</td>
<td>3.3 ± 4.1</td>
</tr>
</tbody>
</table>

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

### TABLE 2

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 3</th>
<th>Step 3</th>
<th>Step 3</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GM-CSF + IL-4</td>
<td>GM-CSF + IL-4 + TNF-α</td>
<td>GM-CSF + IL-4 + TNF-α</td>
<td>GM-CSF + IL-4 + TNF-α</td>
<td>GM-CSF + IL-4 + TNF-α</td>
</tr>
<tr>
<td>R1 gated cells</td>
<td>NA</td>
<td>58.8 ± 12.3</td>
<td>45.5 ± 12.1</td>
<td>46.7 ± 14.9</td>
<td>39.9 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>CD1a</td>
<td>3.3 ± 2.1</td>
<td>82.9 ± 12.4</td>
<td>66.9 ± 24.0</td>
<td>78.2 ± 7.7</td>
<td>30.3 ± 27.1</td>
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</tr>
<tr>
<td>AMFI</td>
<td>750.2 ± 700.7</td>
<td>74.8 ± 60.8</td>
<td>148.3 ± 161.9</td>
<td>77.1 ± 72.1</td>
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<tr>
<td>CD14</td>
<td>12.6 ± 7.1</td>
<td>25.6 ± 7.5</td>
<td>71.1 ± 12.2</td>
<td>39.0 ± 19.3</td>
<td>19.8 ± 15.1</td>
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<tr>
<td>AMFI</td>
<td>14.7 ± 4.2</td>
<td>27.6 ± 15.5</td>
<td>55.3 ± 38.1</td>
<td>31.5 ± 29.0</td>
<td>40.7 ± 50.8</td>
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<tr>
<td>CD16</td>
<td>&lt;1</td>
<td>87.6 ± 7.7</td>
<td>&lt;2</td>
<td>84.7 ± 4.2</td>
<td>17.3 ± 15.4</td>
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<tr>
<td>AMFI</td>
<td>460.3 ± 352.0</td>
<td>213.8 ± 160.1</td>
<td>40.2 ± 39.1</td>
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<td></td>
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</tr>
<tr>
<td>CD83</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
<td>CD11c</td>
<td>60.0 ± 14.2</td>
<td>94.1 ± 5.3</td>
<td>98.0 ± 1.6</td>
<td>93.7 ± 3.3</td>
<td>91.0 ± 8.5</td>
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<tr>
<td>AMFI</td>
<td>132.1 ± 59.9</td>
<td>282.3 ± 37.2</td>
<td>202.3 ± 19.8</td>
<td>237.6 ± 17.8</td>
<td>97.4 ± 41.8</td>
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<tr>
<td>CD11b</td>
<td>59.4 ± 13.1</td>
<td>67.4 ± 29.0</td>
<td>48.8 ± 24.9</td>
<td>56.0 ± 5.4</td>
<td>59.6 ± 8.4</td>
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</tr>
<tr>
<td>AMFI</td>
<td>69.3 ± 23.0</td>
<td>52.0 ± 33.6</td>
<td>24.6 ± 14.3</td>
<td>47.9 ± 32.5</td>
<td>40.1 ± 53.5</td>
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</table>
### TABLE 2-continued

Phenotypic analysis of DCs induced by different cytokine combinations*

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 3</th>
<th>Step 3</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GM-CSF</td>
<td>IL-4</td>
<td>GM-CSF</td>
<td>TNF-α</td>
<td>IL-4 + TNF-α</td>
</tr>
<tr>
<td>CD123 %</td>
<td>35.5 ± 14.6</td>
<td>58.8 ± 12.3</td>
<td>63.5 ± 16.6</td>
<td>45.1 ± 7.9</td>
<td>29.4 ± 18.6</td>
</tr>
<tr>
<td>AMFI</td>
<td>27.8 ± 15.2</td>
<td>35.9 ± 14.6</td>
<td>28.3 ± 12.6</td>
<td>33.9 ± 20.1</td>
<td>18.9 ± 15.3</td>
</tr>
<tr>
<td>HLA-ABC</td>
<td>76.0 ± 8.8</td>
<td>90.3 ± 8.4</td>
<td>91.8 ± 4.1</td>
<td>84.8 ± 5.3</td>
<td>99.2 ± 6.9</td>
</tr>
<tr>
<td>HLA-DR %</td>
<td>14.9 ± 12.0</td>
<td>90.1 ± 6.3</td>
<td>90.1 ± 4.1</td>
<td>82.1 ± 8.0</td>
<td>89.4 ± 7.7</td>
</tr>
<tr>
<td>CD80 %</td>
<td>35.1 ± 3.1</td>
<td>93.4 ± 3.5</td>
<td>85.4 ± 7.3</td>
<td>90.1 ± 2.9</td>
<td>82.4 ± 14.1</td>
</tr>
<tr>
<td>CD86 %</td>
<td>60.2 ± 24.3</td>
<td>1767.4 ± 1122.3</td>
<td>138.5 ± 94.6</td>
<td>439 ± 131.0</td>
<td>125.3 ± 107.2</td>
</tr>
<tr>
<td>CD80 %</td>
<td>7.9 ± 7.8</td>
<td>81.2 ± 21.8</td>
<td>84.8 ± 10.7</td>
<td>81.8 ± 11.6</td>
<td>81.6 ± 19.3</td>
</tr>
<tr>
<td>CD40 %</td>
<td>4.6 ± 4.4</td>
<td>46.4 ± 16.9</td>
<td>43.3 ± 23.7</td>
<td>57.0 ± 1.6</td>
<td>53.9 ± 26.8</td>
</tr>
<tr>
<td>HLA-DR %</td>
<td>621.2 ± 402.9</td>
<td>1289 ± 80.4</td>
<td>2958 ± 353.7</td>
<td>610 ± 13.2</td>
<td></td>
</tr>
<tr>
<td>Cell subset</td>
<td>Step 2</td>
<td>Step 3</td>
<td>Step 3</td>
<td>Step 3</td>
<td>Step 3</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>IL-4</td>
<td>GM-CSF</td>
<td>TNF-α</td>
<td>GM-CSF + IL-4 + TNF-α</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IFN-α</td>
</tr>
</tbody>
</table>

*Results are mean ± SD of 4 to 5 independent experiments; for step 3 cultures % and AMFI of HLA gated cells calculated.

### TABLE 3

Antigen-presenting capacity of HLA-derived DCs*

<table>
<thead>
<tr>
<th>T Cells</th>
<th>DC</th>
<th>CMV</th>
<th>Proliferation (cpm)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>652 ± 129</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>17225 ± 579</td>
<td>224 ± 26.7</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>20303 ± 1279</td>
<td>326 ± 11.8</td>
</tr>
</tbody>
</table>

*HLA-A02 HLA-derived dendritic cells (cells obtained in step 3 with GM-CSF + IL-4) incubated overnight with or without CMV virus and then added to the HLA-A0201 restricted allogeneic T cell clone with specificity to CMV pp65. Results expressed as a mean ± SD of triplicates.

We claim:

1. A culture of human regulatory dendritic cells in which majority of the cells express CD1a low, CD80<sup>hi</sup>, CD86<sup>hi</sup>, CD9<sup>hi</sup> phenotype, display diminished ability to induce the proliferation of naive T cells, and have tolerogenic properties.

* * * * *