Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro

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Summary

Background **Differentiated cells derived from pluripotent human embryonic stem (hES) cells offer the opportunity for new transplantation therapies. However, hES cells and their differentiated progeny express highly polymorphic MHC molecules that serve as major graft rejection antigens to the immune system of allogeneic hosts. To achieve sustained engraftment of donor cells, strategies must be developed to overcome graft rejection without broadly suppressing host immunity. One approach entails induction of donor-specific immune tolerance by establishing chimeric engraftment in hosts with haemopoietic cells derived from an existing hES cell line. We aimed to develop methods to efficiently differentiate hES cells to haemopoietic cells, including immune-modulating leucocytes, a prerequisite of the tolerance induction strategies applying to hES cell-mediated transplantation.**

Methods **We developed a method to generate a broad range of haemopoietic cells from hES-generated embryonic bodies in the absence of murine stromal feeder cells. Embryonic bodies were further cultured in the presence of haemopoietic cytokines. In addition to flow cytometric analyses of haemopoietic cell markers, we analysed the hES cell-derived haemopoietic cells by colony-forming assays (for erythroid and myeloid progenitor cells), cytochemical staining, and mixed leucocyte reactions to determine the functional capacity of the generated antigen-presenting cells.**

Findings **12 independent experiments were done. When selected growth factors were added, leucocytes expressing CD45 were generated and released into culture media for 6–7 weeks. Under the condition used, both erythroid and myeloid progenitor cells were generated. About 25% of the generated leucocytes acquired MHC class II and costimulatory molecule expression. These hES-derived, MHC class II+ leucocytes resembled dendritic cells and macrophages, and they functioned as antigen-presenting cells capable of eliciting allogeneic CD4 and CD8 T-cell responses in culture.**

Interpretation **The hES cell-derived antigen-presenting cells could be used to regulate alloreactive T cells and induce immune tolerance for improvement of the transplant acceptance of hES-cell derivatives.**

Introduction

Human embryonic stem (hES) cells are pluripotent diploid cells that can proliferate in culture indefinitely.[1,2] From these cells we might be able to develop new transplantation therapies to replace diseased or aged cells or tissues.[1–5] To this end, researchers need to develop methods with which they can derive from hES cells their required cell types, such as cardiomyocytes or haemopoietic cells, and overcome immune-mediated rejection when these cells are transplanted into hosts that are genetically different (allogeneic) from the hES cell line in use. The HLA system has a central role in the initiation and development of immune rejection. The most important genes are *HLA-A*, *HLA-B*, and *HLA-C* (collectively called class I) and *HLA-DR* and *HLA-DQ* (class II).[6] Class I genes are expressed in virtually all somatic cells whereas expression of class II genes is restricted largely to cells of the immune system, such as dendritic cells, macrophages, and other antigen-presenting cells.[7]

MHC molecules are essential in antigen-specific immune activation or tolerance induction because they bind antigenic peptides and present them to a specific T-cell receptor complex. MHC class I molecules preferentially present antigens to CD8+ cytotoxic T cells, whereas CD4+ helper T cells preferentially recognise peptides presented by MHC class II molecules.[7] The peptides presented by MHC complex molecules can come from an external source—eg, viral proteins—but mostly they are derived from endogenous proteins encoded by either the nuclear or mitochondrial genome. MHC-restricted antigen presentation by antigen-presenting cells provides the first signal needed to stimulate a specific clone of naive T cells and determines response specificity. However, the ultimate outcome of immune response—either sustained activation (immunogenicity) or tolerance induction—is dictated by other signals from complex interactions between antigen-presenting cells and T cells of various developmental or physiological types. During normal maturation of the haemopoietic and immune systems, tolerance develops to self proteins or antigens. Highly polymorphic MHC molecules (in unmatched allogeneic donor cells) are themselves major foreign antigens presented either directly by donor or indirectly by host antigen-presenting cells, and alloreactive T cells confronting non-identical HLA molecules proliferate vigorously leading to donor cell rejection. Therefore, the

matching of MHC molecules (HLA-A, B, C, DR, and DQ) is necessary to minimise immune rejection. However, polymorphisms in many other non-HLA endogenous proteins still provide sources of minor histocompatibility antigens, including highly polymorphic mitochondrial and H-Y gene products, resulting in rejection even in HLA-matched individuals.[8]

Currently, immunosuppressive drugs such as ciclosporin are administered to transplant recipients to prevent acute and chronic immune-mediated rejection of allogeneic bone marrow and organ transplants even with best possible MHC matching. These drugs, which inhibit all types of T cells non-specifically, result in many side-effects, particularly after long-term use. Fortunately, new developments with haemopoietic chimerism to induce immune tolerance have helped us to greatly reduce or avoid allogeneic rejection responses.[8,9] These new strategies include use of haemopoietic stem cells that could reset or reconstitute the haemopoietic and immune systems in myeloablated or non-myeloablated hosts, use of immature dendritic cells or other types of antigen-presenting cells to induce tolerance, and use of transduced dendritic cells with specific genes that will specifically kill or inactivate alloreactive T cells.[7–9] The unique properties of ES cells (unlimited growth in culture and pluripotency) allow exploration of new strategies to engineer ES cell-derived donor tissues matching those of the recipients. Several strategies that potentially improve hES cell transplant acceptance have been proposed[10,11] but they all have their advantages and disadvantages. First, we could generate parthenogenetic hES cells if they can be produced from oocytes, but this strategy is restricted to premenopausal females. Second, creation of a patient-specific hES cell by individual somatic cell nuclear transfer into enucleated oocytes (therapeutic cloning) is possible but the presence of the mitochondrial genome in oocytes can contribute minor polymorphic rejection antigens, and the current protocol is far from efficient.[12] Third, to achieve best possible MHC matching we could establish large banks of HLA-defined and highly diversified hES cell lines, but this strategy might not be sufficient since minor rejection antigens, are still present and difficult to define. Finally, we could establish immune tolerance (after maximum MHC matching between the patient and an ES cell line in hES cell banks) by preinjection or coinjection of haemopoietic cells derived from the donor ES cells. This approach is based on the idea that we can induce immune tolerance to donor-specific antigens by donor haemopoietic cells and promising results in combined allogeneic haemopoietic cell and organ transplantations.[7–9]

We aimed to develop methods to efficiently differentiate hES cells to haemopoietic cells, including immune-modulating leucocytes, a prerequisite of the tolerance induction strategies applying to hES cell-mediated transplantation.

Methods

Cell culture and differentiation

We cultured the H1 hES cell line (passage 22; WiCell Research Institute, Wisconsin, MI, USA) on primary mouse embryonic fibroblasts that we used as feeder cells. We used karyotypically normal hES cells (passage 30–80). The procedure for karyotyping and culturing H1 hES cells on primary mouse embryonic fibroblasts or human feeder cells has been previously described.[13] In brief, the hES cell culture medium consists of 80% (v/v) knockout DMEM (Dulbecco's modified eagle medium), 20% (v/v) of the knockout serum replacement, 2 mmol/L L-glutamine, 0·1 mmol/L non-essential aminoacids (all from Invitrogen, Carlsbad, CA, USA), 0·1 mmol/L β mercaptoethanol (Sigma, St Louis, MI, USA), and 4 μg/L basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA).

Before differentiation, we passaged hES cells at a high density (1/1 to 1/3 split ratios) onto Matrigel (Becton Dickson Labware, Bedford, MA, USA), as previously described.[14] After reaching the full size or confluency, hES cell colonies were incubated with dispase (0·2 g/L, Invitrogen) at 37°C for 45–60 min. Under this condition, we lifted ES colonies intact after digestion and separated them away from the residual feeder cells. We resuspended the harvested hES cell colonies in the hES cell medium in the absence of basic fibroblast growth factor; we also added fetal bovine serum (20% final; StemCell Technologies, Vancouver, Canada). To prevent cell attachment to plastic and to induce formation of embryonic bodies, we cultured ES colonies in ultralow-attachment plates (Corning Costar, Cambridge, MA, USA). About 0·1–0·5 million ES cells were incubated in every well of six-well plates and formed 20–30 embryonic bodies. Cystic embryonic bodies emerged after 5–20 days in suspension cells. When harvested at day 10–20, about 50–80% embryonic bodies were cystic (on average every embryonic body contained about 10 000 cells).

To generate a broad range of haemopoietic cells, including dendritic cells and other antigen-presenting cells, we adapted a protocol developed previously for mouse ES cells.[15] We transferred whole embryonic bodies formed in suspension onto tissue culture plates (without dispersion) and allowed them to differentiate into haemopoietic and other cell types in fetal bovine serum-containing media. This medium contained 80% knockout DMEM, 2 mmol/L glutamine, 0·1 mmol/L β mercaptoethanol, 0·1 mmol/L non-essential aminoacids, and 20% fetal bovine serum. Furthermore, to stimulate production of haemopoietic progenitor cells and dendritic cells, we added: stem cell factor (100 μg/L), FLT3 ligand (50 μg/L), and thrombopoietin (20 μg/L), which are cytokines widely used to maintain human postnatal haemopoietic stem cells and to expand committed progenitor cells;[16] interleukin 3 (20 μg/L); granulocyte-macrophage

colony-stimulating factor (100 μg/L); and, to enhance possible maturation of lymphoid cells and dendritic cells, we added interleukin 4 (20 μg/L).[7] We purchased all cytokines from Peprotech.

Flow cytometric analysis

We harvested undifferentiated adherent hES cells and adherent cells in haemopoietic cell cultures with non-enzymatic cell dissociation solution (Invitrogen). Cells in embryonic bodies were digested with 0·4 U/mL collagenase B (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 37°C for 2 h. We stained harvested suspension or adherent cells with antigen-specific monoclonal antibodies or their isotype controls (mouse IgG1 or IgG2a). The following R-phycoerythrin-conjugated monoclonal antibodies were used for flow cytometric analysis: antibodies against HLA-DR, CD3, CD5, CD14, CD19, CD33, CD83, CD80, and CD86 (Becton Dickinson PharMingen, San Diego, CA, USA); glycophorin A (Immunotech); and CD2, CD7, CD22, CD31, CD34, and CD45 (Caltag Laboratories, Burlingame, CA, USA). In two-colour (antigen) analyses, we used fluorescein-isothiocyanate (FITC)-conjugated CD45 or its isotype (mouse IgG1) control antibody (Caltag) in conjunction with a phycoerythrin-conjugated antibody.

We did fluorescence-activated cell sorter (FACS) analysis with a FACScan or FACSCalibur analyser (Becton Dickinson). We set up the machine such that 10 000 events (cells) were counted and analysed as a test population. The fluorescence intensity (reflecting antigen density on cell surface) from the binding of a specific antibody (conjugated with a fluorochrome) is recorded for each of 10 000 cells in four orders of magnitude. In histograms, cell numbers are plotted as a function of variable fluorescence intensities. The percentage of antigen-expressing cells with specific fluorescence signals above background (≤1% of 10 000 collected events) is automatically calculated by the FACS machine with BD CellQuest Pro software (Becton Dickinson). In dot plots (fluorescence intensities of a given antigen *vs* that of the second antigen or side scatter), every dot represents one of 10 000 cells (or in proportion) analysed automatically by the machine. Since the denominator is 10 000 events, the SE is small.

Cell assays

For haemopoietic colony-forming assays, single cells from either non-adherent (suspension) or adherent cell fractions were plated in methylcellulose media (Marrow-Gro, Quality Biological, Gaithersburg, MD, USA), as previously described.[16] We plated up to 5×10^4 cells in every 35-mm plate in duplicate. After 14 days of culture, we counted colonies resembling either erythroid burst colony-forming units or granulocyte-monocyte colony-forming units. We assessed morphological differentiation of colony-derived progeny on cytospin slides by standard Wright-Giemsa staining or by a modified version with Diff-quick staining kit (Fisher Scientific Company, Swanee, GA, USA). We viewed the cells under a ×100 oil immersion lens. Similarly, to identify different cell types we stained cells harvested

Marker (other names)	Major presence on postnatal human cells	Undifferentiated hES cells*	Cells within embryonic bodies†	Haemopoietic progeny‡
CD2	T and natural killer cells, certain blood myeloid cells	–	N/D	+ (27–37%)
CD3	T cells	–	N/D	–
CD14	Monocytes and macrophages	–	N/D	+ (up to 20%)
CD16	Neutrophils; natural killer cells	–	N/D	+ (up to 15%)
CD19	B cells	–	N/D	–
CD31 (PE-CAM)	Haemopoietic and endothelial cells	–	+	+ (up to 10%)
CD33	Myeloid cells	–	N/D	+ (up to 69%)
CD34	Stem/progenitor cells; endothelial cells	–	+ (5–10%)	+ (up to 3%)
CD40	Antigen-presenting cells such as B cells, dendritic cells, and macrophages	–	N/D	+ (~15%)
CD45 (LCA)	Pan leucocytes	–	–	+ (>90%)
CD56 (N-CAM)	Natural killer cells; neutrophils; neural cells	–	N/D	–
CD80 (B7.1, costimulatory molecule)	Antigen-presenting cells such as dendritic cells, B cells, and macrophages	–	N/D	+ (~16%)
CD83	Mature dendritic cells	–	N/D	+ (8%)
CD86 (B7.2, costimulatory molecule)	Antigen-presenting cells such as dendritic cells, B cells, and macrophages	–	N/D	+ (~30%)
CD90 (Thy-1)	Stem/progenitor cells; thymocytes; non-haemopoietic cells	+	N/D	N/D
CD105 (endoglin)	Haemopoietic and endothelial cells; fibroblasts	–	N/D	N/D
CD117 (C-KIT/SCFR)	Stem/progenitor cells; mast cells	+	+	N/D
CD133 (AC133)	Stem/progenitor cells	+	N/D	N/D
VEGFR2 (FLK1, KDR)	Stem/progenitor cells; endothelial cells	+	N/D	N/D
MHC I (HLA-ABC)	All nucleated cells	+	+	+ (100%)
MHC II (HLA-DR)	Antigen-presenting cells	–	–	+ (~25%)

N/D=not determined. *FACS analysis of undifferentiated (SSEA4+) hES cells. –=<2% cells display signals above background. Non-enzymatic or trypsin/EDTA mediated cell dissociation methods gave similar results. †FACS analysis or section staining when cystic embryonic bodies were first seen. ‡FACS analysis of non-adherent cells harvested at various timepoints from our haemopoietic cultures.

Table: **Expression of cell surface markers on undifferentiated hES cells and their haemopoietic progeny**

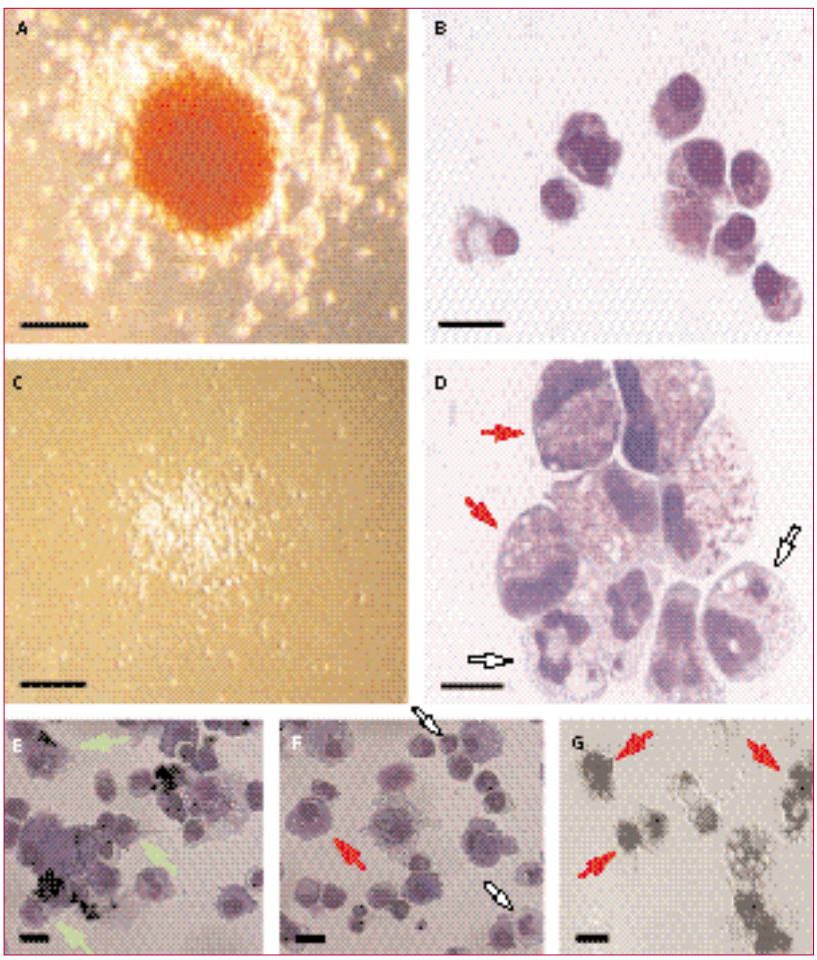

Figure 1: **Photomicrographs of colony-forming progenitor cells and other hES-derived haemopoietic cells**
(A) Erythroid burst colony-forming units. (B) Diff-quick stained individual erythroid burst colony-forming units containing nucleated, polychromophilic, or orthochromophilic erythroblast cells typical of those seen in cells derived from bone marrow.[18] (C) Myeloid colony. (D) Diff-quick stained myeloid colonies containing monocytes (red arrows) and granulocytes (white arrows). (E and F) Wright-Giemsa staining of non-adherent haemopoietic cells. Green arrows indicate cells resembling dendritic cells, red arrows point to macrophage-like cells, and white arrows show cells resembling granulocytes. (G) α naphthyl acetate esterase staining for macrophages. Positive staining for this enzyme is seen in the cytoplasm containing vacuoles . Most cells did not stain positively. Scale bars: 200 μm in A and B; 20 μm in C–G.

from suspension cultures by Wright-Giemsa staining on cytospin slides. The presence of macrophages was further confirmed by their expression of α naphthyl acetate esterase with a staining kit (procedure 91; Sigma).

Differentiated haemopoietic cells obtained from ES cell derivatives in suspension were further cultured for 4 days with granulocyte-macrophage colony-stimulating factor (150 μg/L) and interleukin 4 (20 μg/L) in RPMI-1640 medium with 10% fetal bovine serum. Harvested cells were briefly activated by tumour necrosis factor α (20 μg/L) and prostaglandin E2 (10 mg/L) for 4 h, and they were irradiated at 30 Gy to block cell proliferation. We used these cells as stimulators in mixed leucocyte reaction assays to measure antigen-presenting cell-dependent allogeneic T-cell proliferation, as previously described.[16] Briefly, peripheral blood mononuclear cells from healthy adult donors were seeded (2×10^5 cells per well, constant) in 96-well plates as responders, with serially diluted hES cell progeny as stimulators (stimulator/responder ratios were 1/8, 1/16, and 1/32) in replicates (n=3–5) in every experiment with mononuclear cells from different donors. As control antigen-presenting cells we used human cord-blood leucocytes, undifferentiated hES cells, and embryonic body-derived hES cells before haemopoietic differentiation as stimulators after irradiation. In selected experiments, purified human CD4+ or CD8+ T cells (10^5 per well) were used to replace mononuclear cells as responders (n=3). With the MACS CD4 or CD8 T-cell purification systems (Miltenyi Biotec, Auburn, CA, USA) more than 99% of purified cells expressed CD4 or CD8 (from about 45% and 15%, respectively, in total mononuclear cells). After 3 days of coculture, we pulsed cells with 1 μCi per well of ^{3}H-thymidine and harvested them 18–20 h later with a Packard Micromate cell harvester (Packard BioScience, Meriden, CT, USA). We measured ^{3}H-thymidine incorporation (counts per minute, cpm) with a Packard Matrix 96 direct β counter, as previously described.[16]

Statistical analysis

We used Microsoft Excel (version X for Macintosh) software for data management (calculation of mean and SD), scatter plots, and Student's *t* tests (two-sided, unequal variance). When the number of replicates (n) was small (n=3–5) in every group in individual experiments (not pooled data from several similar experiments), we used the non-parametric Mann-Whitney test (also known as Wilcoxon two-sample test). This test was run unsupervised with the SAS 8.2 package (SAS Institute, Cary, NC, USA). We judged findings to be significant if p (null hypothesis) was 0·05 or less by both tests.

Role of the funding source

The sponsor had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit for publication.

Results

The table summarises the expression of 21 surface markers on undifferentiated hES cells. Some commonly used markers associated with haemopoietic progenitor cells and committed lineages were also expressed in undifferentiated hES cells, including Thy-1/CD90 and MHC class I. However, CD45 (leucocyte common antigen), CD34, and MHC class II (such as HLA-DR) were not expressed in undifferentiated hES cells defined as SSEA4+ cells. We therefore chose acquired expression of these markers (such as CD45 and MHC class II) to monitor differentiation towards haemopoietic lineages.

Embryonic bodies attached firmly to culture plates within 2 days. During the next 2 weeks, many types of adherent cells proliferated rapidly then migrated out and formed adherent cells surrounding embryonic bodies. After 5 days, haemopoietic-like cell clusters emerged on the edge of the attached embryonic body cell masses, apparently in tight association with the newly formed adherent cells. These clusters were similar to those seen in the mouse ES cell system and to the early events of haemopoietic differentiation from rhesus monkey ES cells.[15,17] Most of these haemopoietic cell-like clusters disappeared from adherent cell layers in the following 5 weeks after they reached a diameter of 0·5 mm. Eventually, these adherent blastic cells disappeared and most probably moved into suspension in a manner typical of fetal and postnatal haemopoietic cells.

Within 7–10 days after embryonic bodies were plated, haemopoietic-like cells started to appear in suspension. Most non-adherent cells appeared as single cells, but small cell clusters with dendritic appearance were also visible. These non-adherent cells were collected weekly (week 2–6); mean total number of harvested cells was 2·32 million per well (SD 0·80; n=4), containing about 40 embryonic bodies. Starting at week 2 when sufficient numbers of cells were available, both non-adherent and adherent cell fractions were analysed by colony-forming assays (figure 1) and FACS analysis for haemopoietic marker expression (figure 2). Observed erythroid colonies resembled erythroid burst colony-forming units; granulocyte-monocyte colony-forming units were also detected in both non-adherent and adherent cell fractions (figure 1). At all timepoints, more granulocyte-monocyte colony-forming units (4–5 fold) were seen than erythroid burst colony-forming units (data not shown). In one representative experiment, frequencies of total colony-forming cells (mean [SD], n=2) measured every week were: 35·5 (1·5), 18·5 (1·5), 14 (2), 32 (2), and 10 (1) per 10^5 non-adherent cells; and 77 (2), 49 (1), 18 (0), 15 (1), and 2 (0) per 10^5 adherent cells; for weeks 2–6, respectively.

Figures 2 and 3 show FACS analyses of non-adherent and adherent cells harvested at week 2. Nearly all

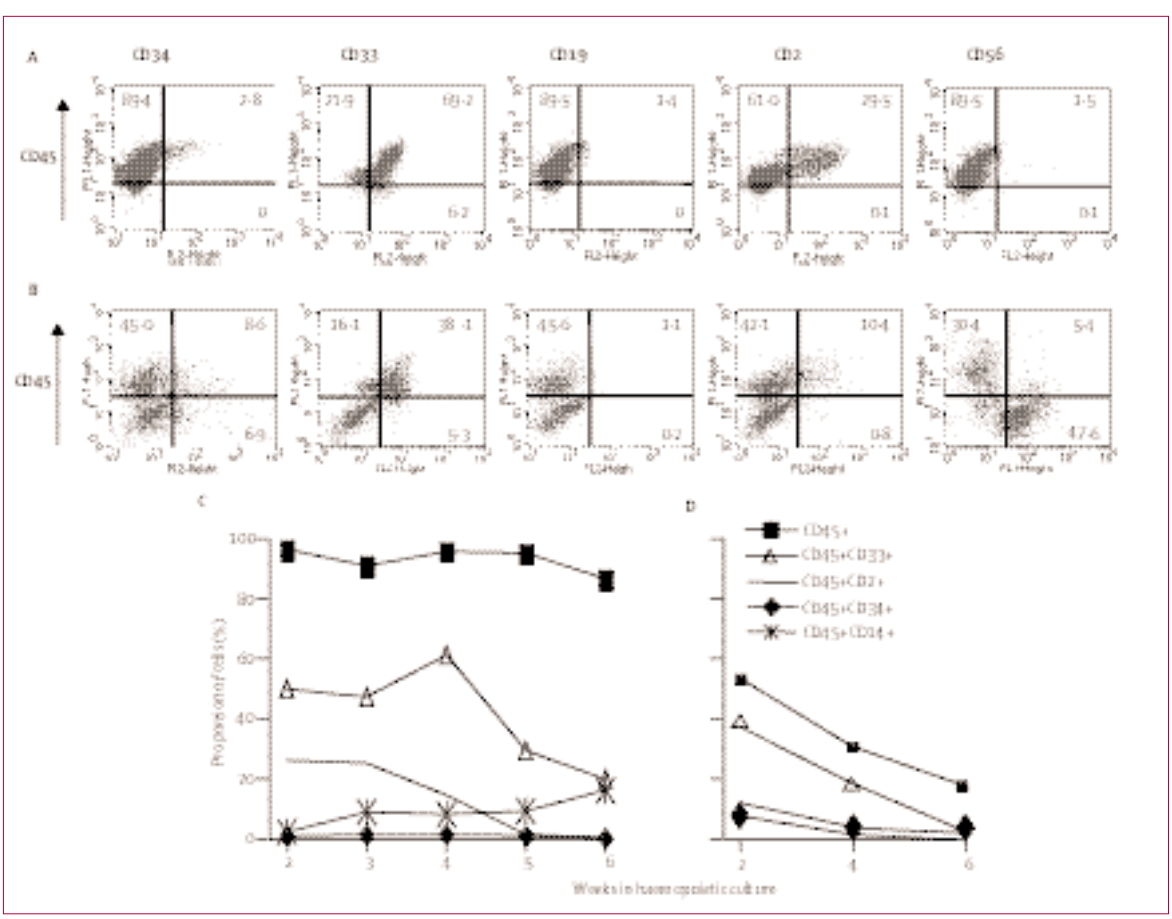

Figure 2: **FACS analysis of multiple types of hES-derived haemopoietic cells**
Non-adherent (A) or adherent cells (B) harvested at week 2. Committed haemopoietic cells were identified by CD45 (*y* axis) together with other lineage markers (*x* axis), indicated at the top of every panel. Background (≤1%) was identified by staining with isotype-matched control antibodies. Percentages of positively stained cells are based on 10 000 cells counted automatically by the machine. Percentages of cells in (C) non-adherent and (D) adherent cell fractions at week 2 and later timepoints.

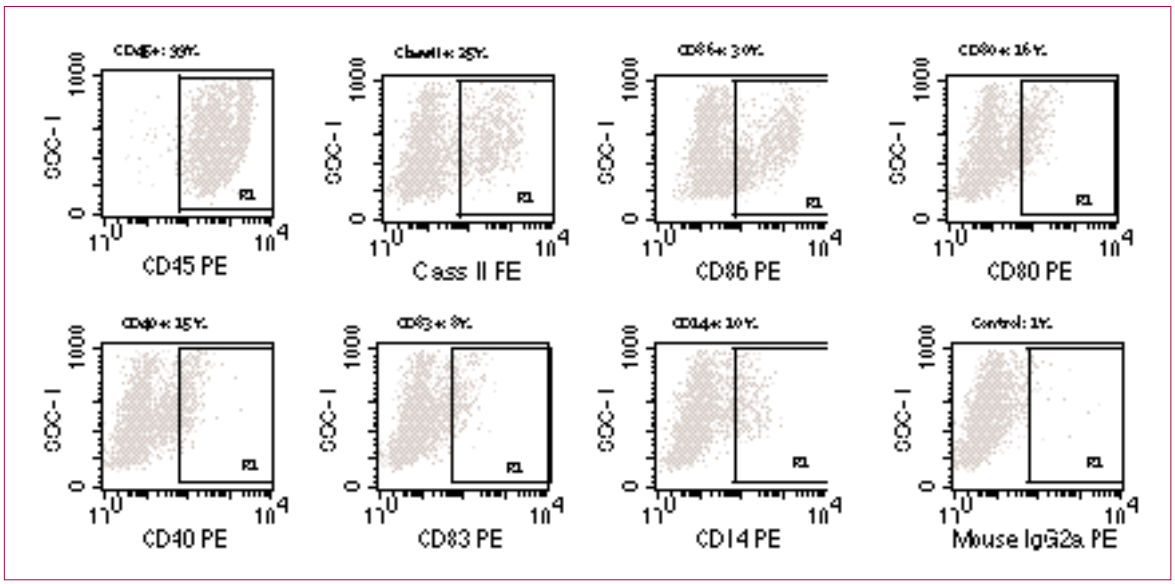

Figure 3: **FACS analysis of ES-derived leucocytes after stimulation**
Side scatter (SSC-H; *y* axis) was plotted against expression of various leucocyte markers (*x* axis). The positive signal above background (shown in the lower right corner) was gated (R1) accordingly. Percentage of positively stained cells (in R1, out of 10 000 cells counted) is denoted at the top of every panel.

non-adherent cells were CD45+ (96%), confirming their haemopoietic nature. Notably, 53·6% of adherent cells were also CD45+, indicating that many haemopoietic cells were also present after non-adherent cell harvest. More CD45+CD34+ (haemopoietic stem and progenitor) cells were recorded in the adherent cell fraction (8·6%) than in the suspension (non-adherent cell) fraction (2·8%) at week 2. The percentages of these cells in the non-adherent and adherent cell fractions fell to background levels (≤1%) at weeks 3 and 6, respectively (figure 2). CD34+CD45– cells were also noted in the adherent fraction, which were probably endothelial cells; these cells remained during the analysis period (data not shown).

CD33+CD45+ cells (characteristic of myeloid lineages) were readily detectable in both the non-adherent and adherent cell fractions (figure 2) as were low percentages (about 5%) of CD14+CD45+ cells (data not shown). Under the culture conditions, non-significant numbers of B lymphoid (CD19+) cells were detected in each fraction. Unexpectedly, CD45+ cells coexpressing CD2 (a marker usually seen on T cells and natural killer lymphoid cells) were detected in both fractions (figure 2). The reproducible presence of high percentage (27–37%) CD2+ non-adherent cells in every experiment (n=5) prompted us to further determine whether they could be lymphocytes. However, the isolated non-adherent cells did not express other lymphoid markers such as CD3, CD5, and CD7 (data not shown). Although about 50% of CD2+ cells expressed CD16 (in both natural killer cells and granulocytes) they did not have other markers associated with natural killer cells such as CD56 (figure 2) and CD94 (data not shown). Since CD2+ monocytes and dendritic cells are reported in blood, CD2 is no longer regarded as an exclusive lymphocyte (natural killer and T) cell marker.[19,20] These data are consistent with the notion that CD2+CD16+ cells are probably myeloid in origin.

At later timepoints of haemopoietic differentiation (weeks 3–6), more non-adherent cells than adherent cells expressed CD14 (figure 2) and MHC class II and costimulatory markers such as CD80 or CD86 (see below). The MHC class II complex is selectively expressed in such antigen-presenting cells as dendritic cells, macrophages, and B cells, whereas nearly all nucleated cells express MHC class I. Since we did not detect (CD19+) B cells, we directly analysed the presence of macrophages and dendritic cells in hES-derived leucocytes found in suspension. Wright-Giemsa staining revealed the presence of many cell types, including morphologically distinct cells resembling dendritic cells, macrophages, and granulocytes (figure 1). The presence of macrophages was further confirmed by cytoplasmic expression of α naphthyl acetate esterase (figure 1).

We further analysed the non-adherent cell population after brief activation of dendritic cells and antigen-presenting cells with tumour necrosis factor α and prostaglandin E2. About 25% of cells expressed moderate to high amounts of MHC class II (HLA-DR) and the costimulatory molecule CD86 (figure 3). Cells expressing CD40 (a marker for antigen-presenting cells), CD83 (a dendritic cell marker), or CD14 (a macrophage and monocyte marker) with a high side scatter were also detected (figure 3), confirming the findings on Wright-Giemsa staining that dendritic cells

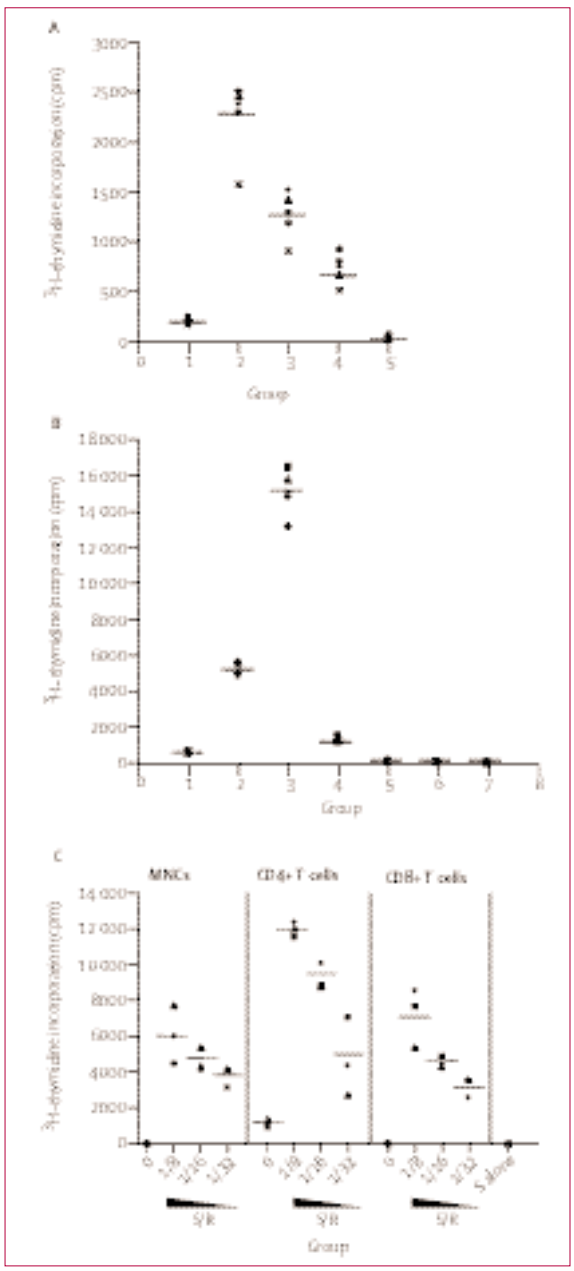

Figure 4: **Mixed leucocyte reaction assays**
(A) 2×10^5 human blood leucocytes (responders [R], n=5) seeded without (1) or with (2–4) hES cell-derived haemopoietic cells used as antigen-presenting cells (stimulators [S]) to stimulate T-cell proliferation. S/R ratios (2–4) were 1/8, 1/16, and 1/32, respectively. (5) Maximum number of S (25 000 cells, irradiated and mitotically inactive) seeded alone as a negative control. Horizontal bar=mean value. For all comparisons, p=0·0122, by Mann-Whitney test. (B) Blood leucocytes (R, n=5) seeded alone (1) or with irradiated antigen-presenting cells (S; S/R=1/8) in groups (2–4). (2) hES-derived haemopoietic cells, as above; (3) allogeneic blood leucocytes from cord blood; (4) non-haemopoietic progeny of hES cells. (5-7) Corresponding unseeded cells in (2–4). For all comparisons, p=0·0122, by Mann-Whitney test. (C) Purified CD4+ or CD8+ T cells (10^5 cells per well, n=3) used as responders (R), in comparison with total mononuclear cells (MNCs, 2×10^5 cells per well). Stimulators (S) are hES cell-derived haemopoietic cells as antigen-presenting cells with different responders (R). S/R ratios: 0 (R alone), 1/8, 1/16, and 1/32. S alone: irradiated antigen-presenting cells (maximum dose, 25 000 cells).

and macrophages were present (figure 1). However, most dendritic cells and macrophages seemed immature or not fully activated (figure 3).[7]

To test whether these hES cell-derived haemopoietic cells consisting of MHC class II+ cells could indeed function as antigen-presenting cells, we did mixed leucocyte reaction assays to measure (allogeneic) T lymphocyte reaction. The non-adherent haemopoietic derivatives of hES cells were briefly activated, irradiated, and used as antigen-presenting cells (stimulators) in mixture with blood leucocytes as a source of responding T cells (figure 4). Dose-dependent proliferation of responding T cells was recorded as ^{3}H-thymidine incorporation. The hES cell-derived haemopoietic cells (about 25% expressing MHC class II) stimulated T-cell proliferation significantly at a dose as low as 1/32 (figure 4); activities of the hES-derived leucocytes harvested at weeks 3–5 were similar (data not shown). However, the antigen-presenting cell activities were always lower (2–5-fold) than those of irradiated postnatal blood leucocytes from three different donors (figure 4).

We also tested antigen-presenting cell activities of undifferentiated hES cells or their progeny after differentiation mediated by embryonic bodies. Differentiated hES cells before the directed haemopoietic differentiation step showed a low but detectable amount of antigen-presenting cell activity (figure 4); undifferentiated cells did not show activity (data not shown). We next did the same assay with purified CD4 or CD8 T cells that require antigen-presenting cells expressing MHC class II or class I antigen complexes, respectively. Figure 4 shows the results of a representative experiment using the same batch of hES-derived haemopoietic derivatives as antigen-presenting cells and 10^5 CD4 T cells, 10^5 CD8 T cells, or 2×10^5 unfractionated mononuclear cells (containing about 45% CD4 and 15% CD8 T cells) as responders. Highly purified CD4 T cells seemed to show a greater stimulation in response to hES-derived antigen-presenting cells than CD8 T cells and T cells in mononuclear cell mixtures (figure 4).

Discussion

We have provided strong evidence that hES cell-derived leucocytes can function as antigen-presenting cells and directly stimulate allogeneic CD4 and CD8 T cells. We also have extended previous analysis of haemopoietic markers expressed on the H1 hES cell line.[21–23]

Kaufman and colleagues[21] provided evidence of haemopoietic differentiation from hES cell lines by showing the formation of erythroid and myeloid progenitor cells in culture. Our method generated multiple lines of haemopoietic cells from hES cells in culture without use of exogenously added stromal feeder cells of either animal or human origin. The absence of feeder cells makes easy not only counting and analysis of hES cell derivatives but also study of growth factor

requirements of hES cell-initiated haemopoiesis. The absence of murine stromal cells further reduces the risk of rodent pathogen transmission to hES cell derivatives generated in coculture, especially when they are destined for clinical use. In our culture system, human adherent cells generated from cystic embryonic bodies seemed to be sufficient in the presence of haemopoietic cytokines. This finding accords with those of a study to derive haemopoietic cells after embryonic body formation.[24] The developmental stages of haemopoietic differentiation from hES cells are similar to those shown with mouse ES cells with the embryonic body formation approach.[3,15]

Similar to findings of previous studies,[21,24] we noted the formation of erythroid and myeloid progenitor cells in our hES cell differentiation system. Moreover, we also saw the generation of immune-modulating leucocytes such as dendritic cells and macrophages. About 25% of cells in suspension acquired expression of MHC class II and expressed CD80 or CD86 costimulatory molecules. More macrophages than dendritic cells were present in this population, based on both morphological and FACS analyses. Collectively, these hES cell-derived leucocytes functioned as antigen-presenting cells in stimulation of purified allogeneic CD4 and CD8 T cells in mixed leucocyte reaction assays. Our culture system of hES cell-initiated haemopoiesis provides a foundation for future improvements to study early developmental events of human blood and immune cell formation. Our findings also serve as a step to investigate induction of immune tolerance with hES cell-derived haemopoietic cells.

In addition to their uniqueness as a model system to study human cell biology and immunology, differentiated cells derived from pluripotent hES cells offer the opportunity for new transplantation therapies. To achieve sustained engraftment of hES-derived donor cells, strategies must be developed to overcome graft rejection without broadly suppressing host immunity. Creation of a patient-specific ES cell line by undertaking patient's somatic cell nuclear transfer is now feasible[12] but inefficient, maybe insufficient, and associated with ethical concerns. An alternative approach to avoid graft rejection entails induction of donor-specific immune tolerance to cells and tissues from a selected hES cell line. To achieve the best graft acceptance we should select from hES cell banks a cell line, the HLA type of which is the closest possible match to that of the patient. Since multiple cell types could be derived from the same ES cell line, to derive MHC-identical haemopoietic cells (for tolerance induction) and a second (therapeutic) tissue such as pancreatic islet cells or cardiomyocytes is possible.

Haemopoietic cells are fairly easy to engraft after intravenous injection and can induce immune tolerance and form stable chimerism in allogeneic but MHC-matched hosts.[8,9,25] Because multiple cell types derived from one hES cell line are genetically identical (including HLA genes), engraftment of the ES cell-derived haemopoietic cells might lead to induction of immune tolerance and permit better subsequent graft acceptance of the second cell type.[10,11] This approach is not specific to HLA types of individual patients or hES cell lines, and has been used in transplantation of postnatal tissues or organs.[8–11,25] It is also substantiated by findings with rat ES-like cells that MHC class II+ leucocytes were generated in vivo and induced long-term acceptance of cardiac graft (with a haplotype identical to the rat ES-like cells) in a fully MHC-mismatched recipient rat.[26]

However, undifferentiated hES cells do not express MHC class II and could not function as antigen-presenting cells to stimulate T cells. Commitment to haemopoietic differentiation is required to obtain MHC class II expression and raised antigen-presenting cell activities in stimulation of T cells in culture. Because undifferentiated hES cells can form tumour (teratoma) and lack antigen-presenting cell activity, direct injection of hES cells into recipients to induce immune tolerance is not a safe approach. For safe and effective induction, we will probably need to differentiate hES cells towards haemopoietic commitment in vitro before transplantation. In the future, we will need to test our ability to generate transplantable antigen-presenting cells or haemopoietic stem cells (that subsequently generate antigen-presenting cells in vivo after transplantation) from differentiated hES cells.

Contributors

X Zhan and G Dravid contributed equally to this work. X Zhan and L Cheng initiated the project. G Dravid was responsible for cell analysis and antigen-presenting cell functional assays. Z Ye and H Hammond provided support at every stage of this project. M Shamblott contributed analysis of cell types within embryonic bodies. J Gearhart provided insight into many issues of ES cell transplantation. L Cheng conceived and supervised the project, and wrote the report.

Conflict of interest statement

All authors are employees of Johns Hopkins University and declare no conflict of interest.

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