Indirect immune recognition of mouse embryonic stem cell–derived hematopoietic progenitors in vitro

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The clinical use of embryonic stem cell (ESC)–derived hematopoietic progenitors (ESHPs) requires the generation of ESHPs that produce mature hematopoietic cells and do not induce immune rejection after transplantation. We compared the developmental maturity and immunogenicity of ESHPs generated using two methods: embryoid body (EB) formation and culture of ESCs with the OP9 bone marrow stromal cell line (ESC-OP9). ESHPs derived from EBs displayed an immature hematopoietic phenotype and were devoid of immunogenicity marker expression. In contrast, ESHPs derived via ESC-OP9 displayed a mature phenotype and expressed high levels of some immunostimulatory molecules. ESHPs alone could not stimulate CD4+ T lymphocyte proliferation directly. However, preferential phagocytosis of ESHPs and T cell proliferation were observed in the presence of antigen-presenting cells, consistent with a model of indirect immune recognition of ESHPs. These results suggest that depletion of host CD4+ T lymphocytes or antigen-presenting cells may be necessary for successful ESHP transplantation.

Recent advancements in embryonic stem cell (ESC) culture methods have resulted in the generation of specific tissue types in vitro. Despite these advancements, increasing the efficiency of production, developmental compatibility, and survival of ESC-derived tissues after transplantation must still be achieved [1]. ESCs and their derivatives can be recognized by the immune system in transplanted hosts [2–8]. However, a gap exists in the knowledge of the mechanisms by which ESC-derived tissues are recognized by the adult immune system.

In adults, hematopoietic stem cells (HSCs) produce all cells of the blood and the immune system. HSC transplantation (HSCT) is used in the clinic for the treatment of immunodeficiencies, autoimmune disease, and leukemia. In addition to its use as a blood replacement therapy, HSCT also has been used as a means to induce immunologic tolerance to donor antigens across allogeneic barriers [9]. If ESC-derived hematopoietic progenitors (ESHPs) derived in vitro are functionally similar to adult HSCs and if the host immune system will accept them, it is possible that the same benefits of HSCT transplantation can be achieved with ESHPs.

No single universally accepted culture protocol to generate ESHPs currently exists [10], and it is unclear whether the ESHPs generated by different methods will be equivalent in function, viability, and immunogenicity. In this study, we hypothesized that the culture method used to generate ESHPs would result in differences in the emergence of mature ESHPs, as well as the expression of cell surface proteins involved in immune system recognition and activation. In addition, we tested the ability of ESHPs to stimulate syngeneic and allogeneic T lymphocytes in vitro. Using phagocytosis and antigen presentation assays, we confirmed that indirect presentation by macrophages is a pathway by which ESHP antigens can stimulate adult T lymphocytes.

Experimental procedures

ES cell line maintenance and culture
Mouse ESC-D3 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were expanded and maintained on mitomycin C-treated STO cell (mSTO) monolayers in DMEM (Invitrogen, Carlsbad, CA,
USA) media containing 15% ES-qualified fetal bovine serum (FBS; Gemini Bio Products, West Sacramento, CA, USA), 0.15 mmol/L monothioglycerol (Sigma-Aldrich, St. Louis, MO, USA), 1x penicillin–streptomycin (Pen/Strep; Invitrogen) and 1000 U/mL leukemia inhibitory factor (LIF; Millipore, Billerica, MA, USA) to prevent differentiation, as described [11,12]. ESCs were passaged every 2 to 3 days by trypsinization of colonies and replating 0.5 × 10^6 ESCs onto new mSTO cell monolayers. Culture media was changed daily. Before differentiation, 0.5 × 10^6 ESCs were transferred to 0.1% gelatin coated plates, and cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) media containing the same supplements and LIF as listed above. Cells were incubated in a humidified incubator at 37°C with 5% CO₂.

**ES cell differentiation**

For formation of ESHPs via EBs [11,13], ESCs were plated in a pluronic acid–coated 60-mm petri dishes or six-well plates in IMDM media containing 1% methylcellulose (Fluka, Germany), 15% FBS, 2 mmol/L glutamine, 200 μg/mL transferrin, 0.5 mmol/L ascorbic acid (Sigma-Aldrich), 0.45 mmol/L monothioglycerol and 1x penicillin–streptomycin. ESCs were plated at a density of 5,000–20,000 cells/ml. EBs were fed on days 5 and 10.

For formation of ESHPs by the ESC-OP9 method, OP9 cells (ATCC) were cultured to 80% confluency in α-MEM media (Invitrogen) containing 20% FBS (Atlanta Biologicals, Norcross, GA, USA) and 1x penicillin–streptomycin. ESCs were initially plated on the OP9 monolayers at 500 or 867 ESCs/mm², in 100- or 150-mm–diameter tissue culture dishes, respectively, in the presence of Flt3L and IL7. Cell cultures that required more than 7 days of differentiation were transferred onto new OP9 monolayers on day 7 and were fed on days 8.

**Mice**

The UC Merced Institutional Animal Care and Use Committee approved all animal procedures. Mice of the 129 and BALB/c strains were purchased from The Jackson Laboratory. Mice were housed in specific pathogen-free conditions with autoclaved food and sterile water. To obtain embryonic tissues, timed matings of breeder mice were set up overnight, with embryonic day 0.5 indicating the observation of vaginal plugs. Embryo stages were confirmed by comparison to staging criteria [15].

**Flow cytometry**

Embryoid bodies were harvested and digested in collagenase media, which is composed of M199 containing 0.125% w/v Collagenase D and 0.1% v/v DNase I (both from Roche) for 60 min at 37°C, followed by dissociation with a syringe and a 21G needle. Cells were washed with an equal volume of M199+ Media, which consists of 2% FBS in Medium 199 (Invitrogen), and centrifuged at 400 rpm for 10 min. After dissociation, cells were filtered through nylon mesh with a pore size of 64 μm (Small Parts Inc., Logansport, IN, USA). Cells from ESC-OP9 cultures were harvested from plates by vigorous pipetting, collagenase digestion, or both. The yolk sac (YS), placenta (PL), and fetal liver (FL) were removed from mouse embryos as described previously [16]. Embryonic tissues were digested in collagenase media for 60 min at 37°C. The bone marrow from tibiae and femora and spleen from the adult mother was also collected, as described [17].

A maximum of 10⁶ viable cells was aliquoted for staining. Cells were stained at 4°C for 30 min in 2.4G2 (anti-CD16/CD32) hybridoma supernatant to block Fc receptors. Cells were then stained with specific antibody cocktails for 30 min in a total volume of 100 μL. Antibody-specific staining was determined using isotype-matched control antibody staining, fluorescence minus-one controls, or comparison to unstained cells.

Cells were sorted or analyzed on BD FACS Aria II or LSR II flow cytometers. Live, singlet cell populations were gated based on light scatter properties and low 4,6-diamino-2-phenylindole (DAPI) staining. Flow cytometry data analysis was performed using FlowJo (Tree Star, Ashland, OR, USA). For functional assays, CD41⁺ ESHPs were first enriched with a “yield” sort followed by one to two “purity” sorts, or were enriched with three rounds of positive selection using the EasySep Positive Selection Kit (Stemcell Technologies, Vancouver, BC, Canada) followed by a purity sort on the FACS Aria II.

**One-way mixed lymphocyte reaction assays**

Spleens from BALB/c (H-2b) and 129 (H-2b) mice were harvested and gently crushed with the base of a 5-mL syringe. Red blood cells were lysed using ACK buffer. BALB/c responder splenocytes were washed twice in 1x PBS at 37°C and resuspended at 5 × 10^6 cells/mL in a 15-mL conical tube. One μL of 5-mmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes), diluted in DMSO, was added per milliliter of splenocytes. Cells were then incubated for 10 min at 37°C. CFSE labeling was quenched by washing the cells in DMEM containing 5% FBS at 37°C and centrifuging at 2000 rpm for 10 min. Cells were then incubated for an additional 30 min at 37°C and washed twice in warm media. Responder cells were plated at 2 × 10⁵ cells per well. Antigen-presenting cells (“stimulators”) were irradiated with 2000 rad with a 137Cs irradiator (J. L. Shepherd and Associates, San Fernando, CA, USA) and plated at 10⁴ cells per well. This dose was experimentally determined to be the lowest possible number of stimulator cells from which a statistically significant allogeneic response could be detected (Supplementary Figure E1, online only, available at www.expem.org). Ten thousand FACS-sorted CD41⁺ ESHPs from ESC-OP9 day 16 cultures were added to the
appropriate culture wells. Mixed lymphocyte reactions were analyzed after 5 days of incubation.

For analysis, all cells were harvested and incubated with 2.4G2 supernatant for 20 min before staining. Cells were then washed and stained with CD4 APC-Cy7 (clone GK1.5) (BioLegend, San Diego, CA, USA). Live CD4+ cells were gated and analyzed for cell division via their CFSE fluorescence intensity. CFSE^high cells were considered to be undivided, whereas cells with low to intermediate CFSE fluorescence intensity (CFSE^low/int) were considered to have divided. The percentage of stimulated responder cells in each mixed lymphocyte reaction (MLR) culture was calculated as follows:

%CD4+ cells that divided =
\[
\frac{\%CD4^+ \text{CFSE}^{\text{low/int}}}{\%CD4^+ \text{CFSE}^{\text{high}} + \%CD4^+ \text{CFSE}^{\text{int}}}
\]

The stimulation index (SI) for each MLR culture was calculated as follows:

\[
\text{SI} = \frac{\%CD4^+ \text{cells that divided}}{\%CD4^+ \text{background response}}
\]

“Background response” was equated to the percentage of CFSE^low/int+ CD4+ cells that were observed in response to syngeneic stimulator cells alone. One-tailed Student t test assuming unequal variance between groups was performed with Microsoft Excel, and differences were considered statistically significant if p ≤ 0.05.

In vitro phagocytosis assays

Spleens were harvested from BALB/c mice, and single-cell suspensions were prepared. Macrophages were stained with anti-F4/80-biotin (clone BM8, BioLegend), and were isolated using three rounds of positive selection with the EasySep Biotin Positive Selection Kit (StemCell Technologies) to a purity greater than 95%. Twenty thousand F4/80^+ macrophages were plated per well. T cells were isolated from additional BALB/c spleens using Mouse CD4^+ T Cell Enrichment Columns (R&D Systems) and then labeled with CFSE as described earlier and plated at 1 × 10^5 CD4^+ T cells per well. Ten thousand ESHPs and Lin^- BM cells were added to a specific well. Anti-MHC II (3.75 μg, clone 14-4-4S; eBioscience, San Diego, CA, USA) was added to respective wells to block indirect antigen presentation by macrophages to the T cells. After 4 days, cells were harvested, blocked with 2.4G2 supernatant, and stained with anti-CD4 APC (RM4.5; BioLegend) and DAPI as a viability marker. Cells were analyzed by flow cytometry as described in the previous section, One-way mixed lymphocyte reaction assays.

Results

ESHPs generated using OP9 coculture display a mature hematopoietic progenitor cell surface phenotype

To determine the hematopoietic progenitor phenotype of ESHPs, we used EB and ESC-OP9 methods and analyzed them on days 7 and 16 of culture. These cultures exhibited distinct morphologies, as expected [18] (Fig. 1A). ESHPs display a “hybrid” intermediate phenotype between adult and fetal hematopoietic stem cells [19,20]. CD41 has been established as the first marker of embryonic hematopoiesis and is progressively downregulated concomitant with increased expression of CD45 [21–24]. CD41 is then re-expressed in the adult and is a marker of myeloid-based hematopoietic stem cells [25]. We used the prescribed developmental pattern of CD41 and CD45 expression to compare directly the maturation kinetics of ESHPs generated using EB and ESC-OP9 culture methods, and to compare this to hematopoietic progenitors in primary embryonic and adult tissues [20,24]. ESHPs from EBs displayed low expression of CD41 on day 7, which remained relatively constant as cultures progressed to day 16, and CD45 expression remained low (Fig. 1B [top row] and 1C). ESHPs from ESC-OP9 cocultures transitioned from the CD41^-CD45^ population at day 7 to CD41^-CD45^ and CD41^+CD45^- populations by day 16 (Fig. 1B [middle row] and 1C). This pattern of development resembled that found in hematopoietic populations transitioning in the yolk sac, fetal liver, and bone marrow.
Figure 1. Effect of culture method on ESHP developmental kinetics and yield. (A) Morphologic differences between EB (right) and ESC-OP9 (left) cultures at day 7 (upper panels) and day 16 (lower panels). (B) CD41 and CD45 expression on ESHPs generated via EB (top row) and ESC-OP9 (middle row) from days 7 to 16. Expression in embryonic and adult hematopoietic tissues is also shown (bottom row). See also Supplementary Figure E2 (online only, available at www.exphem.org). (C) Percentages of CD41⁻CD45⁺, CD41⁺CD45⁻, and CD41⁺CD45⁺ ESHP populations in EB and ESC-OP9 cultures and adult bone marrow. Results from day 7 (D7) and day 16 (D16) cultures represent the average percentages of nine independent experiments. Higher percentages of CD41⁻CD45⁺ populations (light grey bars) and CD41⁺CD45⁻ populations (dark grey bars) were observed in D16 ESC-OP9 cultures compared with D16 EBs (p < 0.05).
(Fig. 1B, bottom row). CD41⁺ ESHPs did not express the lineage-specific markers CD4, CD8, CD11b, Gr1, Ter119, CD19, F4/80, or DX5 (data not shown).

The ESC-OP9 method produced 7.12-fold higher percentages of CD41⁺CD45⁺ ESHPs and 3.02-fold higher percentages of CD41⁻CD45⁺ ESHPs than the EB method at day 16 (< 0.05; Fig. 1C). CD41⁺ ES-HP populations sorted from cultures demonstrated clear multilineage hematopoietic potential in colony-forming assays (Supplementary Figures E2C and E2D, online only, available at www.exphem.org). However, ESHPs generated via ESC-OP9 produced significantly more colonies than those from EB cultures (Supplementary Figure E2C, online only, available at www.exphem.org). Transplantation of day 16 ESC-OP9 ESHPs into immune-compromised hosts demonstrated short-term hematopoietic engraftment in the bone marrow with multilineage potential (data not shown).

We further analyzed ESHPs for the expression of genes and proteins that are expressed in adult HSCs and these data are shown in Supplementary Figure E2 (online only, available at www.exphem.org). CD150 and CKIT are two biomarkers that are highly expressed on long-term HSCs in the adult mouse [26]. Cd150 messenger RNA (mRNA) expression in ESC-OP9 day 16 ESHPs was higher than that in adult HSCs, and this difference was also detectable by flow cytometry (Supplementary Figures E2A and E2B, online only, available at www.exphem.org). CKIT surface protein expression on CD41⁺ ESC-OP9 day 16 ESHPs was the highest, compared with the other CD41⁺ ESHPs (Supplementary Figure E2B, online only, available at www.exphem.org). However, Ckit mRNA levels did not correspond to the CKIT surface protein levels (Supplementary Figure E2A, online only, available at www.exphem.org). CD34, SCA1, and AA4.1, three other hematopoietic progenitor cell markers [20,27], were expressed on CD41⁺ ESC-OP9 day 16 ESHPs at similar or higher levels, as compared with adult BM cells (Supplementary Figure E2B, online only, available at www.exphem.org). Hoxb4, which encodes for a transcription factor that confers improved HSC self-renewal and differentiation capability in ESCs and adult HSCs [28–30], and Cxcr4, which encodes a chemokine receptor that is involved in HSC migration [29–31] and retention of quiescent adult HSCs in the bone marrow [31–33], were expressed at significantly lower mRNA levels in most ESHPs, regardless of the culture method used to generate them (Supplementary Figure E2A, online only, available at www.exphem.org).

Taken together, these data suggest that the ESC-OP9 method produces ESHPs in sufficient numbers that are more similar in developmental phenotype to hematopoietic progenitor cells in the fetal liver and adult bone marrow.

Distinct patterns of immunogenicity biomarker expression are evident in ESHPs generated by EB versus ESC-OP9 methods

The immunogenicity of undifferentiated ESC and their derivatives has been an active area of research [2,3,5,6,34,35]. However, the effects of different ESC culture methods on the potential immunogenicity of specific ESC-derived cell lineages are still unclear, and they could have implications on the fate of ESC derivatives after transplantation. Given our observation of distinct expression patterns of hematopoietic progenitor cell surface proteins and developmental profiles (Fig. 1 and Supplementary Figure E2 (online only, available at www.exphem.org)), we hypothesized that the culture method or duration of culture could also affect ESHP immunogenicity profiles. To test this idea, we selected a panel of immunogenicity biomarkers and examined their expression on ESHPs by flow cytometry.

Major histocompatibility complex class I (MHC I) is expressed on all mature nucleated cells, and it is a primary molecule used by the immune system to recognize what is “self” versus what is “non-self” by CD8⁺ cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and macrophages [36]. Direct recognition of MHC I by CTL and NK cells could result in CTL/NK cell activation or NK cell inhibition [37,38]. Therefore, patterns of MHC I expression on ESHPs could predict their susceptibility to immune rejection or acceptance after transplantation. We compared MHC I (H-2Kb) expression on CD41⁺ ESHPs from EB and ESC-OP9 cultures (Fig. 2), as well as embryonic and adult hematopoietic tissues (data not shown). H-2Kb expression was undetectable on CD41⁺ ESHPs in the yolk sac of EB day 7 and day 16 cultures (Fig. 2, top row). In contrast, H-2Kb expression was highest on CD41⁺ cells in ESC-OP9 day 16 cultures (Fig. 2). MHC class II is expressed by antigen-presenting cells and is recognized by CD4⁺ T cells, resulting in T cell activation. Expression of MHC class II was not detected on all ESHPs examined (Fig. 2, second row), but was observed on a small population in the adult BM (data not shown), suggesting that ESHPs would not be able to directly present antigens to CD4⁺ T cells. Uregulation of the nonclassical MHC molecule Qa-1b was observed on CD41⁺ ESHPs generated via ESC-OP9 (Fig. 2), but Qa-2, another nonclassical MHC protein, was not expressed on any ESHP population.

CD80 and CD86 are normally expressed on antigen-presenting cells that provide costimulatory signals through CD28 binding to CD40⁺ helper T lymphocytes stimulated by major histocompatibility complex MHC class II (MHC II) through the T cell receptor (TCR) [39,40]. CD86 has also been described recently as a marker expressed on adult HSCs with lymphopoietic potential [41]. Expression of CD80 and CD86 was not detected on EB-derived CD41⁺ ESHPs to any significant degree (Fig. 2, fifth and
Figure 2. Expression of markers of immunogenicity on ESHPs. Analysis of electronically gated CD41\(^+\) cells generated in EB or ESC-OP9 cultures are shown. T lymphocyte interacting markers include MHC I, MHC II, CD80 and CD86, and NK receptor ligands are Qa-1b, Qa-2, RAE-1, H60 or MULT-1. Antigen-specific staining (black lines) and isotype control staining (grey shaded regions) are shown as overlays. See also Supplementary Figure E3 (online only, available at www.exphem.org).
sixth rows). In contrast, CD80+ cells were evident in CD41+ ESHPs generated by ESC-OP9 culture on day 7, but were not evident at day 16 (Fig. 2, fifth row). Reciprocally, CD86 expression was not observed on ESHPs in day 7 ESC-OP9 cultures, but was significantly increased in day 16 ESC-OP9 cultures (Fig. 2, sixth row).

The high MHC I expression and CD86 on ESHPs from day 16 ESC-OP9 cultures correlated with the expression of CD45, as we observed upregulation of MHC I and CD86 on both CD41+CD45+ and CD41−CD45+ populations, similar to the expression observed on CD45+ subpopulations in the fetal liver (Supplementary Figure E3, online only, available at www.exphem.org).

Self versus non-self recognition by NK cells is mediated by a combination of activating and inhibitory signaling through distinct NK cell receptors [42]. We observed no expression of the NK stimulatory receptor ligands retinoic acid early inducible gene (RAE-1), H60, or murine ULBP-like transcript 1 (MULT-1), in any ESHP population (Fig. 2, seventh to ninth rows). This observation suggests that ESHPs do not initiate NK cell activation using these receptors. Only ESC-OP9-derived ESHPs expressed the NK inhibitory ligand Qa-1b, and ESC-OP9 day 16 ESHPs express high levels of MHC I (Fig. 2).

In summary, the expression patterns of the immunogenicity biomarkers MHC I, CD80, and CD86 on ESHPs are distinct and dependent on the ESC culture method, which could translate into the activation or inhibition of different cells of the immune system, leading to their rejection after transplantation.

**Allogeneic ESHPs cannot stimulate CD4+ T lymphocytes in the absence of mature antigen-presenting cells**

Our results suggested that ESHPs from day16 ESC-OP9 cultures were the most mature hematopoietic progenitors amongst the ESHPs that we generated (Fig. 1 and Supplementary Figure E2, online only, available at www.exphem.org). These ESHPs also expressed high levels of MHC I and CD86 and low levels of MHC II and CD80 (Fig. 2). Based on this expression profile, we hypothesized that ESC-OP9 day16 ESHPs could stimulate proliferation of allogeneic CD4+ lymphocytes. However, the absence of MHC II expression on all ESHPs suggested that ESHPs might not directly stimulate CD4+ T helper cells. To test these hypotheses, we used an adapted version of the classic MLR assay to detect T-lymphocyte responses to allogeneic ESHPs [43]. We isolated CD41+ ESHPs from ESC-OP9 day 16 cultures and added them to MLR assays, either alone or in the presence of irradiated antigen-presenting cells obtained from adult mouse spleens (Fig. 3A and B). Responder cells were naive CFSE-labeled spleen cells (which include CD4+ T lymphocytes) from BALB/c (H-2d) mice, which are allogeneic to the ESC-D3 cell line (which is derived from the 129 mouse strain [H-2b]).
Figure 3. Evidence for T-lymphocyte stimulation by allogeneic ESHPs via indirect recognition. (A) Schematic outline of the MLR experimental assay. (B) Proliferative responses of gated CD4⁺ BALB/c cells, as measured by CFSE dilution. Representative flow cytometry plots of three independent experiments are shown. (C) Stimulation indices (mean ± SEM) of BALB/c CD4⁺ T cell responses to different stimulator cell types, normalized to their response to syngeneic cells. (D) Working model of ESHP immune recognition via indirect recognition. See also Supplementary Figure E1 (online only, available at www.exphem.org). *p < 0.05; ***p < 0.001.
Figure 4. Phagocytosis of ESHPs by macrophages. (A) Graphical representation of the phagocytosis assay. (B) Flow cytometry analysis of gated live F4/80\(^+\) macrophages after incubation with CFSE-labeled targets. CFSE-positive gates were drawn based on the analysis of macrophages incubated without targets (\(^a\)). Concatenated data for replicate samples are shown from one of two independent experiments. (C) Percentages of F4/80\(^+\) macrophages that have phagocytosed either Lin\(^-\) BM or ESHP targets. Horizontal lines indicate the mean value for each group. Error bars represent SEM, and the \(p\) values for each comparison are shown. See also Supplementary Figure E4 (online only, available at www.exphem.org).
of the fluid-phase compartment of the endosome. If green CFSE<sup>+</sup> ESHPs were colocalized in the endosome, they would be visualized as yellow [47]. If cell fusion between macrophages and ESHPs occurred, then the green and blue staining dyes would be diffused in no specific area. ESHPs were observed both completely surrounded by macrophages and colocalized within the fluid phase in the endosome, confirming that the presence of both active phagocytosis and endocytosis of ESHPs, and no nonspecific cell fusion (Supplementary Figure E4, online only, available at www.exphem.org). Taken together, these data indicate that in contrast to adult Lin<sup>−</sup> BM cells, ESHPs are preferentially recognized and phagocytosed by macrophages, and that allogeneic differences further enhance the phagocytic response.

Because we showed that ESHPs were preferentially phagocytosed compared with adult Lin<sup>−</sup> BM cells, we next investigated the ability of allogeneic F4/80<sup>+</sup> macrophages to present ESHP antigens to CD4<sup>+</sup> lymphocytes (Fig. 5). A model of the experimental design and expected results are shown in Figure 5A. When cultured with syngeneic BALB/c F4/80<sup>+</sup> macrophages and allogeneic 129 ESHPs, BALB/c CD4<sup>+</sup> lymphocytes proliferated robustly. In contrast, little T cell proliferation was observed in response to syngeneic BALB/c F4/80<sup>+</sup> macrophages and allogeneic 129 Lin<sup>−</sup> BM cells (Fig. 5B and C). To test whether indirect presentation of ESHPs by BALB/c macrophages resulted in CD4<sup>+</sup> T cell proliferation, we added blocking MHC II (I-E) antibody 14-4-4S [48] to the cultures. T cell proliferation in response to ESHPs was significantly reduced in the presence of blocking MHC II antibody (Fig. 5B and C). In contrast, no change in proliferative response was detected when blocking antibody was added to wells containing Lin<sup>−</sup> BM cells. These data confirm that phagocytosis of ESHPs by macrophages leads to indirect ESHP antigen presentation and T cell stimulation in vitro.

**Discussion**

Our results clearly demonstrate that the ESHPs produced via ESC-OP9 for 16 days are functional hematopoietic progenitors that display many features of adult hematopoietic progenitors in the bone marrow, and are produced in relatively high numbers using this method. Therefore, these ESHPs could be good candidates for an alternative to adult hematopoietic progenitors for therapies. However, ESC-OP9 day 16 ESHPs express immunogenicity proteins MHC I, Qa1-b, and CD86, and they stimulate CD4<sup>+</sup> T lymphocytes, likely via an indirect recognition mechanism. The roles of direct versus indirect recognition of foreign tissues in the acute and chronic rejection of transplanted tissues have been well characterized [49]. “Direct recognition” refers to T cell responses that are initiated by direct binding of the T cell receptor to the allogeneic MHC on the surface of a stimulating cell. In contrast, “indirect recognition” refers to T cell responses that are initiated by the proteolytic processing and presentation of allogeneic antigenic peptides in the groove of MHC II that is expressed on professional antigen presenting cells. Our mixed lymphocyte assay results (Fig. 3) show that T cell responses to allogeneic ESHPs were only significantly higher than background controls when T cells were stimulated with an admixture of ESHPs plus primary syngeneic or allogeneic splenocytes. Our phagocytosis assays suggest that ESHPs are readily phagocytosed by macrophages (Fig. 4), and our antigen-presenting assay results (Fig. 5) demonstrate that CD4<sup>+</sup> T cells recognize allogeneic ESHP antigens using indirect recognition—that is, as antigenic peptides that are processed after phagocytosis of ESHPs by antigen-presenting cells that are then presented on MHC class II for recognition by CD4<sup>+</sup> T cells. The lack of MHC class II expression on ESHPs themselves and the lack of significant CD4<sup>+</sup> T cell stimulation in the presence of allogeneic ESHPs alone also support the idea that indirect recognition is the primary mechanism by which allogeneic ESHPs may be recognized by the immune system [50]. It has been noted that inhibition of indirect recognition pathways can enhance allograft survival in some settings [49]. Therefore, it is possible that immune recognition of allogeneic ESHPs could also be prevented by a similar inhibitory strategy in vivo, and such studies are currently underway in our laboratory.

The mechanism by which macrophages specifically respond to ESHPs but not Lin<sup>−</sup> BM cells is still not understood. Our results suggest that embryonic antigens may be specifically recognized by adult macrophages. In support of this idea, several articles from the 1980s and early 1990s showed a unique relationship between macrophages and embryo-derived teratocarcinoma cells, as well as cells derived from the inner cell mass of the early blastocyst [51–54]. In those studies, the teratocarcinoma cells were taken up live by the macrophages and subsequently killed intracellularly. Although teratocarcinoma cells are not equivalent to ESHPs, we hypothesize that there are some similarities between the two cell types that make them recognizable by macrophages. Our data also suggest that ESC-OP9-derived ESHPs, especially those cultured to day 16, express MHC I and CD86 proteins at levels that could possibly activate CD8<sup>+</sup> T cell responses and inhibit NK cell responses (Fig. 2). These hypotheses have yet to be formally tested, but at least one group has shown that NK cells are a barrier to engraftment of ESC-derived hematopoietic progenitors in adult mice [6]. Our results, in conjunction with recent reports of the immunogenicity of other ESC-derived tissues [55–57], as well as the immunogenicity of induced pluripotent stem cells (iPSCs) [58], bolster the need to screen derivatives of ESCs and iPSCs for their ability to induce an immune response after transplantation.
Figure 5. Indirect antigen presentation of ESHPs by F4/80⁺ macrophages. (A) Graphical representation of the antigen presentation assay. (B) Flow cytometry analysis of gated live CD4⁺ CFSE-labeled T lymphocytes. CFSE⁺ gates were drawn based on unstimulated CD4⁺ T cells (shown in gray). T cell proliferation in cultures without anti-class II blocking antibody (left panels) and with anti-class II antibody (right panels) were compared. Concatenated data for three to four replicate samples from one experiment are shown (heavy black line). (C) Percent of CD4⁺ lymphocytes that have divided in response to antigen presentation by macrophages. The error bars represent SEM, and the p values for each comparison are shown.
Our data clearly demonstrate that the ESC culture method alone can drastically influence the maturity, total cell yield, and potential immunogenicity of the ESHP population that is generated. As new protocols for directed ESC differentiation into specific lineages are generated or optimized, or are applied to distinct ESC or iPSC lines, our results demonstrate that it is important to consider the potential immunogenicity of clearly defined ESC derivatives that are proposed for cellular therapy. We also conclude that ESHPs are preferentially recognized by adult macrophages, in contrast to adult Lin−BM cells. Our results suggest that determination of ESHP immunogenicity profiles, as well as understanding the mechanisms by which embryonic versus adult antigens are recognized by macrophages, may improve the success of ESHP survival and function in vivo after transplant into an adult host.

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Conflict of interest disclosure
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References


Supplemental experimental procedures

Quantitative polymerase chain reaction

Cell pellets were either snap frozen and stored at −80°C or placed directly into RLT buffer (QIAGEN, Hilden, Germany). RNA was prepared using the Rneasy Plus Micro Kit (QIAGEN). cDNA was reverse-transcribed from mRNA using the Superscript III kit with oligo dT primers (Invitrogen). Quantitative polymerase chain reaction (PCR) was performed as described [59]. All adult HSCs (Lineage− Sca-1+ c-Kit+ IL-7Rα−) samples were pooled together to obtain the ΔCt for the calibrator samples, and from this value, the ΔΔCt was calculated for each gene of interest. To determine the fold difference in gene expression relative to the HSCs, 2−ΔΔCt was calculated, and the mean relative fold difference and standard deviation were plotted for each sample group (GraphPad Prism, San Diego, CA, USA). A minimum of two biological replicates were tested in duplicate for each gene. Outliers were removed when the group’s coefficient of variance was greater than 10% [60]. Primer sequences are listed in Table E1. Differences between means was determined by two-tailed t test, assuming unequal variance using Microsoft Excel Software (Microsoft, Redmond, WA), and was considered statistically significant if p < 0.05.

Table E1. PCR primers and sequences

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
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Methylcellulose-based hematopoietic colony forming assays

One thousand sorted ESHPs were added to 1 mL methylcellulose cytokine media in 35mm plates. The methylcellulose cytokine media contained 1% methylcellulose, 15% FBS, 0.2 mg/mL transferrin, 0.01 mg/mL insulin (Sigma-Aldrich), 0.05 μg/mL stem cell factor (Peprotech, Rocky Hill, NJ, USA), 0.01 μg/mL IL-3 (Peprotech), 0.01 μg/mL IL-6 (Peprotech), and 0.003 U/mL erythropoietin (Peprotech) or Methocult GF M3434 (STEMCELL Technologies). Cells were incubated for 12 days, harvested, and cytospun (400 rpm for 8 min at room temperature) onto microscope slides. Slides were fixed for 2 min in ice cold methanol and then dried for 5 min. Slides were then stained with May-Grunwald (Fluka) for 3 min, rinsed with water, and then stained with Giemsa (Fisher Scientific, Waltham, MA, USA) for 20 min. Slides were air-dried and coverslips were mounted using Permount (Fisher Scientific) [61].

Visualization of phagocytosis by fluorescent microscopy

Macrophages were stained with F4/80 Biotin (clone BM8) (BioLegend). Macrophages were isolated using three rounds of positive selection EasySep Bio Selection (STEMCELL Technologies) with a purity of >95%. Four hundred thousand macrophages were plated in 12 well plates with glass coverslips with 1x104 CFSE-labeled ESHPs. Fifty μg of Alexa Fluor 633 hydrazide salt (a fluid phase marker; Life Technologies, Waltham, MA, USA) was added per well [62]. Cells were incubated for 3 hours at 37°C. Cells were washed three times with 1 x PBS, fixed with 4% paraformaldehyde, and stained with Streptavidin conjugated to Pacific Blue (Invitrogen, San Diego, CA, USA) to stain F4/80-positive macrophages. Coverslips were mounted to slides with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA, USA). Cells were imaged using an Olympus BX51 fluorescent microscope with oil immersion.

Supplementary references

60. Strober W. Wright-Giemsa and nonspecific esterase staining of cells. Curr Protoc Cytom. 2001; Appendix 3:Appendix 3D.
Supplementary Figure E1. Experimental determination of the responder to target ratio for the MLR. During optimization of the MLR assay to test responses to ESHPs, it was clear that the number of ESHP stimulators obtained for this assay would be limited (Thompson and Manilay, unpublished results). To determine the lowest number of allogeneic stimulators that could be used to obtain a detectable response, CFSE-labeled splenocytes from B6 (H-2^b) were cultured with specific dilutions of allogeneic irradiated splenocyte stimulators from 129 (H-2^b, major histocompatibility matched, but minor antigen mismatched) and BALB/c (H-2^d, major and minor histocompatibility mismatch). (A) Stimulation indices were determined for each stimulator cell dose and statistical analysis performed, as described in the experimental procedures of the main report. (B) Flow cytometry plots of the CFSE dilution assays at each stimulator dose.
Supplementary Figure E2. Developmental profiles and colony forming ability of ES-HPs. (A) Quantitative PCR analysis of CD150, c-kit, HOXB4 and CXCR4 mRNA expression in ESHPs. Mean relative fold differences in gene expression ± SD in ESHPs compared with adult LSK HSCs are shown, and are representative of two to four independent experiments. Individual p values between samples that demonstrated statistically significant (p < 0.05) differences in expression are shown. (B) Expression of developmental cell surface marker on ESHPs compared with embryonic and adult hematopoietic tissues, as measured by flow cytometry. For analysis, CD41+ cells were gated, and positive marker staining was determined by comparison with unstained controls (consisting of a pool of cells from each tissue type). (C) Results of CFU assays that were seeded with sorted CD41+ ESHPs from day 16 EB and ESC-OP9 cultures, LSK cells from embryonic day 18 FL or LSKs from adult BM. (D) Photos of CFUs generated from day 16 CD41+ ESHPs, as viewed by phase contrast (top), and of CFUs after cytopinning and staining with May-Grunwald/Giesma (bottom). CFU = colony forming unit; FL = fetal liver; LSK = Lineage- Sca1+ c-Kit+; PCR = polymerase chain reaction; SD = standard deviation.
Supplementary Figure E3. Expression of immunogenicity markers during ESHP maturation. Flow cytometric analysis of CD80, CD86, MHC I, and MHC II expression on gated CD41⁺ CD45⁻, CD41⁺ CD45⁺, and CD41⁻ CD45⁺ cells from day 16 ESHPs from ESC-OP9 cultures are shown. Data shown are representative of three to five experiments.
Supplementary Figure E4. ESHPs are phagocytosed by macrophages. BALB/c macrophages were plated onto coverslips with CFSE labeled ESHPs and an Alexa Fluor 633 hydrazide salt (colored red) was placed into the media to label the fluid phase of the endosome. Cells were washed and counter stained with anti-F4/80-biotin followed by Streptavidin Pacific Blue to label macrophages. The arrows show where fluid phase and ESHPs colocalize, indicating macropinocytosis.