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Thompson, Heather Linn

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Immunogenicity of Embryonic Stem Cell Derived Hematopoietic Progenitors

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

In

Quantitative and Systems Biology
with emphases in Stem Cell Biology and Immunology

by

Heather Linn Thompson

Committee in charge:
Dr. David Ojcius, Chair
Dr. Jennifer O. Manilay
Dr. Maria Pallavicini
Dr. Marcos García-Ojeda

2014
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________________________________________________________________
Dr. Jennifer O. Manilay
________________________________________________________________
Dr. Marcos García-Ojeda
________________________________________________________________
Dr. Maria Pallavicini
________________________________________________________________
Dr. David Ojcius, Chair

University of California, Merced
2014
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**List of Abbreviations**

ACK – ammonium-chloride-potassium  
AEC – 3-amino-9-ethylcarbazole  
AGM – aorta-gonad-mesonephros  
APC – antigen presenting cell  
APC – allophycocyanin  
B/c – Balb/c  
B6 – C57Bl/6  
BM – bone marrow  
BMC – bone marrow cells  
BMT – bone marrow transplant  
CAR – CXCL12 abundant reticular cells  
CFSE – carboxyfluorescein diacetate succinimidyl ester  
CFU – colony forming unit  
CLL – clodronate loaded liposomes  
CsA – cyclosporine A  
CTL – Cytotoxic T Lymphocyte  
CXCL12 – chemokine C-X-C motif ligand 12  
D – day of differentiation  
DAPI – 4’6-diamidino-2-phenylindole  
Dc – dendritic cell  
DP – double positive  
E – embryonic day  
EB – embryoid body  
EPOCH – EB derived, passed on OP9 and treated with ectopic Cdx4 and HoxB4  
ESC – embryonic stem cell  
ESC-OP9 embryonic stem cells cocultured on OP9 stromal cells  
ES-HP – embryonic stem cell derived hematopoietic progenitor  
FITC -- fluorescein  
FL – fetal liver  
γc – IL-2 and IL-15 common gamma chain  
G-CSF – granulocyte colony stimulating factor  
GFP – green fluorescent protein  
GM-CSF – granulocyte macrophage colony stimulating factor  
hESC – human embryonic stem cell  
HLH -- hemophagocytic lymphohistocytosis  
HPC – hematopoietic progenitor cell  
HSC – hematopoietic stem cell  
ICM – inner cell mass  
IHC – immunohistochemistry  
IPCC – insulin producing cell clusters  
iPSC – induced pluripotent stem cells
ITAM – immunoreceptor tyrosine based activation motif
ITIM – immunoreceptor tyrosine based inhibitory motif
LIF – leukemia inhibitory factor
LIFR – leukemia inhibitory factor receptor
Lin-BM – Lineage negative bone marrow
LSK – lineage negative, SCA-1+, C-Kit+
LT – long term
NK – natural killer
MHC – major histocompatibility complex
MSC – mesenchymal stem cell
mSTO – mitomycin C treated STO cells
Mφ -- macrophage
mESC – mouse embryonic stem cell
M-CSF – macrophage colony stimulating factor, also known as CSF-1
MIF – macrophage inhibitory factor
MLR – mixed lymphocyte reaction
MTG – monothioglycerol
NK – natural killer
NOD -- Non-Obese Diabetic mice
NSG -- NOD. Severe Combined Immunodeficiency. Common Gamma Chain knockout
PBS – phosphate buffered saline
P-sp – para-aorta-splanchnopleura
PE – R-phycerierythrin
Pen/Strep – Penicillin-Streptomycin
PL – placenta
PLL – phosphate buffered saline loaded liposomes
RAG – recombinase activating gene
RBC – red blood cell
SCID – severe combined immunodeficiency
SP – single positive
ST – short term
TAP -- transporter associated with antigen processing
T-reg – regulatory T cells
TS – trophoblast stem cell
WT – wild type
XEN – extra-embryonic endoderm stem cell
YS – yolk sac
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Curriculum Vitae

Education:

1998-2001 Marian University (formerly Marian College), Indianapolis, Indiana
   Bachelor of Science, Biology

2008-2014 University of California, Merced
   Ph.D. in Quantitative and Systems Biology
   Emphases in Stem Cell Biology and Immunology

Professional Research Experience:

8/2008-Present, University of California, Merced, Natural Sciences
   Thesis Advisor, Dr. Jennifer O. Manilay
   Title: Graduate Student & Ph.D. Candidate
   Fields of Study: Immunology, Stem Cell Biology & Developmental Biology

   PI, Dr. Gabriela Loots
   Title: Biomedical Scientist
   Fields of Study: Developmental Biology & Genomics

   PI, Dr. Lisa Stubbs
   Title: Biomedical Scientist
   Fields of Study: Developmental Biology & Genomics

9/2001-1/2005, University of California, San Francisco
   PI, Dr. Jeffrey A. Bluestone
   Title: Staff Research Associate
   Fields of Study: Immunology & Autoimmune Disease

Teaching:

8/2013-12/2013, University of California, Merced, Natural Sciences
   Microbiology, Biology 120
   Title: Graduate Student Teaching Assistant

8/2008-5/2009, University of California, Merced, Natural Sciences
   Biology 1
   Title: Graduate Student Teaching Assistant
Publications:


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Abstract of the Dissertation

Immunogenicity of Embryonic Stem Cell Derived Hematopoietic Progenitors

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Heather Linn Thompson

Doctor of Philosophy

University of California, Merced, 2014

Dr. David Ojcius, Chair
Dr. Jennifer O. Manilay
Dr. Maria Pallavicini
Dr. Marcos Garcia-Ojeda
Embryonic stem cells (ESCs) have an amazing potential, in that they can be used to generate any cell in the adult human body. The difficulty has been in translating this theoretical potential to practical, clinically relevant therapies. The goal of my work is to identify key immune barriers to embryonic stem cell derived hematopoietic progenitors (ES-HPs) after transplantation. Understanding the mechanisms of this rejection will also be used to improve ES-HP engraftment after transplantation. Cells derived from ESCs, including ES-HPs are often difficult to find, even shortly after transplantation. To determine potential mechanisms of immunogenicity, we compared development and immunogenicity markers on ES-HPs derived using the embryoid body method and coculture on the bone marrow stromal cell line OP9 and compared these to both adult and fetal tissues. From those results we tested the functional immunogenicity of ES-HPs in vitro using mixed lymphocyte reactions, phagocytosis assays, and an antigen presenting cell assay. We transplanted ES-HPs into NSG mice lacking B, T and NK cells to test interactions with host macrophages. To test directly if macrophages were a barrier to ES-HPs, we depleted mice of macrophages with clodronate loaded liposomes prior to transplantation. We found that using the OP9 method generated ES-HPs at day 16, resembled those found in late fetal development through adult bone marrow stages. As our ES-HPs matured they expressed MHC-I, but not MHC-II. The expression of costimulatory molecules CD80 and CD86 was temporally dependent on ES-HPs. NK activating ligands were not expressed on ES-HPs. Macrophages inhibitory ligands CD47 and CD200 had low to no expression on ES-HPs. In vitro assays of day 16 ES-HPs from the OP9 method revealed they could stimulate T cells in the context of F4/80+ macrophages and that this stimulation was dependent on host MHC-II. Macrophages preferentially phagocytosed ESCs and ES-HPs compared to lineage negative bone marrow. After in vivo transplantation into NSG mice, ES-HPs achieved a low level of chimerism. However, there was an increase in weight in comparison to untransplanted NSG and bone marrow transplanted controls. This increase could not be accounted for by contributions of ES-HP derived cells. Instead we found a significant increase in host F4/80+ macrophages. Depletion of macrophages improved chimerism of ES-HPs in the spleen and we also observed an increase in chimerism in the bone marrow, but it did not reach statistical significance. From our work, we can conclude that 1) ES-HPs interact with the adult host immune system, 2) ES-HP transplantation increases the number of host derived F4/80+ macrophages in the spleen and 3) Host F4/80 macrophages preferentially phagocytose ES-HPs compared to lineage negative bone marrow. This suggests that macrophages are a barrier to ES-HP engraftment in adult host. This mechanism through which ES-HPs are rejected is distinct from the mechanisms that reject adult bone marrow. Importantly, this research could provide clues about how stem cell and fetal cell populations interact with the adult immune system and could lead to conditioning therapies appropriate for these unique rejection mechanisms. Consequently, this research could therefore improve the therapeutic potential of stem cell therapies for use in regenerative medicine.
Chapter 1: Introduction

The potential of embryonic stem cells (ESCs) for therapeutic uses is extraordinary [1]. Their potential stems from their ability to differentiate into the three basic germ layers (ectoderm, endoderm, mesoderm) and consequently into any of the two hundred cell types present in the adult human body. Since ESCs can undergo self-renewal indefinitely, they represent an unlimited supply of cells [1-3]. Currently, there are many challenges for translating the basic biology of these cells into clinically relevant therapies (Fig 1.1).

1.1 Overview of Early Cell Fate Decisions, Embryonic Stem Cells, and Their Relatives

By the blastocyst stage, (3.5D in mouse) three distinct cell types are already present: trophectoderm and the epiblast containing both the early inter-cell mass and primitive endoderm [4]. Interestingly, each of these cell types has its own form of stem cells which can self-renew; trophectoderm has trophoblast stem cells, extra-embryonic endoderm stem cell (XEN) cells can be isolated from the primitive endoderm, and ESC are isolated from the inner cell mass [4]. The trophectoderm becomes trophoblast, which further develops into extra-embryonic tissues such as the placenta, while the inner cell mass (ICM) becomes the embryo proper [4].

Murine ESCs were initially isolated from of ICM of blastocyst in 1981 [5,6]. The therapeutic potential of these cells was unlocked in 1998, when human ESC lines were generated by Dr. James A. Thomson [7]. After that finding, any cell in the adult human body could be generated from these human ESC lines and excitement grew in anticipation of how these cells could be used to replace disease or damaged tissue.

Even before these ESC lines were generated, developmental biologist had become curious about pluripotent tumor cells. These tumor cells included teratomas, embryonal carcinoma (EC) and teratocarcinomas, which all had the ability to form all three basic germ layers (ectoderm, endoderm, and mesoderm) [3]. Stevens and Little noticed that 1% of male 129 mice developed spontaneous testicular teratomas [8]. While these tumor cells are malignant, their differentiated products are not [9,10]. This allowed for understanding mechanisms of pluripotency and development of proper culture conditions for propagation of pluripotent cells to be established even before the isolation of the first ESC line [3]. These early cultures utilized fibroblast cell line coculture system. The fibroblasts provided a signal that prevented pluripotent cells from differentiating [3]. Later it was identified that Leukemia Inhibitory Factor (LIF) was the active component maintaining pluripotency in mouse ESCs [3,11,12]. LIF binds to the LIFR, which recruits gp130 [3]. Dimmerization of gp130 mediates downstream signaling through the signal transducer and activator of transcription, STAT3 [3]. STAT3 promotes self-renewal of ESCs [3].
To derive mouse ESC lines female mice are super-ovulated and mated, early blastocyst are flushed from the uterus and hatched from early blastocyst [5] (Figure 1.2). ESCs are then cultured on mitotically inactive fibroblasts in the presence of LIF to maintain pluripotency [13,14]. Assessment of ESC colony morphology and expression of SSEA-1 and Oct-4 in mice, and SSEA-3, TRA-1-60 and TRA-1-81 in humans can be informative on the state of pluripotency [2,12]. Functional pluripotency is often tested by injecting large numbers of ESC into immunodeficient mice or by injecting ESC into the early blastocyst to see if they contribute to all three germ layers [3]. The most stringent test is tetraploid complementation [15], in which ESCs being tested for potency are injected into 4n host blastocyst. The 4n cells cannot contribute to the embryo proper, consequently the embryo is composed of the cells being tested [15].

Not all ESCs are created equal nor do they require the same culture conditions. 129 ESCs have tremendous pluripotent capacity, which makes 129 derived ESCs easy to work with, and consequently most of the ESC lines are derived from 129 mice [16]. ESCs have also been derived from CBA, C57Bl/6, Balb/c and NOD mice, but these lines are more difficult to work with [16]. For example, Sharova et al. showed that C57Bl/6 ESCs had a greater tendency to lose pluripotency in comparison to 129 ESCs [17], not surprisingly 129 ESCs showed upregulation of genes associated with pluripotency compared to C57Bl/6 ESC lines [17]. Culture conditions had to be altered for NOD ESCs for them to be germ-line competent [18]. Similarly, human ESC lines have varying levels of genes associated with pluripotency and consequently varying behavior [19]. Brinkman’s group suggests that ESCs themselves are a heterogeneous group of cells in at least three stages [20].

While human and mouse ESCs share many similarities they also have some critical differences in their biology. It should be noted that LIF is required for maintaining pluripotency with mouse ESCs, but not human [12]. Mouse ESCs express abundant levels of LIFR, whereas human ESCs lack expression of LIFR [12]. Another important difference is that human ESCs have more potency than mouse ESCs since they are also able to differentiate into cells of the trophoblast [12].
Figure 1.1: Challenges in using ESCs and their derivatives for regenerative medicine.

ESCs must be differentiated before transplantation so that their tumor forming potential is limited, but not terminally differentiated. After transplantation these cells must be able to integrate properly into the adult host, and home to the correct location and engraft into adult tissue properly. Finally, ESCs and their derivate must be able to interact with the host immune system to avoid immune rejection.
Figure 1.2: ESC line derivation and maintenance.
Embryonic stem cells are derived from the inner cell mass of the early blastocyst and can be propagated in vitro on mitotically inactivated fibroblast in the presence of Leukemia Inhibitory Factor (LIF).
1.2 Overview of Hematopoiesis

Hematopoietic Stem Cells (HSCs) have the remarkable ability to produce all the blood and immune lineages throughout the lifespan of an organism [21]. Within the embryo, hematopoietic development occurs at multiple distinct anatomical locations [22] (Figure 1.3). HSCs for clinical use can be found in the bone marrow, umbilical cord blood, as well as mobilized from the bone marrow into the peripheral blood by G-CSF for easier isolation [1]. In the clinic, they can be used to treat a variety of hematopoietic cancers, immune deficiencies, and more recently, autoimmune disease [1,2,23,24]. However there are several barriers to HSC transplantation, which will be discussed in the following section, while this section will focus on HSC development.

1.2A Embryonic Hematopoiesis

During embryonic development, the hematopoietic system is one of the earliest, yet most complex tissues to form [25]. The complexity of hematopoietic development is underscored by the variety of tissues where these cells originate, and the diversity of tissues where they mature. Each tissue has a unique microenvironment created by cytokines, growth factors, chemokines, and cell contact dependent signals for the developing hematopoietic system [26]. Currently, there is considerable debate regarding the true origin of HSCs. The hypothesized sites of origin include extra-embryonic tissues (yolk sac and placenta) or intra-embryonic regions (including the para-aorta-splanchnopleura and aorta-gonad-mesonephros [27]. These carefully orchestrated events produce a small pool of stem cells that are responsible for the continuous production of hematopoietic cells throughout the life of an individual [28].

In 1917, Florence Sabin described hematopoietic cells, with masses containing hemoglobin budding off the endothelial lining of blood vessels in preparations from chick yolk sac (YS) [29]. The YS is formed from visceral endoderm and extra-embryonic mesoderm forming a membrane that surrounds the developing embryo [30]. The wave of hematopoiesis occurring in the YS is termed ‘primitive’ [31], since it produces special erythrocytes. At this stage the erythrocytes appear to be ‘primitive’ since they resemble that of lower vertebrates [28]; these erythrocytes still have their nuclei, being large in shape, and producing the embryonic form of hemoglobin $\xi^e_2$. This embryonic hemoglobin has a higher affinity for oxygen than adult hemoglobin and is therefore able to deliver oxygen to the developing embryo [31]. The hematopoietic progenitors (HP) isolated from the yolk sac had both erythroid and myeloid potential, but had poor lymphoid potential, and importantly could not reconstitute an adult mouse [32]. However if YS hematopoietic cells were first cocultured with stromal layers from the aorta-gonad-mesonephros region (AGM), then they had the capacity to reconstitute an adult mouse [33]. This suggests that hematopoietic cells from the
YS are missing signals from the environment, but that these signals can be acquired.

The yolk sac was assumed as the origin of all hematopoiesis in developing mouse [32], until 1968 when the Herzenberg’s group showed the embryo proper, specifically the caudal half (including the aorta-gonad-mesonephros region) at E9 could reconstitute lethally irradiated mice [34]. Similarly, Godin et al. showed that SCID mice could be reconstituted with cells from the para-aorta-splanchnopleura (P-sp) at E8, the precursor that will form the AGM [28]. The importance of this is that the P-sp isolated at E8 is before the onset of circulation, meaning it could not have contaminating cells from the YS. Although, it did require several days of culture to mature the cells, since no hematopoietic cells were capable of reconstituting an adult mouse before E8.5 [35]. The next wave of hematopoiesis begins at the aorta-gonad-mesonephros (AGM) region within the developing embryo proper [32]. Hematopoietic cell clusters bud into circulation off sub-aortic patches on the ventral wall of the dorsal aorta [36]. At E10 cells from the AGM have potential to develop into both myeloid and lymphoid lineages and the capacity for long-term reconstitution when adoptively transferred into adult mice [32]. Interestingly, hematopoietic cells isolated from the AGM show higher levels of reconstitution when injected directly into the bone marrow cavity [27,37]. Currently, the AGM is viewed as the principal site of definitive hematopoiesis [27].

The placenta forms as a result of the allantois and chorionic plate fusing [38]. As early as 1945, the relationship between blood and placenta was known. In that year Ray Owen found a set of fraternal twin cattle that shared a placenta and consequently shared blood types for the rest of their lives [23]. Since the placenta is directly downstream of aortic circulation, AGM derived hematopoietic cells colonize the placenta [22]. Hematopoietic cells with reconstituting ability can first detected in the placenta at E10 [34]. To test if the placenta was capable of de novo hematopoietic development, Ncx-/- embryos, which lack sodium calcium exchanger-1, were studied. Ncx-/- embryos lack a heartbeat and consequently circulation. In Ncx-/- embryos the placenta has hematopoietic cells, albeit less than those with intact circulation [39]. Within the placenta, the pool of definitive hematopoietic cells greatly expands [22].

Regardless of their original site, hematopoietic cells enter the circulation and colonize the fetal liver (FL) [22,27,40]. Similar to the placenta hematopoietic cells expand and mature in the FL [27] and cells originating at other sites go to the FL to acquire additional maturation cues [41]. Interestingly, no hematopoietic cells are thought to originate in the FL, currently it is assumed they are brought to the FL via circulation [22]. At E11.5, long-term (LT) HSCs can be detected in the fetal liver [42]. From the FL, hematopoietic cells colonize the fetal spleen and fetal bone marrow beginning at E15 [22,27]. Dzierzak et al. suggest that a few HSCs colonize the limb bud well before the formation of bone, this suggests that the developing bone may be in part seeded with HSC [43]. The bone marrow
remains the primary site for hematopoiesis for the rest of the life for mammalian organisms [22,27].
Figure 1.3: The complex journey of hematopoietic development. The inner cell mass gives rise to intra-embryonic hematopoiesis in the AGM, while the trophoblast and yolk sac give rise to erythroid-myeloid cells. Independent of their origins, these early hematopoietic cells enters circulation, colonize the fetal liver. Afterwards some with the capacity to become HSCs will colonize the bone marrow. The bone marrow is the primary site of hematopoiesis in the adult.
In 1945, the atomic bomb blasts at Hiroshima and Nagasaki exposed civilian populations to radiation, and consequently those people who did not die immediately, subsequently died of hematopoietic failure [23]. Later, Till and McCulloch identified the bone marrow as being sensitive to radiation [44]. Further, Till and McCulloch along with Becker described cells from the bone marrow that were capable of self-renewal [45]. Together with Siminovitch, they created a model of stem cell development; they found that hematopoietic stem cells (HSCs) were capable of self-renewal, could differentiate into all the blood lineages, and lastly the source of these stem cells could be exhausted by serial transplantation [46-48]. Today, this is the basis for how we define stem cells: 1) are capable of self-renewal and 2) can differentiate into other cell types [2,15].

The bone marrow cavity is the primary site for HSCs in adult life and it provides a specialized niche [22,49-52]. Schofield first proposed the stem cell niche in 1978 [53]. Since then, many cell types have been identified as supporting hematopoietic stem cells in the adult bone marrow including stromal cells (including fibroblast, mesenchymal stem cells (MSC) and CAR cells), endothelial cells [54], osteocytes, osteoblast, as well as other hematopoietic cells [55,56]. MSCs by definition have the ability to differentiate into osteocytes, adipocytes, and chondrocytes [57]. While the BM niche has been extensively studied, the complexity makes teasing apart the individual contributions of each of these cell types difficult [51,54]. Signals from the niche instruct HSCs to self-renew, remain in quiescence, differentiate, and initiate cell death [49]. HSCs are primarily found in the endosteum, which is a highly vascularized zone between the bone and bone marrow [58]. Haylock et al. found that spatial differences in relationship to the endosteal region could result in different functional abilities for HSCs. Using the same cell surface markers, they found that HSCs in close contact with the bone displayed greater in vivo hematopoietic potential, as well as a greater homing efficiency compared to HSCs centrally located in the bone marrow [59]. This suggests a special relationship between osteoblasts and HSCs. Within the HSC niche, osteoblast secrete soluble factors such as angiopoietin, thrombopoietin, CXCL12, and are also capable of delivering cell contact dependent signals such as Notch1 to maintain and support HSCs [58].

Adult HSCs come in two forms, the long term HSC which can self-renew continuously over a long period of time [60] and short term HSC which self-renew for three to six weeks. In contrast hematopoietic progenitors do not self-renew, but have the capacity to differentiate into multiple lineages (Fig. 1.3) [61]. The adult HSC that is capable of LT-reconstitution has been experimentally defined as lineage (Lin)\(^{-}\)c-Kit\(^{+}\)Sca-1\(^{+}\)FLK2\(^{-}\)CD34\(^{-}\)CD150\(^{-}\)CD41\(^{-}\)IL7R\(\alpha\)^- , while ST-HSC have been defined as lineage (Lin)\(^{-}\)c-Kit\(^{+}\)Sca-1\(^{+}\)FLK2\(^{-}\)CD34\(^{+}\)CD150\(^{+}\)CD41\(^{-}\)IL7R\(\alpha\)^+.
[49]. ST-HSCs have the capacity to reconstitute the hematopoietic compartment for approximately 10 weeks [23]. Even within this highly defined population of adult HSCs, several groups have described markers that are indicative of lineage bias within the hematopoietic stem cell population. Within the adult HSC, CD86 has recently been identified as a marker for HSC with greater lymphoid potential, which declines with age [62,63]; conversely CD41 has recently been identified as a marker for HSC with myeloid bias that increases with age [64].
LT-HSCs have been identified as (Lin)^-c-Kit^+Sca-1^+FLK2^-CD34^-CD150^-CD41^-IL7R^α^- and can either self renew or differentiate. LT-HSCs can reconstitute for the lifetime of an organism, while ST-HSCs can reconstitute for around 10 weeks. ST-HSCs are defined as (Lin)^-c-Kit^+Sca-1^+FLK2^+CD34^+CD150^-CD41^-IL7R^α^-.

Both LT-HSCs and ST-HSCs can give rise to multi-potent progenitors (MPP), which can then give rise to more restricted progenitors such as the common myeloid progenitor (CMP) and common lymphoid progenitors (CLP). These can then give rise to lineage-restricted progenitors and finally mature effector cells.

**Figure 1.4: Classic LT-HSC to lineage commitment.**

Myeloid Lineages: Granulocytes, Monocytes, Macrophages

Lymphoid Lineages: NK, T cells, B cells
1.2C Challenges of in vitro Hematopoietic Development from ESCs

The derivation of hematopoietic stem and/or progenitor cells from ESCs has been exceedingly challenging [1,40]. Hematopoiesis itself is an elegant and complex process during embryonic development, occurring at a variety of temporally distinct locations and microenvironments [1,27,32,41,52]. Embryonic stem cell derived hematopoietic progenitors (ES-HPs) need to have several characteristics to be successful: they need differentiated enough so that their tumor forming potential is limited, but conversely not terminally differentiated, so they can self-renew and provide an ongoing supply of hematopoietic cells, they also need to be able to migrate to and integrate into the correct microenvironment in the adult (BM), and they need to be able to withstand the adult host immune system [1,27]. Generation of ES-HPs that behave similar to adult HSCs has been a tremendously difficult hurdle for the field of stem cell biology.

Two general methods have been used to generate ES-HPs from ESCs. The first is generation of embryoid bodies (EBs), in which cells are either suspended in hanging drops or in viscous media such as methylcellulose [56]. EBs spontaneously form into a three-dimensional spheres of cells containing all three basic germ layers found in the developing embryo. The other method involves co-culturing ESCs on bone marrow stromal cells. Bone marrow stromal cells can support varying degrees of hematopoiesis [52]. RP.0.10, ST2, MS-5, and OP9 cell lines are commonly used [56,65]. OP9 are the most commonly used cells for hematopoietic development because these cells were derived from mice lacking M-CSF and consequently these cocultures are not taken over by macrophages [66]. These methods have been used alone and in combination to generate ES-HPs [67]. ES-HPs generated using these methods have occasionally led to ES-HPs able to reconstitution mice in vivo; this maybe low frequency of success is dependent of serum or on how the cells have been handled [56]. Little to no reconstitution of adult mice occurs without injection into the bone marrow cavity or transfection of ESCs with HoxB4 or BCR/ABL [56,68].

BCR/ABL was transfected into ESCs, then differentiated into ES-HPs. These ES-HPs were able to self-renew and consequently able to reconstitute an adult host immune system. Further BCR/ABL ESCs were able to differentiate into both myeloid and lymphoid lineages [68]. Transfection with the BCR/ABL oncogene caused the recipients to die of a myeloproliferative disorder [68]. Similarly, HoxB4 (a helix-loop-helix transcription factor) promotes self-renewal of HSCs and ES-HPs [69]. Transfection of HSCs by HoxB4 in larger animals resulted in leukemia [70]; this is now used as a model for leukemia in dogs [71]. Although ES-HPs often express HoxB4 it is often at levels too low to establish self-renewal [65]. Matsumoto found that HoxB4 must be continuously on to
maintain reconstitution of ES-HPs \textit{in vivo} [72]. Although, HoxB4 transfection greatly improves the ability of ES-HPs, it is not necessary in normal hematopoietic development. HoxB4 knockout mice display only mild hematopoietic defects [73].

Interestingly, ESC can indeed contribute directly to the hematopoietic system in chimeric mice when ESCs were injected into blastocyst [21,74,75]. Importantly, this suggests there is no underlying defect that would prevent them from developing properly if they were given the correct developmental cues. ESCs do have the potential to form both myeloid and lymphoid lineages \textit{in vitro}, however, despite their potential ES-HPs derived \textit{in vitro} often fail to engraft in adult mice \textit{in vivo} [1,76,77]. To overcome this limitation several groups have genetically modified their ESC prior to differentiation [78-80], while others have transplanted their cells into the liver of newborn mice [76], or transplanted cells directly into the femur of adult mice [24,81]. Several groups have been able to transplant ES-HPs; however there are conflicting reports about how well these cells regenerate the adult host hematopoietic system, and how well tolerated these cells are by the adult host immune system [1]. An additional hurdle is identifying applicable cell surface markers, since ES-HPs have characteristics of both embryonic and adult hematopoiesis. Commonly used markers can be found in Table 1.1 and their distribution on hematopoietic populations is in Table 1.2.
# Table 1.1: Markers Commonly Used to Define Hematopoietic Stem Cells and Progenitors

<table>
<thead>
<tr>
<th>Cell Surface Markers</th>
<th>Alternate Name or Biological Function</th>
<th>Fetal Population</th>
<th>Adult Population</th>
<th>ES-HP Population</th>
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<tr>
<td>Flk-1</td>
<td>Vascular endothelial growth factor receptor. Fetal Liver Kinase [82]</td>
<td>Found on mesoderm in the YS, as well as early vascular and hematopoietic cells in the early embryo and in the FL [82-84]</td>
<td>Flk-1-/- cells were unable to contribute to adult hematopoiesis after chimeric embryo formation [84]</td>
<td>Early expression on colonies capable of colony formation in vitro [85], although it is not necessary for erythroid colony formation in vitro [84]</td>
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<tr>
<td>CD41</td>
<td>Integrin $\alpha_{IIb}\beta_{3}$</td>
<td>Marks transition to hematopoietic fate [86]</td>
<td>Megakaryocytes, HPCs, and HSCs [64,87]</td>
<td>Marks ES-HP derived using the EPOCH method with potential for long-term reconstitution [80]</td>
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<tr>
<td>AA4.1</td>
<td>Homologous to a complement component receptor in humans C1qRP [88] CD93 [65]</td>
<td>Marks definitive in P-sp and AGM and primitive and endothelial hematopoietic populations in FL from E11 and the AGM as early as E9 [32,88]</td>
<td>Pro-B cells and some multi-potent stem cells [88] and is low to undetectable on adult HSCs [65].</td>
<td>AA4.1+ cells have colony forming ability [65]. One paper showed they have reconstitution ability [89] and one paper demonstrated the lack reconstitution potential [65].</td>
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<tr>
<td>CD34</td>
<td>Surface glycoprophosphate [90]</td>
<td>Expressed on c-kit+ cells in the AGM at E10 and c-kit+ in the FL at E11 [91].</td>
<td>Expressed on ST-HSC and HPC populations [92] and small vessel endothelial cells [90].</td>
<td>Expressed on ES-HP using the EPOCH System [93]</td>
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<tr>
<td>CD150</td>
<td>Signaling lymphocyte activation molecule family 1 (slamf1) [94] Surface glycoprotein [94] Stimulatory T/B cell costimulatory receptor [94]</td>
<td>Expressed on very few cells in the AGM at E11.5 [80] Expressed on LT-HSC in the FL E14.5 [95]</td>
<td>Expressed on quiescent HSC in the adult bone marrow [96]</td>
<td>Expressed on ES-HP [80].</td>
</tr>
<tr>
<td><strong>Cell Surface Markers</strong></td>
<td><strong>Alternate Name or Biological Function</strong></td>
<td><strong>Fetal Population</strong></td>
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<tr>
<td>CD48</td>
<td>Singling lymphocyte activation molecule family 2 (samf2) Receptor for CD244 [97]</td>
<td>CD48 negative population enriched for repopulating ability in E11.5 AGM and E12.5 PL [80] CD48 negative population enriched for HSC activity in FL E14.5 [98]</td>
<td>Expressed on restricted progenitors in the adult, but not on highly purified HSCs [54]</td>
<td>Using ES-HPs derived using the EPOCH method CD48 negative reconstituted 2/3 mice and CD48+ reconstituted 5/6 mice. CD48 was less important in determining potential than in fetal tissues and BM [80].</td>
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<tr>
<td>CD244</td>
<td>2B4 [97] SLAM family member [97]</td>
<td>CD244 negative population enriched for HSC activity in the FL E14.5 [98]</td>
<td>Expressed by a small population of MPP, but not HSC [54]</td>
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<td>CD45</td>
<td>Protein tyrosine phosphatase [100] Common leukocyte antigen [101]</td>
<td>CD41+ precedes CD45+, in the fetal liver CD41 begins to be down regulated [86] Adult engrafting cells change from CD45- to CD45+ between E10.5-11.5 [37,102]</td>
<td>Expressed on all mature hematopoietic populations, except red blood cells [102]</td>
<td>CD41+ precedes CD45+ expression in EBs [86] Used to define ES-HPs that can from ESC and from EBs capable of reconstituting mice [81] Expressed on ES-HP using the EPOCH system [93]</td>
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### Table 1.2: Markers Expressed on Hematopoietic Populations with Multi-Lineage Repopulating Activity

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* denotes if that marker is used to define the population
1.3 Transplantation Biology

1.3A Transplant Rejection versus Transplantation Tolerance

The immune system is remarkable for its ability to identify self from non-
self, or even self from altered self [105]. Transplantation is the act of transferring
cells, tissues or organs from one individual or location to another individual or
location [106]. Once tissue is transplanted into a recipient, the tissue can be
recognized as foreign and be rejected [105,107], or it may not be seen by the
immune system or ignored [108-110], and lastly, it maybe actively accepted
(transplant tolerance) [105]. In this section, I will review transplantation
immunology in general, then review what is known about bone marrow
transplantation, and lastly I will review what is known about immune responses to
ESCs and their derivatives.

T Cell Responses

Much of the work focusing on transplant rejection has focused on T cells
[111]. Major histocompatibility complex (MHC) molecules are one of the most
diverse functional gene families described in vertebrates [106]. Both Allogeneic
MHC-I and MHC-II are potent stimulators of allogeneic T cell responses [106].
MHC molecules are responsible for presenting peptides to T cells. MHC-II
molecules on professional antigen presenting cells (APCs) present exogenous
peptides to CD4+ T cells [106]. MHC-I molecules primarily present intracellular
peptides, but have also been found to cross-present peptides of extracellular
origin to CD8+ T cells [106].

T cells recognize antigens in the context of MHC molecules via direct or
indirect pathways [111-114] (Figure 1.4). In direct responses, APCs transferred in
the transplanted tissue present donor antigens in the context of donor APCs.
Conversely, during indirect recognition donor antigens are picked up and
presented by host APCs. Both direct and indirect-presentation have been
implicated in transplant rejection. Direct presentation elicits a rapid and strong
response in comparison to indirect presentation, while indirect presentation
requires antigen processing [106].

Costimulatory signals provide important cues for the immune system to
decide how to respond or regulate to a potential immune response to a particular
antigen [115]. The first signal (“Signal 1”) for T cells is delivered through MHC
molecules presenting peptides to a corresponding T cell receptor (TCR) on the T
cell. The second signal (“Signal 2”) is provided from costimulatory molecules
[115]. CD80 and CD86 can promote T cell activation through interaction with
CD28. Conversely they can interact with CTLA-4 to inhibit T cell responses
[115]. Although this system is more complex than this, as signaling through CD28
can also promote the development of regulatory T cells, which help modulate immune responses [116].

While T cells are prominent players in transplant rejection they can also mediate immune tolerance. Tolerance is the lack of immune reactivity to an antigen or tissue without the requirement for immunosuppression, while retaining the ability to mount an effective immune response to all other foreign antigens [117]. T cell tolerance can be achieved by either central or peripheral mechanisms [117]. Central tolerance occurs in the thymus, where potentially auto-reactive T cells are deleted, and where high affinity T cell clones become regulatory T cells (T-regs) [117]. In the periphery, T cells may undergo clonal deletion, anergy, immune ignorance, or regulation by T-regs to maintain tolerance [117].

**NK Cell Responses**

NK cells are innate lymphocytes that protect against transformed cells, virally infected cells, as well as intracellular parasites [111,118]. NK cell activity is regulated by the milieu of signals received through NK cell receptors [119]. Activating ligands such as RAE-1, Mult-1, and H60 are upregulated by DNA damage or other sources of cellular stress [119]. NK cells also respond to “missing self” such as tumors or virally infected cells that down regulate MHC-I [2,111]. In transplantation, NK cells from F1 generation mice, can reject cells from either of the parental lines because they can determine the correct level of MHC-I that should be expressed on the cell surface. Self is determined by the level of MHC-I binding to the NK inhibitory receptor Ly49 family members [118]. While NK cells are not sufficient to reject solid organs, they play a critical role in rejection of bone marrow transplants [118,120]. NK cells are also radio-resistant compared to other lymphocyte population, this makes them particularly problematic for bone marrow transplantation [118].

**Macrophage Responses**

Ilya Metchnikoff introduced the world to the cellular theory of immunity and phagocytic theory in 1882-1884, for which he was awarded the Nobel Prize in 1908 [121]. More recent immunology has focused on acquired immunity after Paul Ehrlich discovered antibodies. Metchnikoff was a trained as a developmental biologist and embryologist, and he worked on the development of invertebrate germ layers in his thesis [121]. Importantly, Metchnikoff made connections between phylogenetic and ontogenetic history of cells where phagocytosis was used for cell eating in lower organisms, but evolved to a specialized role in defense and homeostasis in higher organisms [121]. It is now well-appreciated that macrophages are highly heterogeneous. Yolk sac hematopoiesis provides one source of tissue-derived macrophages, while bone marrow derived hematopoiesis generates monocytes capable of differentiating into macrophages [122-124]. The current role of different macrophage
populations in tissue homeostasis and the coordination of immune response is currently an active area of research [124].

Immediately following allogeneic transplantation, cells of the innate immune system (including macrophages) infiltrate the graft [111]. Macrophages alone are sufficient to reject xenogeneic bone marrow [125]. Zecher et al. was one of the first studies characterizing the innate immune system in rejection of allogeneic cells in the absence of adaptive immunity [126]. They noticed allogeneic dependent responses when splenocytes were injected into RAG-/- mice lacking B cells and T cells, further this swelling was independent of NK cells [126]. The infiltrate consisted of macrophages, monocytes, as well as polymorphonuclear neutrophils [126]. Further, there was some degree of recall when the mice were re-challenged or when monocytes from recipients were adoptively transferred in mice challenged with allogeneic splenocytes [126]. Depletion of monocytes and macrophages using clodronate-loaded liposomes decreased the recall response [126].

The field as a whole is just beginning to understand how macrophages understand self from non-self, and consequently how they know what to tolerate as self [126]. What has become clear is that macrophages play a critical role in determining the fate of the immune response. Similar to NK cells, macrophages respond to a combination of signals on a potential cell. Several ligand-receptor pairs have been characterized for their ability to inhibit phagocytosis or macrophage activation: these include CD47-SIR-alpha, CD200-CD200R, and MHC-I-Ly49Q [127]. Additionally, NKG2D ligands, as well as other signals of cellular stress or transformation can deliver activating signals to macrophages [128-130].

CD47 is a ligand that binds to SIRP-alpha on macrophages to deliver an inhibitory or ‘don’t eat me signal’ to macrophages through an immunoreceptor tyrosine based inhibitory motif (ITIM) [131]. Loss of macrophage control is the hallmark of macrophage activation syndrome in familial hemophagocytic lymphohistocytosis (HLH) and is commonly associated with autoimmune diseases and loss of tolerance [132]. Recently, CD47 downregulation has been described in the HSCs of HLH patients and may account for the reduction in bone marrow cells [132]. Splenic pulp macrophages regulated clearance of CD47 deficient red blood cells [133]. Both macrophages and CD47-SIRP-α system have a critical role in tumor surveillance [130,134]. Splenectomy or elimination of macrophages with liposome encapsulated drugs prevented the elimination of CD47-/- red blood cells [133].

CD200 (Ox-2) is expressed on a wide variety of tissues including hematopoietic cells, neurons, endothelial cells, and the trophoblast, whereas its receptor CD200R is expressed only on myeloid and lymphoid cells [135]. CD200R delivers an inhibitory signal to macrophages [135]. CD200 knockout animals had elevated levels macrophages in the red pulp of the spleen and these
macrophages had increased expression of F4/80 on their cell surface [135,136].
These animals were also more susceptible to a variety of autoimmune diseases
including experimental autoimmune encephalomyelitis (EAE), and collagen
induced arthritis [135]. In transplantation, disruption of CD200-CD200R
interactions resulted in graft rejection [135,136].
1.3B The Original Stem Cell Transplant – Bone Marrow

Transplantation of bone marrow cells is known to create a state of immunological tolerance between host and donor tissues. Mixed chimerism was first observed by Owen in 1946 when he noticed that cattle that shared a placenta (dizygotic twins), but were genetically distinct, shared blood systems for the rest of their life [137]. Medawar, had extensive experience as a surgeon with skin grafts had noticed that secondary grafts rejected much faster than the primary skin graft [107].Billingham while working with Medawar combined these observations and transplanted skin from the opposing twins skin and showed that the cattle that shared a placenta were indeed tolerant to each others skin grafts [138]. In studies since this time, it has been shown that even low levels of hematopoietic chimerism under appropriate conditioning regimes can induce transplantation tolerance in both mice and man [139]. In recent years this has made its way into the clinic, where hematopoietic stem cell transplants have been able to induce tolerance to kidney transplants from the same donor [140].

T Cells

Mixed chimerism by bone marrow derived hematopoietic cells can even induce T cell tolerance across xenogeneic barriers [141]. T cell tolerance induced by hematopoietic cells induces a state of non-responsiveness in mixed lymphocyte reactions (MLR). Since a high frequency (5-10%) of the T cells may be reactive against donor, deletion of donor and host reactive T cell clones in the thymus is required to develop central tolerance [110,141]. Achieving this deletion requires donor-derived antigen presenting cells (APC) migrating to the thymus [141]. In addition to clonal deletion, high levels of both donor and recipient T regulatory cells can be found in tolerant BM chimeras [117].

NK Cells

While NK cells can be problematic for bone marrow transplantation because they are radio-resistant and can recognize changes in the level of MHC-I, they can be educated to understand what is self versus non-self in mixed chimeras [118,142]. BM transplantation resulting in mixed hematopoietic chimerism also results in NK cell tolerance. Ly-49 molecules are the receptors on NK cells for MHC-I molecules. In BM chimeras, the cells in the environment where NK develop determines how much of each Ly-49 molecule to express on the cells surface [118,142].

Macrophages

While macrophages are clearly a barrier for xenogeneic bone marrow transplantation [125], less is known about how macrophages tell self from non-
self in allogeneic settings. CD47 deficient bone marrow could not reconstitute
the hematopoietic system of wild-type mice expressing CD47 [143]. Similar to
NK cells, macrophage activation is regulated by the balance of inhibitory and
activating signals [144]. Wang et al. examined macrophage tolerance in bone
marrow and found that wild-type macrophages could be tolerized to most CD47
knockout hematopoietic cells [144]. They found that in these chimeras, tolerance
could be achieved to most CD47 knockout cells with the exception of red blood
cells, they suggest that the non-red blood cell hematopoietic cells might have
additional inhibitory signals present on other hematopoietic cells, that they could
deliver to macrophages in what they termed ‘split-tolerance’ [144]. This group
also teased apart the non-hematopoietic and hematopoietic components that
induced macrophage tolerance and found that it was the stromal cell component
that was responsible for educating macrophage that CD47 as should be seen as
self [144].

Whatever the immune cell type being tolerized, the bone marrow has an
important role in the recognition of self versus non-self. Non-myeloablative
conditioning combined with bone marrow transplant has been shown to improve
the outcome of subsequently transplanted solid organ grafts [145]. Further, in
settings such as autoimmune disease where tolerance is broken, bone marrow
transplantation can alter the course of disease by re-educating the immune
system [23].
Figure 1.5: Direct and indirect presentation of ES-HP peptides to CD4+ T cells.

**Top.** In direct presentation, the donor cell can present antigens using its own MHC molecules. **Bottom.** In indirect presentation, the donor cell is taken up by an antigen-presenting cell such as a dendritic cell or macrophage and processed. Donor antigens are presented on host MHC molecules to T cells.
1.3C Immune Responses to ESCs and their Derivatives

Syngeneic 129 mice could mount an effective antibody response against teratoma cell lines, suggesting that these cells have antigenic determinants not present in the adult organism, leading to the conclusion that these cells have critical differences than of those normally present in the adult organism, such as embryonic antigens [146].

Conversely, initial studies suggested that ESCs and their derivatives were immune privileged [147-149]. Several groups showed that ESCs had little to no expression of MHC-I [147,150], which is a highly potent molecule in transplant rejection. This led to the hypothesis that ESCs and their derivatives would have immune privilege after transplantation into an adult host. However, as these cells were differentiated towards specific tissue types or if they are treated with IFN-γ, MHC-I was upregulated [79,149,151].

More recently, this idea has been challenged, as several groups have recently shown rejection of ESCs (Table 1.3), as well as ESC-derived cell populations by the adult host immune system (Table 1.4) [152-156]. Both innate and adaptive branches of the mammalian immune system are potential barriers for ESCs and ESC derived tissues [1,2].

ESCs without differentiation are difficult for the adaptive branch of adult immune system to recognize [147,157,158], however they are recognized by innate components of the immune system [155,156]. Several group have shown that NK cells are able to reject ESCs and their derivatives indirectly; engraftment is increased in Rag2-/-, γc−/− compared to Rag2-/--. Since, the difference between these two mouse lines is that NK cells are absence in Rag2-/-, γc−/−, NK cells are implicated [155,156], while others have shown ESCs and their derivatives are resistant to NK cell mediated lysis in vitro [147,149].

The most well characterized adaptive immune response to ESCs and their progeny is the T cell response. However, it is still remarkably controversial if ESCs are able to elicit a T cell response [1]. Some groups have shown T cell mediated rejection of ESCs and their derivatives in vivo [153,159]. Further, Pearl et al. showed rejection could be mitigated by use of immuno-suppressive drugs or by costimulatory blockade [159]. Koch et al. found that ESCs inhibited T cell proliferation and that this effect was mediated by TGF-β in vitro [157]. Lui et al. showed that transplanted EBs mediated induction of T-regulatory cells (T-regs); however if T-regs were depleted then activated macrophages and CD8+ T cells were present in the rejecting grafts [160].

Innate recognition by antigen presenting cells (APCs) can cause removal of ESCs and their derivatives, and they can present antigens to adaptive branches of the immune system, thus serving a role as a key coordinator for the
adult host immune response. Macrophages have controversial roles in tissue regeneration and repair [124]. This suggests that they have a special role with stem and progenitor cells [124]. Immediately proceeding injury, macrophage depletion can improve recovery by limiting damage; conversely macrophage depletion in the recovery phase can impair in liver, kidney, and skeletal muscle regeneration [124]. Macrophages also serve as key coordinators of immune responses, instructing T cells how to respond to certain antigens [161]. Several groups have noticed strong recruitment of macrophages in response to the inner cell mass of the early blastocyst, ESC transplants, or transplants of cells differentiated from ESC [160,162,163]. Macrophages may reject ESCs and their derivatives directly or they may coordinate the T cell response.

The variety of immune responses to ESCs and their derivatives is diverse; this makes the rules of engagement for their use difficult to interpret. Understanding how and when they are rejected compared to how and when they can induce tolerance is key in translating their potential to clinical application.
<table>
<thead>
<tr>
<th>Author</th>
<th>Population</th>
<th>Tolerance-Ignorance</th>
<th>Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bonde, et al., 2006 [149]</td>
<td>Mouse 129 ESC lines RW-4 and R1 and C57BL/6</td>
<td>Poor susceptibility to NK cell and CTL lysis in vitro. ESCs also expressed FasL and induced apoptosis in T cells after transplant. ESCs were able to induce mixed chimerism.</td>
<td>ESCs disappeared within 4 weeks of transplant.</td>
</tr>
<tr>
<td>Dressel, et al., 2008 [164]</td>
<td>Mouse 129 ESC (MPI-II)</td>
<td>Teratoma formation in syngeneic mice.</td>
<td>Teratoma rejection in allogeneic and xenogeneic mice, including minor mismatches.</td>
</tr>
<tr>
<td>Koch, et al., 2008 [157]</td>
<td>Various mouse ESC lines (C57BL/6, BALB/c x 129, R1, and 129SvJ)</td>
<td>If greater than 5x10^6 ESCs were injected subcutaneously in allogeneic mice the ESCs did not reject, but it did not confer allogeneic transplantation tolerance.</td>
<td>If less than 5x10^6 ESCs were injected subcutaneously in allogeneic mice the cells disappeared.</td>
</tr>
<tr>
<td>Koch, et al., 2008 [157]</td>
<td>Various mouse ESC lines (C57BL/6, BALB/c x 129, R1, and 129SvJ)</td>
<td>ESCs resisted lysis by both syngeneic and allogeneic NK cells.</td>
<td></td>
</tr>
<tr>
<td>Koch, et al., 2008 [157]</td>
<td>Various mouse ESC lines (C57BL/6, BALB/c x 129, R1, and 129SvJ)</td>
<td>ESCs inhibited T cell proliferation to allogeneic DCs, anti-CD3 and anti-CD28 or concanavalin A in a dose dependent manner. This inhibition could be transferred by ESCs conditioned media or a transwell system. Mediated by TGF-beta</td>
<td></td>
</tr>
<tr>
<td>Dressel, et al., 2008 [164]</td>
<td>Mouse 129 ESC (MPI-II)</td>
<td>Xenogeneic NK cells readily lysed ESCs, but not differentiated cells. Symgeneic mouse NK cells could also kill ESCs, but only if they were stimulated with IL-2.</td>
<td></td>
</tr>
<tr>
<td>Dressel, et al., 2008 [164]</td>
<td>Mouse 129 ESC (MPI-II)</td>
<td>ESCs express RAE-1, but not MHC-I. RAE-1 was not expressed on differentiated cells and MHC-I was barely detectable.</td>
<td></td>
</tr>
<tr>
<td>Mohib et al., 2012 [158]</td>
<td>Variety of mouse ESC lines (D3, J1, and B6)</td>
<td>ESC derived factors inhibited T cell proliferation and activation. Induced T-regulatory cells.</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.3: Immune Responses to ESCs**
<table>
<thead>
<tr>
<th>Author</th>
<th>Population</th>
<th>Tolerance-Ignorance</th>
<th>Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kofidis et al., 2005 [152]</td>
<td>Mouse D3-eGFP</td>
<td></td>
<td>Increased CD3+ T cell infiltration by histology and increased alloantibody responses after transplantation in the heart.</td>
</tr>
<tr>
<td>Swijnenburg et al., 2005 [153]</td>
<td>Mouse D3</td>
<td></td>
<td>As ESCs differentiated in the heart, increased immune response in allogeneic recipients was detected.</td>
</tr>
<tr>
<td>Pearl et al., 2011 [159]</td>
<td>Mouse D3 H7 Human</td>
<td></td>
<td>Costimulatory blockade increased survival of ESCs after allogeneic and xenogeneic transplants.</td>
</tr>
<tr>
<td>Fandrich et al., 2002 [165]</td>
<td>Rat ESC</td>
<td>Achieved hematopoietic chimerism and transplant tolerance</td>
<td></td>
</tr>
<tr>
<td>Zhao et al., 2011 [166]</td>
<td>129 ESC</td>
<td></td>
<td>CD3+, CD4+ T cell infiltrate in teratomas in B6 recipient mice</td>
</tr>
<tr>
<td>Zhao et al., 2011 [166]</td>
<td>129 iPS lines</td>
<td></td>
<td>CD3+, CD4+ T cell infiltrate in teratomas in B6 recipient mice</td>
</tr>
<tr>
<td>Wu et al., 2008 [167]</td>
<td>ESF134 (C57Bl/6)</td>
<td>Low levels of MHC-I (H2-kb) expressed even after 48 hours of differentiation. No MHC II was detectable. Although MHC-I increased after 4 days of transplantation in the kidney capsule, no T cell response above sham could be detected.</td>
<td>MHC-I (H2-kb) levels increased after being exposed to 1,000 U/ml IFN-γ. Rejected when transplanted into fully allogeneic host. No additional proliferation occurred using CD8+ TCR for H2-kb.</td>
</tr>
</tbody>
</table>
Table 1.4: Immune Responses to ESC Differentiated Populations

<table>
<thead>
<tr>
<th>Author</th>
<th>Population</th>
<th>Tolerance-Ignorance</th>
<th>Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dressel et al., 2008</td>
<td>Mouse 129 ESC differentiated by coculture on PA6 cells for 14 days to neural lineages</td>
<td>In syngeneic transplants a high percentage of the differentiated cells survived.</td>
<td>In allogeneic and xenogeneic recipients, rejection of differentiated cells occurred. Treatment with CsA only slightly increased cell survival.</td>
</tr>
<tr>
<td>Bonde et al., 2008</td>
<td>Mouse 129 ESC transduced with HoxB4</td>
<td>ES-HPs induced transplantation tolerance to cardiac grafts.</td>
<td>Engraftment increased in Rag2-/-, γc-/- mice compared to syngeneic mice, suggesting involvement of NK cells.</td>
</tr>
<tr>
<td>Burt et al., 2004</td>
<td>Mouse R1 ESC EB formation 7-10 days to hematopoietic fate (CD45+).</td>
<td>Able to induce transplant tolerance.</td>
<td>Chimerism higher with increased radiation, this could be space or host immune response. Also intra-bone marrow transplant had higher levels of chimerism. This could bypass defects in migration or bypass host immune response.</td>
</tr>
<tr>
<td>Verda et al., 2008</td>
<td>ESF121, ESF116, ESF191, ESF166, ESF75. EB formation 14 days and transplanted in kidney capsule.</td>
<td>Accepted in allogeneic CBA.RAG-/- .</td>
<td>Rejected in allogeneic mice.</td>
</tr>
<tr>
<td>Robertson et al., 2007</td>
<td>EB formation 14 days and transplanted in kidney capsule.</td>
<td>Tolerance in immune competent host could be achieved with co-receptor blockade.</td>
<td>Rejected at a higher rate in female recipients. Mediated by CD8+ T cells.</td>
</tr>
<tr>
<td>Lui et al., 2010</td>
<td>EB from single MHC-I mismatch could survive in transplanted mice.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearl et al., 2011</td>
<td>Human H7 ESC teratoma, which was subsequently transplanted into immuno-competent host.</td>
<td></td>
<td>Costimulatory blockade increased survival of differentiated product.</td>
</tr>
<tr>
<td>Author</td>
<td>Population</td>
<td>Tolerance-Ignorance</td>
<td>Rejection</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------------------------------------------------------------------</td>
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<td>-----------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Wu et al., 2008 [167]</td>
<td>Mouse ESF134 (C57Bl/6) and ESF122 (CBA) differentiated into insulin producing cell clusters (IPCC)</td>
<td>Even in syngeneic. Rag-/- mice normal glycemia was restored for only 15 days.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>In syngeneic immune competent mice normal glycemia was restored for only a few days.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>In fully allogeneic immune competent mice normal glycemia was not restored.</td>
<td></td>
</tr>
</tbody>
</table>
1.5 Objective of Study and Hypotheses Tested

The main goal of my dissertation work was to understand how ES-HPs develop and interact with the adult host immune system. I proposed to examine the interaction of ES-HPs with the adult immune system by completing the following objectives:

1. **To generate ES-HPs from ESCs and define the potential of ES-HPs to generate hematopoietic lineages.** ESCs in theory can be differentiated into any cell type in the body, but the practical ability for ESC culture systems to recapitulate in vivo development has been inadequate. I used two different culture methods to generate ES-HPs. I am comparing these ES-HPs to hematopoietic populations isolated from fetal and adult hematopoietic organs. Few studies demonstrating a systematic comparison of *in vitro* derived and naturally developing hematopoietic progenitors and stem cells, as well as a direct comparison of ES-HP culture methods have been published. Since developmental cues from the environment regulate ES-HP development, I tested two classical methods of generating ES-HPs in vitro (embryoid bodies and coculture on the stromal cell layer OP9). To verify that ES-HPs were true progenitors, I examined developmental markers, as well as their developmental potential using colony forming assays, and ES-HP transplantation into immunodeficient mice.

2. **Examine the markers of immunogenicity during ES-HP development to predict host immune responses.** Currently, how the adult immune system will respond to cells differentiated from ESC sources is under intense debate. I hypothesize that expression of markers of immunogenicity provides a screening method to determine which components of the adult immune system are capable of provoking an immune response to ES-HPs isolated at different developmental stages, as well as derived using different culture methods. I performed flow cytometry at different time points, and using different culture methods to predict the functional immunogenicity *in vitro* and *in vivo* using 11 markers.

3. **Evaluate the functional immunogenicity of ES-HPs in vitro.** Understanding immunogenicity can be difficult based on markers of immunogenicity alone, because markers sometimes have dual roles in activation and inhibition within the immune system. To validate that host immune responses to ES-HPs can be predicted based on their immunogenicity marker profile, we will utilize assays measuring the proliferation of CD4+ T cells (mixed lymphocyte reaction), phagocytosis assays to evaluate interactions of ES-HPs with macrophages, or antigen presenting assays to evaluate the functional responses between macrophages, T cells, and ES-HPs or adult tissue.

4. **Evaluate ES-HPs after transplantation into adult host.** The transplantation studies proposed here are the most important functional portion of the project. I hypothesize that the adult immune system is a potential barrier to
ES-HPs. To test this hypothesis, I transplanted ES-HPs into adult mice. This will serve two purposes: first it will allow us to determine the hematopoietic potential of ES-HPs and second it will allow us to determine how well ES-HPs are tolerated by the adult host immune system.
Chapter 2. Development of ES-HPs in vitro

2.1 Introduction

Since ESCs in theory can make any cell in the adult body, they should be able to make functional hematopoietic progenitors and stem cells. While generating fully differentiated hematopoietic cells has been achieved from ESCs [170,171], generating multi-lineage reconstituting HSCs derived from ESCs has been problematic for the field [65,77].

One challenge is determining what cell-surface markers would be expressed on an ES-HPs, as the cell-surface marker phenotype that defines cell populations with the long term engraftment potential varies significantly between embryonic, adult, and ESCs derived populations (Table 1.1 and 1.2) [80,172]. HSCs from the adult bone marrow have been well characterized as lineage marker negative, c-Kit+, Sca-1+, IL-7Ralpha-, CD150+, CD48-, CD244- [54,98], while no markers are known to exclusively mark definitive HSCs in the early embryo [32,80]. Since the markers between adult and embryonic hematopoiesis are dependent on their location, it is difficult to predict what markers might be expressed on ES-HPs.

Two methods are generally used to make ES-HPs from ESC. The first method uses embryoid bodies (EBs); ESCs are plated either in hanging drops or methylcellulose to form EBs. The three basic germ layers spontaneously form in three-dimensional spheres. The other method employs bone marrow stromal cells to coax ESCs towards a hematopoietic fate. It has been recognized that hematopoietic development and lineage commitment can be studied using this method. OP9 is a bone marrow stromal cell line that was developed from mice lacking M-CSF, which prevented macrophages from taking over these cultures [173]. Further, OP9 supported lymphoid development from both HSCs and ESCs [170]. ES-HPs have been made with EBs and coculture systems, alone and in combination.

We directly compared markers and development potential of EB and ESC-OP9 coculture derived ES-HPs to embryonic tissue and adult bone marrow. We found that maturation using the ESC-OP9 system showed strong developmental kinetics, and we could culture enough to enable further characterization of these cells.
2.2 Materials and Methods

ESC Culture

Undifferentiated D3 ESCs (derived from 129 mice with an MHC haplotype H-2<sup>b</sup>) were purchased from ATCC (Manassas, VA, USA). ESCs were maintained and expanded on a mitomycin-C treated fibroblast STO (mSTOs) cell line also from ATCC in 6-well tissue culture treated plates. ESCs were cultured in DMEM media contained 15% ES-qualified fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA, USA), supplemented with 0.15mM monothioglycerol (MTG) (Sigma-Aldrich, St. Louis, MO, USA), 1x Penicillin-Streptomycin (1x Pen/Strep) (Invitrogen) and 1000 U/ml Leukemia Inhibitory Factor (LIF, Millipore, Billerica, MA, USA) to maintain ESCs in an undifferentiated and pluripotent state [13,174]. ESCs were passaged or fed daily. ESCs were passaged every 2 to 3 days by trypsinization (0.25% Trypsin-EDTA (Invitrogen)) of colonies and replating 0.5x10<sup>6</sup> ESCs onto new mSTO cells in the presence of ESCs cell media. Two days before differentiation cells were transferred to 0.1% gelatin coated plated to wean them from the feeder cells in IMDM (Invitrogen) media supplemented as described above. Cells were incubated in a humidified, 5% CO<sub>2</sub> incubator at 37°C.

ESC Differentiation

ESCs were differentiated into EBs by plating ESCs were plated in pluronic acid-coated 60mm petri dishes or 6-well plates in IMDM media with 1% methylcellulose (Fluka, Germany), 15% FBS (Atlanta Biologicals), 2mM glutamine, 200 ug/ml transferrin, 0.5 mM ascorbic acid (Sigma), 0.45 mM MTG, and 1x Pen/Strep [13]. ESCs were plated at a density of 5,000 to 20,000 cells per ml and subsequently fed at Day 5 and 10 post-plating [174].

For differentiation using coculture on bone marrow stromal cells, OP9 cells (ATCC) were cultured to 80% confluency in alpha-MEM media (Invitrogen) containing 20% FBS (Atlanta Biologicals) and 1x Pen/Step. ESCs were seeded onto OP9 layers at 130,000 cells per plate in 150mm diameter tissue culture plates in the presence of the cytokines Flt3L and IL-7 (Peprotech) both at 5 ng/ml in 20 ml of media per plate. Cocultures were fed at Day 4 post plating, by adding 10 ml of new media as listed above plus cytokines at 10 ng/ml instead of 5 ng/ml. For cultures longer than 7 days, at Day 7 cells were harvested from coculture and filtered through 64-µm nylon mesh (Small Parts Inc.) and replated onto fresh OP9 monolayers, and fed again on Day 11.

Antibodies, Flow Cytometry, and Cell Sorting
To prepare cells for flow cytometry and sorting, EBs and cocultures were harvested and dissociated in Collagenase Media containing M199 (Invitrogen), 0.125% w/v Collagenase D, and 0.1% v/v DNase I (from Roche) for 60 minutes at 37°C on a rocker, followed by mechanical dissociation with a syringe and 21G needle or P1000 pipette. Cells were washed with an equal volume of wash media (M199+2%FBS). All University of California, Merced institutional animal care and use policies and procedures were followed. Embryonic tissues were obtained from 129 mice (Jax Mice) were set-up overnight. If plug was observed, embryonic day was set at E0.5. Embryos collected were compared to Theiler to confirm stage of development [175]. Cells from embryonic tissues (yolk sac, fetal liver and placenta) were isolated and digested using the Collagenase Media above. Adult splenocytes and bone marrow were isolated as previously described [118].

0.5-1x10^6 viable cells were aliquoted for staining. Fc receptors were blocked using 2.4G2 (anti-CD16/CD32) (ATCC) supernatant for 30 minutes at 4°C. Antibody specific cocktails were then added to the appropriate tubes for 30 minutes at 4°C. To determine antigen specificity Isotype-matched control antibody staining was used for antigens where small shifts could be significant, otherwise fluorescence minus-one controls, or unstained cells were used to determine background. Cells were subsequently analyzed on BD FACS Aria II, III or LSR II flow cytometers (Milpitas, CA). Populations were gated based on forward and side light scatter properties and low level of DAPI staining to determine live, singlet cells. FlowJo (Tree Star) was used to perform data analysis. To obtain ES-HPs with stringent purity, ES-HPs were sorted using a yield sort followed by a purity sort or were isolated using 3 rounds of positive selection using EasySep kits (StemCell Technologies) followed by a purity sort on the BD Aria II or Aria III.

Colony-Forming Unit Assays

Sorted ES-HPs or specific hematopoietic populations from adult bone marrow or E17.5 fetal liver were added to 1 ml methylcellulose cytokine media in 35mm plates. Methylcellulose cytokine media was prepared 1% methylcellulose, 15% FBS, 0.2 mg/ml transferrin, 0.01 mg/ml insulin (Sigma), 0.05 mg/ml SCF (Peprotech), 0.01 mg/ml IL-3 (Peprotech), 0.01 µg/ml IL-6 (Peprotech), and 0.003 U/ml EPO (Peprotech) or Methocult GF M3434 (STEMCELL Technologies). Cells were incubated for 12 days and analyzed by counting colonies based on morphology. After analysis by counting, cells were harvested and cytospun (400 rpm for 8 minutes at room temperature) onto microscope slides. Slides were fixed for 2 minutes in ice-cold methanol and allowed to dry for 5 minutes. Slides were then stained with May-Grunwald (Fluka) for 3 minutes, rinsed with water, followed by staining with Giemsa (Gurr) for 20 minutes. Cover slips were mounted using Permount (Fisher Scientific) [176].
Table 2.1: Antibodies used to examine hematopoietic development

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD41</td>
<td>PE</td>
<td>MWReg30</td>
<td>BioLegend, 133906</td>
</tr>
<tr>
<td>CD41</td>
<td>PE/Cy7</td>
<td>eBioMWReg30</td>
<td>eBioscience, 25-0411-82</td>
</tr>
<tr>
<td>CD45</td>
<td>FITC</td>
<td>30-F11</td>
<td>eBioscience, 11-0451-82</td>
</tr>
<tr>
<td>CD45</td>
<td>PE</td>
<td>30-F11</td>
<td>eBioscience, 12-0451-82</td>
</tr>
<tr>
<td>CD45</td>
<td>PE/Cy7</td>
<td>30-F11</td>
<td>BioLegend, 103113</td>
</tr>
<tr>
<td>Flk-1</td>
<td>Biotin</td>
<td>Avas12a1</td>
<td>eBioscience, 13-5821-82</td>
</tr>
<tr>
<td>Flk-1</td>
<td>PE</td>
<td>Avas12a1</td>
<td>eBioscience, 12-5821-81</td>
</tr>
<tr>
<td>SSEA-1</td>
<td>AlexaFlour647</td>
<td>MC-480</td>
<td>BioLegend, 125607</td>
</tr>
<tr>
<td>SSEA-1</td>
<td>Biotin</td>
<td>MC-480</td>
<td>BioLegend, 12506</td>
</tr>
<tr>
<td>C-kit</td>
<td>APC/Cy7</td>
<td>ACK2</td>
<td>eBioscience, 47-1172-82</td>
</tr>
<tr>
<td>Sca-1</td>
<td>APC</td>
<td>D7</td>
<td>BioLegend 108111</td>
</tr>
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<td>Biotin</td>
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<td>CD48</td>
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<td>HW 48-1</td>
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<tr>
<td>AA4.1</td>
<td>APC</td>
<td>AA4.1</td>
<td>eBioscience, 17-5892-81</td>
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</tbody>
</table>
2.3 Results

ESCs were cultured on mSTO (Fig. 2.1 A) then transferred to gelatin-coated plates two days before starting differentiation. ESCs cultured on mSTO expressed SSEA-1 a marker of pluripotency for mouse ESC (Fig. 2.1 B) [12]. Both the morphology of the colonies and the expression of SSEA-1 on our ESCs suggest that they are pluripotent before differentiation.

To directly compare the effect of culture method on hematopoietic development, we used both EB and ESC-OP9 methods to generate ES-HPs. To verify that we could generate in vitro HSCs and HPCs that resembled HSCs and HPCs found in vivo using the EB and ESC-OP9 method, we used of flow cytometry to look at markers expressed on hematopoietic populations in fetal development and in the adult (Table 1.1), with the ultimate goal of making ES-HPs that resemble LT-HSCs (Table 1.2). We took advantage of two prominent cell surface markers expressed during embryonic development; CD41 which is transiently expressed on all cells destined to become hematopoietic during development [86,172], as well as CD45 which is expressed on all mature hematopoietic cells with the exception of erythrocytes [103] (Fig. 2.2). Since CD41 was expressed early in hematopoietic commitment, we used CD41 positive as the primary gating strategy and then assessed these cells for other hematopoietic and immunogenicity markers using both culture methods (Fig. 2.3 and 2.4). We found CD41 was expressed early in both embryonic and ES-HPs development (Fig. 2.2-2.4 and Table 2.2). In ESC-OP9 derived ES-HPs, CD41 expression transitions to CD41+, CD45+ double positive population at D10; this is followed by an increase in CD45+ populations by D16. Interestingly this pattern resembles expression of these markers in fetal liver, placenta, and adult bone marrow. Whereas in the yolk sac or EB derived populations CD41+ cells are predominate. These cultures had distinct morphologies (Fig. 2.3 A and Fig. 2.4 A). We then examined these cells for developmental hematopoietic markers. At D6 in differentiation process Flk-1 is expressed on CD41+ cells in EB, while it is expressed at both D4 and 6 on cocultured cells (Fig. 2.3 B top). A small amount of c-kit staining is found on early EB generated ES-HPs, but is substantially higher on cocultured cells (Fig. 2.3 B middle). At D4, SCA-1 expression is high on ESC-OP9 cells and is reduced to moderate levels by D6 (Fig. 2.3 B bottom). On EBs SCA-1 expression is moderate at both D4 and D6 (Fig 2.3 B bottom).

Pictures of late hematopoietic development are shown in figure 2.4 A. CD150 and C-kit are both expressed at high levels on murine BM LT-HSCs [54]. Flow cytometry plots were gated on CD41 positive cell. ES-HPs generated using the EB method express C-kit, SCA-1 CD150 and CD48 at D7 (Fig. 2.4 B 1st column). By D16, ES-HPs express higher levels of the markers expressed at D7, plus AA4.1 and CD34 (Fig. 2.4 B 2nd column). Using the coculture method ES-
HPs at D7 express C-kit, SCA-1, AA4.1, CD150, and CD48 (Fig. 2.4 B 4th column). By D16 ES-HPs expressed the same markers as at D7, plus some cells expressed CD34 (Fig. 2.4 B 5th column). We compared our ES-HPs generated in vitro to hematopoietic cells generated during embryonic development, at E14.5 CD41+ cells in the fetal liver express C-Kit, CD150, CD48 and CD34 (Fig. 2.4 B 3rd column). In the adult, CD41+ cells in the bone marrow express C-Kit, AA4.1, CD150, and CD48 (Fig. 2.4 B 6th column), while CD45+ cells express C-kit, SCA-1, AA4.1 and CD48 (Fig. 2.4 B 7th column).

Expressions of developmental markers on ES-HPs are summarized in table 2.2. Importantly the total number of CD41+ ES-HPs generated using the coculture method was 2.09 fold higher per cm² of tissue culture plate area than those obtained by the EB method (data not shown).

To determine if ES-HPs could produce CFU colonies, ES-HPs from both EBs and cocultures were plated at D7 and D16 at various densities and allowed to form hematopoietic colonies. LSK populations from fetal liver E17.5 and adult bone marrow were sorted and plated as controls. ES-HPs generated on OP9 monolayers had more CFU potential when compared with EB generated ES-HPs, although all ES-HP populations had at least some CFU potential (Fig. 2.5 A). Fetal liver and bone marrow LSK cells are highly enriched for HSCs and other progenitor populations, as expected they formed colonies at a much higher rate than ES-HPs (Fig. 2.5 A). Importantly, ES-HPs generated colonies with multilineage potential (Fig. 2.5 A and B).
Figure 2.1: ESC growth and maintenance.

A. D3 ESCs grown on mSTO monolayers. Scale bar = 889 microns. B. Flow cytometry of ESC grown on mSTOs. Black is unstained D3 ESCs and blue is specific staining for SSEA-1.
Figure 2.2: CD41 and CD45 reveal strong developmental kinetics in ES-HPs and embryonic tissues.

Gated on size and live cells. Shown in flow cytometry for CD41 (y-axis) and CD45 (x-axis) expression on ESC-OP9 cocultures (top), and EB (middle) at D7, D10 and D6. Control tissues yolk sac at E12.5, placenta E12.5, fetal liver E12.5 and adult BM (bottom).
Figure 2.3: Early hematopoietic development of ES-HPs.

A. EB (left) and ESC-OP9 (right) coculture pictures taken at D2, 4, and 6 of differentiation. Scale bar= 889 um. B. Early hematopoietic marker expression on early ES-HPs. Gated on CD41+ cells. Specific staining shown in blue with background level of unstained cells shown in black. Hematopoietic cells from control tissue placenta, fetal liver, and bone marrow are also shown.
### Figure 2.4: Late markers of hematopoiesis on ES-HPs.

**A.** EB (left) and ESC-OP9 (right) coculture pictures taken at D7 and 16 of differentiation. Scale bar=254 μm.  
**B.** Late hematopoietic marker expression on early ES-HPs. Gated on CD41+ cells. Specific staining shown in blue with background level of unstained cells shown in black. Hematopoietic cells from control tissue placenta, fetal liver, and bone marrow are also shown.
Figure 2.5: ES-HPs are capable of CFU formation and have multi-lineage potential \textit{in vitro}.

\textbf{A.} Quantitation of CFU Colonies from ES-HP cultures and LSK sorted from fetal liver and bone marrow. \textbf{B.} CFU cytospun and stained with Giemsa and May-Grunwald. Scale bar=50 micron.
<table>
<thead>
<tr>
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<th>EB D16</th>
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2.4 Discussion

In this study, we have shown that the method of differentiation produces ES-HPs with expression of different developmental markers. Further, EB generated hematopoietic cells with less colony forming ability than ESC-OP9 generated ES-HPs. Further, CD41 and CD45 expression showed strong developmental kinetics that can be used to evaluate stages of hematopoietic development.

Our results are consistent with McKinney-Freeman et al. who evaluated both cell surface markers on HoxB4 transduced ES-HPs (EPOCH) and compared them to hematopoietic fetal tissues. Our ESC-OP9 at D16 display similar developmental profiles to EPOCH cells [80]. Our ESC-OP9 cocultures generate ES-HPs that generate significantly more CFU colonies than EBs. This suggests that cocultured ES-HPs may have more potential to reconstitute an adult mouse.

While many labs have been able to generate hematopoietic progenitors and terminally differentiated populations from ESCs, few groups have been able to generate ES-HPs that are capable of regenerating the adult host immune system without transfection or direct intra-femoral injection. One hypothesis to explain this is that ES-HPs are a blend of adult and fetal hematopoietic phenotypes and our phenotypic analysis is consistent with this hypothesis. However, even fetal cells down as early as E9 are capable of long-term multi-lineage reconstitution [34]. We hypothesize that other mechanisms may account for the poor reconstitution ability of ES-HPs.

In addition to their developmental stage, we wanted to know how ES-HPs at various developmental stages might be recognized by the adult host immune system, since these interactions could limit their ability to reconstitute an adult host. To test this we screened eleven markers of immunogenicity on ES-HPs, as well as on adult and embryonic tissue to predict what immune barriers they would encounter after transplantation.
Chapter 3. Surface Marker Expression and Prediction of ES-HP Immunogenicity

3.1 Introduction

Similar to any transplantation procedure performed, cell products such as ESCs and their derivatives have potential immunogenicity [177]. The immunogenicity of ESCs and their derivatives is remarkably contentious [1]. Recently, a similar debate has emerged with induced pluripotent stem cells (iPSC) [159,166,178,179]. While some groups find a reduced immune response to ESCs, other groups find that these cells are capable of stimulating an immune response [180] (Table 1.3 and 1.4).

The immune system is complex and multiple cell types have been implicated in rejection of allogeneic tissue [181]. MHC-I presents endogenous antigens to CD8+ T cells, while MHC-II presents exogenous antigens to CD4+ T cells [106]. Allogeneic MHC molecules present a strong signal to T cells, but for efficient activation, costimulatory signals are also needed [182,183]. CD80 (B7-1) and CD86 (B7-2) deliver activating signals through CD28, however they can negatively regulate the T cell responses by binding to CTLA-4 instead [183].

Non-classical MHC molecules, including Qa-1b and Qa-2 are structurally similar to classical MHC molecules, but have fewer polymorphisms [184]. However, they are still able to present a limited repertoire of peptides to CD8 T cells and can also bind receptors on NK cells [184]. Qa-1b can induce minor allogeneic responses, but these responses are weaker than allogeneic responses to classical MHC molecules [185,186]. When Qa-1b is loaded with the signal peptide Qdm it becomes an inhibitory ligand for NK cells through the NKG2GA inhibitory receptor [185]. The majority of Qa-1b molecules are loaded with Qdm, however Qa-1b has also been shown to present peptides from pathogens, mammalian heat-shock protein 60, as well as an epitope from insulin to CD8+ T cells [185]. Interestingly, CD8+ T regulatory cells that are restricted to self-peptides presented by Qa-1b have been reported as a mechanism to limit autoimmunity [187]. HLA-G the human homolog for Qa-2 was originally described in the trophoblast, which suggested a role in maintaining fetal-maternal tolerance [188]. In early embryos Qa-2 is expressed from the 2 cell stage and on both the inner cell mass and trophectoderm of the blastocyst [189,190]. Embryos expressing Qa-2 were shown to have higher survival rates than embryos lacking Qa-2 and also had faster cleavage rates [188-190]. Within the immune system, Qa-2 can deliver inhibitory signals to NK cells and it can also present peptides to T cells in a process dependent on TAP (transporter associated with antigen processing) [191,192].
NK cells are incredibly adept at destroying transformed and damaged cells [119]. In BM transplantation they can destroy cells that do not have the correct level of MHC-I on their surface [118]. NK cells detect the level of responses through activating and inhibitory receptors expressed on their surface [193]. Activating NK receptor ligands in the mouse include Rae-1, H60, and Mult-1 [184]. Interestingly, Rae1 and H60 are expressed in embryonic tissues, but are rarely present in adult tissue unless it has been transformed [184].

CD47 and CD200 are both macrophage inhibitory receptor ligands and function to educate macrophages what is self from what is non-self [135,194]. CD47 deficient hematopoietic cells were readily phagocytosed by macrophages [195]. Similarly, CD47 deficient bone marrow was unable to reconstitute both allogeneic and RAG deficient host, but could reconstitute RAG.CD47 double knockout recipients [195]. CD200 (Ox-2) is a transmembrane glycoprotein expressed on a wide variety of cells including hematopoietic cells, keratinocytes, endothelial cells, central and peripheral nerves, and trophoblast cells [196], while CD200R the receptor is expressed on a wide variety of immune cells including macrophages, neutrophils, dendritic cells, as well as T and B cells [196]. While structurally, CD200 is related to the costimulatory molecules CD80 and CD86, the phenotype of CD200 knockout mice suggests its role in inhibiting macrophages [136,197]. CD200 knockout mice have increased numbers and activation of macrophages, and are more susceptible to several autoimmune diseases [197]. Similar to CD47, CD200 has a role in graft rejection [196]. Skin grafts from CD200 deficient (sex mismatch) mice rejected in less than half of the time of grafts expressing CD200 [196].

To investigate stage-specific immunogenicity we derived ES-HPs using both EB and ESC-OP9 methods and examined them for a broad panel of eleven markers of immunogenicity. Using this method we predicted immune responses to ES-HPs that were distinct from those associated with adult bone marrow.
Table 3.2: List of Immunogenicity markers studied on ESCs and ESCs derived populations

<table>
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<th>Population Defined</th>
<th>MHC - I</th>
<th>MHC - II</th>
<th>CD8 0</th>
<th>CD8 6</th>
<th>Qa -1</th>
<th>Qa -2</th>
<th>Mult -1</th>
<th>Rae -1</th>
<th>H6 0</th>
<th>CD4 7</th>
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<tr>
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<tr>
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</table>
3.2 Materials and Methods

ESC Culture

Undifferentiated D3 ESCs (derived from 129 mice with an MHC haplotype H-2\(^b\)) were purchased from ATCC (Manassas, VA, USA). ESCs were maintained and expanded on a mitomycin-C treated fibroblast STO (mSTOs) cell line also from ATCC in 6-well tissue culture treated plates. ESCs were cultured in DMEM media contained 15% ES-qualified fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA, USA), supplemented with 0.15mM monothioglycerol (Sigma-Aldrich, St. Louis, MO, USA), 1x Penicillin-Streptomycin (1x Pen/Strep) (Invitrogen) and 1000 U/ml Leukemia Inhibitory Factor (LIF, Millipore, Billerica, MA, USA) to maintain ESCs in an undifferentiated and pluripotent state [13]. ESCs were passaged or fed daily. ESCs were passaged every 2 to 3 days by trypsinization (0.25% Trypsin-EDTA (Invitrogen)) of colonies and replating 0.5x10⁶ ESCs onto new mSTO cells in the presence of ESC cell media. Two days preceding differentiation cells were transferred to 0.1% gelatin coated plated to wean them from the feeder cells in IMDM (Invitrogen) media supplemented as described above [13]. Cells were incubated in a humidified, 5% CO₂ incubator at 37°C.

ESC Differentiation

ESC were differentiated into EBs by plating ESCs on pluronic acid-coated 60mm petri dishes or 6-well plates in IMDM media with 1% methylcellulose (Fluka, Germany), 15% FBS (Atlanta Biologicals), 2mM glutamine, 200 ug/ml transferrin, 0.5 mM ascorbic acid (Sigma), 0.45 mM monothioglycerol, and 1x Pen/Strep [13]. ESCs were plated at a density of 5,000 to 20,000 cells per ml and subsequently fed at Day 5 and 10 post-plating.

For differentiation using coculture on bone marrow stromal cells, OP9 cells (ATCC) were cultured to 80% confluency in alpha-MEM media (Invitrogen) containing 20% FBS (Atlanta Biologicals) and 1x Pen/Step. ESCs were seeded onto OP9 monolayers at 130,000 cells per plate in 150mm diameter tissue culture plates in the presence of the cytokines Flt3L and IL-7 (Peprotech) both at 5 ng/ml in 20 ml of media per plate. Cocultures were fed at Day 4 post plating, by adding 10 ml of new media as listed above plus cytokines at 10 ng/ml instead of 5 ng/ml. For cultures longer than 7 days, at Day 7 cells were harvested from coculture and filtered through 64-µm nylon mesh (Small Parts Inc.) and replated onto fresh OP9 monolayers, and fed again on Day 11.

Flow Cytometry
EBs were harvested and digested in “Collagenase Media”, which is comprised of M199 containing 0.125% w/v Collagenase D and 0.1% v/v DNAse I (both from Roche), for 60 minutes at 37°C, followed by dissociation with a syringe and a 21G needle or a P1000 pipette. 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 minutes. After dissociation, cells were filtered through nylon mesh with a pore size of 64 µm (Small Parts Inc., Miramar, FL, USA). Cells from ESC-OP9 cultures were harvested from plates by using cell lifters and vigorous pipetting and/or collagenase digestion. The yolk sac, placenta, and fetal liver were removed from mouse embryos. Embryonic tissues were digested in Collagenase Media for 60 minutes at 37°C. The bone marrow (from tibiae and femur) and spleen from the adult mother was also collected, as described [199]. Live cell numbers were determined using hemacytometer and trypan blue exclusion.

For flow cytometry, a maximum of 1x10⁶ live cells were aliquoted for staining. Fc receptors were blocked with 2.4G2 (anti-CD16/CD32) hybridoma supernatant for 30 minutes on ice. Cells were subsequently stained with specific antibody cocktails for 30 minutes on ice. Antibodies for specific antigens were used as listed in Table 3.2. As controls, isotype controls were at the same dilution as the specific antibody (Table 3.2).
<table>
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<td>miap301</td>
<td>eBioscience 11-0471</td>
<td>Rat IgG2a</td>
<td>Used by macrophages as a 'don't eat signal'.</td>
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</table>
3.3 Results

The purpose of examining ES-HPs using different culture methods and over multiple time points was to understand the relationship between development and potential immunogenicity of ES-HPs.

To test how T lymphocytes could respond to ES-HPs we tested for classical MHC expression (MHC-I, MHC-II), and costimulatory molecule expression (CD80 and CD86), as well as expression of non-classical MHC molecules Qa-1b and Qa-2. While all tissue controls (placenta, fetal liver, and bone marrow) express MHC-I, ES-HPs derived by EB did not express MHC-I during our time course. However in D16 ES-HPs generated using the OP9 method MHC-I is significantly upregulated (Fig. 3.1 top row). This suggests that EBs and ESC-OP9 cultures at early stages (before D16) may be resistant to CTL lysis, since MHC I is used to present peptides to CTLs [106]. At the same time, this very same protection from CTLs may make EB derived ES-HPs more susceptible to NK cell lysis [200]. While MHC-I is expressed on all adult cells except red blood cells, MHC-II is only present on professional antigen presenting cells [106]. We did not observe MHC-II expression on ES-HPs populations (Fig. 3.1, row 2). This suggests that ES-HPs would not be able to present antigen directly to CD4+ T cells.

Costimulatory molecules have multiple functions in immune responses and regulation. For example CD80 (B7-1) and CD86 (B7-2) can prime a T lymphocyte effector response through interactions with CD28. Conversely these same molecules can negatively regulate immune responses through either interactions with CLTA-4 or through the induction of regulatory T cells [116,183]. We found that EBs do not express either CD80 or CD86 (Fig 3.1 row 3 and 4). However, ESC-OP9 derived ES-HPs express CD80 at D7 and express CD86 at D16 (Fig 3.1 row 3 and 4).

To determine how ES-HPs could interact with NK cells, we examined the expression of NK receptor ligands. These included activating receptor ligands retinoic acid early inducible gene (RAE-1), murine ‘ULBP-like transcript 1’ (MULT-1), and H60. These activating ligands are upregulated in response to cellular stress such as DNA damage, transformation, or infection [119]. Qa-1b functions both as an NK receptor ligand through the inhibitory receptor CD94/NKG2A or through the activating CD94/NKG2C and CD94/NKG2E and can present a limited repertoire of peptides to CD8+ T cells [185,189]. Although Qa-1b can engage both inhibitory and activating receptors on NK cells, it preferentially binds to the inhibitory receptor [201]. Similarly Qa-2 can inhibit NK cells through binding NK receptor inhibitory ligands and it can also present peptides to CD8+ T cells [192]. In addition to presenting antigens to CD8+ T cells, MHC-I has a major role in inhibiting NK cell responses [118,119]. We did not observe any upregulation of...
any activating receptor ligands (RAE-1, MULT-1, and H60) (Fig. 3.3), and only saw the presence of inhibitory receptor ligands (MHC-I, Qa-1b, and Qa-2) on cocultured ES-HPs (Fig 3.1 and 3.2). This suggest that syngeneic NK cells would not be sufficiently activated to kill our D16 ESC-OP9 generated ES-HPs.

Several inhibitory receptors have recently been described for macrophages. Given the propensity for ESCs and their derivatives to disappear after transplant, we wanted to examine the expression of these molecules on ES-HPs. The best characterized is CD47, which has recently been described as a ‘don’t eat me’ signal for macrophages; CD47 delivers an inhibitory signal through SIRP-α on macrophages [195]. We examined CD47 on ESC-OP9 grown ES-HPs, and at D7 no CD47 was detectable, while at D16 a minute amount was present compared to strong expression on CD45+ cells in the bone marrow (Figure 3.4 top). These results were also confirmed by qPCR (not shown). We also examined expression of CD200 (Ox-2) on ES-HPs. CD200 has also been characterized as an inhibitory ligand for macrophages [135]. Similar to CD47, CD200 was not detectable of ESC-OP9 ES-HPs at D7, and little to no expression was present on D16 cells (Figure 3.4 bottom row). Together the CD47 and CD200 data suggests that macrophages maybe a barrier to ES-HP transplantation.
Figure 3.1: T cell immunogenicity marker expression.

Specific expression in blue, while isotype controls are shown in black-gray shaded region. **Top.** Expression of H2-Kb (-I). **2nd Row.** Expression of I-Ab (MHC-II). **3rd Row.** Expression of CD80 (B7-1). **Bottom Row.** Expression of CD86 (B7-2).
Figure 3.2: Expression of non-classical MHC molecules on ES-HPs.

Specific expression in blue, while isotype controls are shown in black-gray shaded region. **Top.** Expression of Qa-1. **Bottom.** Expression of Qa-2.
Figure 3.3: Expression of NK activating ligands on ES-HPs.

Specific expression in blue, while isotype controls are shown in black-gray shaded region. **Top.** Expression of Rae-1. **2nd Row.** Expression of H60. **Bottom.** Expression of Mult-1.
Figure 3.4: Expression of macrophage inhibitory molecules on ES-HPs. Specific expression in blue, while isotype controls are shown in black-gray shaded region. **Top.** Expression of CD47. **Bottom Row.** Expression of CD200.
3.4 Discussion

The purpose of this study is to determine the changes in immunogenicity that occurs during the development of hematopoietic progenitors when they are differentiated stepwise from mouse embryonic stem cells (ESCs). While some studies show that ESC derived cell populations may be tolerated better by the adult immune system, the extent to which this protection persists as cells are differentiated is currently unknown.

Understanding the stage-specific immunogenicity of these cells in the context of the adult immune system can improve their use for regenerative medicine. If the immunogenicity of these cells is understood then the host can be specifically conditioned to allow for survival of ESC-derived hematopoietic progenitors (ES-HPs), potentially without the need for broad immunosuppressive therapies.

More recently, CD86 has been reported on HSCs bestowed with greater lymphoid potential [63]. However, the true function of costimulatory B7 molecules on HSCs has yet to be determined [63]. Interestingly, ES-HPs grown on OP9 express CD80 at D7 and CD86 at D16. Consistent with this, ES-HPs grown on OP9 are known to have lymphoid potential, as this system has been extensively used to study B cell and T cell development [170].

MHC-I expression is progressively upregulated on our cocultured ES-HPs. This is consistent with the literature, which suggest that MHC-I is upregulated as ESCs differentiate. In embryonic development, MHC-I is not expressed until E7.5, which is 3 days post-implantation [202]. MHC-I is a major alloantigen recognized during rejection of adult tissues and organs [106]. Wu et al. examined CD8 T cell responses to ESC derived insulin producing cell clusters (IPCC) using a TCR-transgenic model specific for H-2Kb. While allogeneic CD8+ T cells did respond to the IPCC, they were not responding to the ESC derived form of the MHC-I molecule [167]. The role of non-classical MHC molecules was not examined in this model, but maybe an alternate avenue of presentation to CD8+ T cells. It also underscores differences between adult and embryonic tissues in regards to how the immune system can recognize and respond to antigens.

Our initial profiling of possible ES-HP immunogenicity markers was extraordinarily helpful in predicting the host immune response, and we have focused our efforts to examine the mechanisms of host macrophage recognition of ES-HP after transplantation. CD47 and CD200 are macrophage inhibitory receptor ligands that prevent macrophage phagocytosis and activation after engagement with its receptors on target cells [136,195,203,204]. Previously, we found that ES-HPs express lower levels of CD47 than adult hematopoietic stem cells in the bone marrow by qPCR. D16 ES-HPs express higher levels than D7
ES-HPs by qPCR; this was also confirmed by flow cytometry. Corresponding to this observation, we did not detect engraftment of D7 ES-HPs, but we did observe low levels of engraftment of Day 16 ES-HPs. Examination of publically available data shows that CD47 is directly targeted and pulled down by ChIP assays using antibodies to HoxB4 [69,205]. This increase in CD47 may contribute to the increased reconstitution ability of HoxB4 transfected ES-HPs. Similar to CD47, we detected little to no CD200 on ES-HPs. Combined the low expression of both CD47 and CD200 suggests that ES-HPs may be preferentially phagocytosed by macrophages after transplantation.

From our immunogenicity data, we hypothesized that ES-HPs may be preferentially phagocytosed by macrophages. ES-HPs might not be able to present their antigens directly to CD4+ T cells. However, macrophages are professional antigen presenting cells (APCs), consequently they might be able to present ES-HP antigens to CD4+ T cells. We next tested these hypotheses in vitro (Chapter 4).
Chapter 4. *In vitro* immunogenicity of ES-HPs

4.1 Introduction

Predicting the immune response based on cell surface markers for immunogenicity alone can be difficult, as some have multiple roles in immune responses and can in some settings be used for activation, while providing inhibitory signals in other settings [1]. Several assays have been established in the field of transplantation to predict if host and donor are compatible [206,207]. *In vitro* tests have been developed to test CD4 responses (mixed lymphocyte reaction), CD8 responses (cytotoxic T lymphocyte assays), NK cell responses (NK cell lysis assay), and macrophage responses (phagocytosis assays) [1]. Here we focus on CD4+ T cell responses and macrophage responses based on the panel of immunogenicity markers from Chapter 3.

The classic *in vitro* test to determine if CD4 T cells recognize a specific antigen is the mixed lymphocyte reaction (MLR) [206]. The readout of the MLR is T cell proliferation and can be measured by $^3$H-Thymidine, CFSE dilution, or Ki67 protein upregulation [206,208]. In a one-way MLR, antigen-presenting cells (APCs) or splenocytes are irradiated so they do not proliferate, then responding splenocytes or CD4+ cells can be added.

Phagocytosis assays have long been used for testing macrophage responses to microorganisms and for opsonized particles [209]. These assays can readily be adapted to testing mammalian cells after labeling targets with a fluorescent marker [210] and they can be measured by FACS or immunofluorescence [195,211,212].

APCs are cells that are specialized in presenting antigens to T cells, such as dendritic cells, B cells, or macrophages. To test if ES-HPs are taken up by APCs, phagocytosis assays can be used. T cells recognize antigens in the context of MHC molecules using either direct or indirect pathways [106]. With direct CD4+ responses, antigen-presenting cells (APCs) transferred in the transplanted tissue, present donor antigens in the context of donor APCs [106]. Conversely, during indirect recognition, donor antigens are picked up and presented by host APCs [106]. Specific contributions of indirect and direct presentation can be evaluated using antibodies that specifically block host or donor MHC molecules presenting ES-HP antigens.

Few *in vitro* approaches have been used to study ESCs and their derivatives (Table 4.1). Also, none have specifically explored the intimate relationship between APC, T cells, and ES-HPs *in vitro*. Based on markers of immunogenicity expressed on ES-HPs, we set out to validate whether the immunogenicity marker profile allowed us to predict *in vitro* responses to ES-HPs. Based on the expression of markers of immunogenicity, we hypothesize that ES-HPs would be 1) readily phagocytosed by macrophages, 2)
macrophages would be able to cross present ES-HPs antigens to CD4+ T cells, 3) ES-HPs in the context of cross presentation would be able to stimulate CD4+ T cell responses.
Table 4.1: Immune responses to ESCs and ESC derived populations *in vitro*

<table>
<thead>
<tr>
<th>Author</th>
<th>Cell</th>
<th>MLR</th>
<th>CTL</th>
<th>NK</th>
<th>Other</th>
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</thead>
<tbody>
<tr>
<td>Bonde et al., 2006 [149]</td>
<td>129 ESC</td>
<td>Poor</td>
<td>Poor</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td>Bonde et al., 2006 [149]</td>
<td>129 ESC treated with IFN-γ</td>
<td></td>
<td>Poor</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td>Bonde, et al., 2008 [168]</td>
<td>CCE HoxB4+ GFP+, CD45+</td>
<td>Poor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koch, et al., 2009 [157]</td>
<td>C57BL/6, BALB/c x 129, 129 ESC</td>
<td></td>
<td></td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td>Dressel, et al., 2008 [164]</td>
<td>MPI-II, Mouse 129 ESC</td>
<td>Suppressed T cell responses</td>
<td></td>
<td></td>
<td>Killed by xenogeneic NK cells, but not by syngeneic NK cells</td>
</tr>
<tr>
<td>Thompson, et al. [104]</td>
<td>129 D3 ES-HPs</td>
<td>Stimulation in the context of additional APCs. APC assays showing Mφ present ES-HP antigens to CD4+ T cells</td>
<td></td>
<td></td>
<td>Phagocytosis assays showing Mφ preferentially uptake ES-HPs.</td>
</tr>
</tbody>
</table>
4.2 Materials and Methods

ESC Culture

Undifferentiated D3 ESCs (derived from 129 mice with an MHC haplotype H-2^b^) were purchased from ATCC (Manassas, VA, USA). ESCs were maintained and expanded on a mitomycin-C treated fibroblast STO (mSTOs) cell line also from ATCC in 6-well tissue culture treated plates. ESCs were cultured in DMEM media contained 15% ES-qualified fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA, USA), supplemented with 0.15mM monothioglycerol (Sigma-Aldrich, St. Louis, MO, USA), 1x Penicillin-Streptomycin (1x Pen/Strep) (Invitrogen) and 1000 U/ml Leukemia Inhibitory Factor (LIF, Millipore, Billerica, MA, USA) to maintain ESCs in an undifferentiated and pluripotent state [13]. ESCs were passaged or fed daily. ESCs were passaged every 2 to 3 days by trypsinization (0.25% Trypsin-EDTA (Invitrogen)) of colonies and replating 0.5x10^6 ESCs onto new mSTO cells in the presence of ESC cell media. Two days prior to differentiation cells were transferred to 0.1% gelatin coated plated to wean them from the feeder cells in IMDM (Invitrogen) media supplemented as described above [13]. Cells were incubated in a humidified, 5% CO₂ incubator at 37°C.

ESC Differentiation

For differentiation using coculture on bone marrow stromal cells, OP9 cells (ATCC) were cultured to 80% confluency in alpha-MEM media (Invitrogen) containing 20% FBS (Atlanta Biologicals) and 1x Pen/Step. ESCs were seeded onto OP9 layers at 130,000 cells per plate in 150mm diameter tissue culture plates in the presence of the cytokines Flt3L and IL-7 (Peprotech) both at 5 ng/ml in 20 ml of media per plate. Cocultures were fed at D4 post plating, by adding 10 ml of new media as listed above plus cytokines at 10 ng/ml instead of 5 ng/ml. For cultures longer than 7 days, at D7 cells were harvested from coculture and filtered through 64-µm nylon mesh (Small Parts Inc.) and replated onto fresh OP9 monolayers, and fed again on D11.

One-Way Mixed Lymphocyte Reaction (MLR) Assays

Spleens were harvested from 129 (H-2^b^) BALB/c (H-2^d^) and crushed with the base of a 5 m syringe to make a single cell suspension. Cells were spun down at 2000 rpm, for 5 minutes 4°C. Red blood cells were removed by lysis using ACK buffer, followed by filtration through nylon mesh. Responder cells were washed twice in warm 37°C 1x PBS in a centrifuge at room temperature. Responder cells were resuspended at 5x10^6 cells per ml. One ul of 5mM carboxyfluorescein diacetate succinimidyl ester (CFSE) was added per ml of responder cells. Cells were incubated for 10 minutes at 37°C. The staining was
then quenched by washing cells in DMEM plus 5% FBS (DMEM+5%) at 37°C, followed by centrifugation at 2000 rpm for 10 minutes. Cells were then incubated for another 30 minutes at 37°C and washed two additional times. 2x10⁵ responder cells were plated per well. Splenocytes containing additional antigen presenting cells were prepared as stimulators by irradiation with 2000 rads with a ¹³⁷Cs irradiator (J. L. Shepherd and Associates). Stimulators were plated at 1x10⁴ since this was experimental determined to be the lowest dose in which differences between major and minor alloantigens could be detected. 1x10⁴ FACS sorted CD4⁺ ES-HPs from ESC-OP9 cocultures at day 16 were plated into the appropriate wells. After 5 days of incubation at 37°C at 5% CO₂ proliferation of responder cells for each condition were analyzed.

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

\[
\text{% CD4⁺ cells divided} = \frac{\text{% CD4pos CFSElow/int}}{\text{% CD4pos CFSEhigh plus %CD4pos CFSElow/int}}
\]

The stimulation index (SI) for each MLR culture was calculated using the following formula:

\[
\text{SI} = \frac{\text{% CD4pos cells that divided}}{\text{% CD4 "background response"}}
\]

Background or normalize response was equated to the SI for syngeneic stimulator cells alone. Two-tailed Student’s t-test assuming unequal variance between groups was performed with Graph Pad (Prism) and differences were considered statistically significant if p<0.05.

In vitro Phagocytosis Assays

Spleens were harvested from NSG (H-2<sup>g7</sup>), BALB/c (H-2<sup>d</sup>), and 129 (H-2<sup>b</sup>) mice. Single cell suspensions were prepared. Macrophages were enriched by adherence to plastic tissue culture dishes for a minimum of 8 hours in a humidified incubator at 37°C with 5% CO₂. Non-adherent cells were then washed off the plates via gentle pipetting with warm 1xPBS. Adherent cells were removed from plates by trypsinization 5 minutes at 37°C, and counted via hemacytometer. Fifty thousand enriched macrophages were plated per well. CD4₁⁺ ESHPs and adult lineage-negative bone marrow (Lin⁻ BM) were sorted
and labeled with CFSE. Ten thousand labeled cells were plated with the macrophages in each well, and then were incubated for 3 hours at 37°C. Phagocytosis cultures were harvested and stained with anti-F4/80 antibody conjugated to APC (clone BM8) (BioLegend). The percentage of F4/80+ macrophages that phagocytosed targets was determined by the formula:

\[
\% \text{ F4/80 M}\Phi \text{ that have phagocytosed a labeled target} = \frac{(%\text{CFSEposF4/80pos})}{\% \text{ total F4/80pos}} \times 100
\]

Two-tailed Student’s t-test assuming unequal variance between groups was performed on Graph Pad (Prism) and differences were considered statistically significant if \( p \leq 0.05 \).

Phagocytosis Assay Using Immunofluorescence

Coverslips were added to each well, followed by macrophages and ES-HPs as above. Alexa Fluor 633 hydrazide, bis(triethylammonium) salt (Life Technologies, A30634) was added to the media to label the fluid phase (red) [211]. Cells were washed and counter stained with F4/80 biotin followed by Streptavidin conjugated to Pacific Blue. Cells were then imaged under oil immersion.

In vitro Antigen Presenting Assay

BALB/c (H-2\( ^d \)) mice were used to obtain single cell suspensions. Macrophages were stained with F4/80 Biotin (clone BM8) (BioLegend). Easy Sep Bio Selection (Stem Cell Technologies) was used to isolate macrophages from splenocytes with a purity of 95%. Mouse CD4+ T Cell Enrichment Column (R&D Systems) was used to isolate T cells from splenocytes. CFSE was used to label T cells as described above. 0.02x10^6 macrophages, 0.01x10^6 ES-HPs or Lin- BM were added as stimulators with 0.1x10^6 CD4+ T cell per well. Anti-MHC-II (3.75 ug) (clone 14-4-4S)(eBioscience) was added to respective wells to block indirect antigen presentation to T cells. Cells were harvested after 4 days; blocked with 2.4G2 supernatant, stained with CD4 APC (RM4.5) (BioLegend) and DAPI, and run on the ARIA II. Two-tailed Student’s t-test assuming unequal variance between groups was performed on Graph Pad (Prism) and differences were considered statistically significant if \( p \leq 0.05 \).
4.3 Results

Based on proteins expressed on mature hematopoietic cells such as MHC-I and CD86 (Fig. 3.1), we hypothesized that ES-HPs could stimulate a CD4+ T cell response. To test this question directly, we adapted a traditional one-way MLR [206]. As the measure of proliferation we used CFSE because it could be used to discriminate division of a specific population. CFSE\textsuperscript{high} cells represent CD4+ cells that did not divide, while CFSE\textsuperscript{intermediate-low} cells represent CD4+ cells that have undergone one or more rounds of division [213]. To ensure reliability with a limited number of ES-HPs, we first determined the lowest stimulator cell number that could reliably determine the difference between major and minor antigenic differences was 1,000 stimulator cells (Fig. 4.1). We determined that 10,000 stimulator cells were sufficient to determine the difference between major and minor antigens and would be a reasonable number of cells that would allow us to perform experimental replicates.

We then performed one-way MLR using naïve splenocytes responders and compared CD4+ T cell responses to D16 ES-HPs from the ESC-OP9 coculture system, splenocytes, and a combination of splenocytes and ES-HPs. ES-HPs generated less CD4+ T cell proliferation than splenocytes in syngeneic settings (Fig. 4.3), and the addition of syngeneic splenocytes and ES-HPs generated a response that was statistically significant compared to syngeneic splenocytes alone (Fig. 4.3). While ES-HPs generated more response than splenocytes alone in the allogeneic setting it was not significant, allogeneic splenocytes and ES-HPs generated more CD4+ T cell division than allogeneic splenocytes alone (Fig. 4.2), although not statistically significant. Interestingly, CD4+ cells responding to ES-HPs had increased FSC-W, which indicates an increased activation state of CD4+ cells [214]. These cells have also divided fewer times than those responding to splenocytes, which may reflect the need for ES-HPs to be picked up and processed by the addition of splenocytes containing additional APCs before CD4+ T cells can respond to their antigens. Since additional splenocytes and ES-HPs generated a more vigorous response than ES-HPs alone, we hypothesized that ES-HPs were indirectly presented to T cells.

Our immunogenicity marker study also revealed that ES-HPs might be subject to phagocytosis by host macrophages (Fig. 3.4). In order to examine the relationship between host macrophages and ES-HPs directly, I adapted an \textit{in vitro} phagocytosis assay [209]. Target ES-HPs or lineage-negative adult bone marrow were labeled with CFSE, and then incubated with macrophages isolated NSG, Balb/c, or 129 mice. All macrophages preferentially phagocytosed ES-HPs compared to lineage-negative bone marrow (Lin-BM) regardless of their genetic strain (Fig 4.4). To confirm that ES-HPs were phagocytosed and not fused to macrophages, we performed a modified phagocytosis assay with immunofluorescence. To check that macrophages were indeed being
phagocytosed we added a fluorescent salt to mark the fluid phase layer [211]. The fluid phase (red-yellow) around our ES-HPs (green) suggest that ES-HPs are being up taken by phagocytosis (Figure 4.5) [211]. Additionally, macrophage membrane ruffling could be localized to areas near ES-HPs (Figure 4.5 B).

To determine if this was a general finding of ESCs and their derivatives or specific to ES-HPs, I performed a phagocytosis assay comparing Lin-BMCs, ESCs, and ES-HPs. ES-HPs were phagocytosed at the highest rate. ESCs were phagocytosed at greater rates than Lin- BMCs (Fig. 4.6). This suggests that ESCs and their derivatives are more easily phagocytosed than adult cells, such as those from adult bone marrow.

To determine if macrophages were indeed responsible for presentation to CD4+ T cells, we merged our MLR and phagocytosis assay into an antigen-presenting assay. We isolated F4/80+ macrophages to use as our source of antigen presenting cells (APC) and cocultured them with ES-HPs or 129 Lin- BM in the presence or absence of blocking antibodies against responder MHC-II [215]. We observed that ES-HP antigens in the context of APCs stimulate T cells, and that this presentation could be specifically blocked by addition of anti-MHC-II (Fig. 4.7 A. Top and B. Left). In contrast, no difference was observed between wells with F4/80+ macrophages and Lin- BMCs with and without anti-MHC-II antibodies (Fig. 4.7A Bottom and B. Right).
Figure 4.1: Titration of one-way MLR cell dose.
Syngeneic C57BL/6, minor mismatched 129, or fully allogeneic major mismatched stimulator splenocytes were irradiated and added to wells as indicated. **A.** Responder splenocytes were CFSE labeled. Division was detected by CFSE dilution. **B.** Quantitation of CFSE division normalized to response to self (stimulation index SI). Mean ± SEM is shown. *p*<0.05 is considered significant.
Figure 4.2: One-way MLR testing allogeneic responses to ES-HPs.

A. Balb/c responses to media alone, syngeneic Balb/c splenocytes, allogeneic 129 ES-HPs, syngeneic splenocytes plus allogeneic 129 ES-HPs, allogeneic splenocytes, and allogeneic 129 splenocytes plus 129 ES-HPs. B. Quantitation of MLR results. Mean + SEM. p<0.05 is considered significant.
Figure 4.3: One-way MLR testing syngeneic responses to ES-HPs.

A. 129 responses to media alone, syngeneic 129 splenocytes, syngeneic 129 ES-HPs, syngeneic splenocytes plus syngeneic 129 ES-HPs, allogeneic Balb/c splenocytes, and allogeneic Balb/c splenocytes plus syngeneic 129 ES-HPs. B. Quantitation of MLR results. Mean + SEM. p<0.05 is considered significant.
Figure 4.4: F4/80+ macrophages preferentially phagocytose ES-HPs.

A. Histograms showing F4/80+ macrophages that have acquired a CFSE labeled target. Black shaded region shows macrophages alone, while blue open lines show specific staining. 

B. Quantitation of MLR results. Mean + SEM. p<0.05 is considered significant.

<table>
<thead>
<tr>
<th>CFSE Labeled Target</th>
<th>129 Lin-BM</th>
<th>ES-HP</th>
<th>129 Lin-BM</th>
<th>ES-HP</th>
<th>129 Lin-BM</th>
<th>ES-HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac Source:</td>
<td>129</td>
<td>129</td>
<td>Balb/c</td>
<td>Balb/c</td>
<td>NSG</td>
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<tr>
<td>p</td>
<td>0.0003</td>
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<td>p&lt;0.0001</td>
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</table>
Figure 4.5: ES-HPs co-localize with fluid phase marker and membrane ruffling on F4/80+ macrophages.  
A. F4/80+ macrophage in blue, CFSE (Green) labeled ES-HPs, and fluid phase marker in Red.  
B. Bright field image overlaid with CFSE (Green) labeled ES-HPs, and fluid phase marker in Red.
Figure 4.6: Both ESCs and ES-HPs are preferentially phagocytosed by macrophages.

A. Data are shown for cells first gated on FSC and SSC, and then for viability, and expression F4/80+. Histogram depicts macrophages that have internalized CFSE labeled targets. B. Quantification of phagocytosis assay Mean+SEM are shown. p<0.05 was considered significant.
Figure 4.7: Allogeneic F4/80+ macrophages present ES-HP antigens to CD4+ T cells.

A. Histograms showing allogeneic Balb/c CD4+ T cell division in response to ES-HPs or 129 lin- BM. Antibodies specific to Balb/c MHC-II were added to block indirect presentation. B. Quantification of antigen presentation assay (mean + SEM). p<0.05 are considered significant.
4.4 Discussion

We tested the hypothesis that ES-HPs could stimulate CD4+ T cell responses in vitro. We showed that ES-HPs could stimulate CD4+ T cells, but only in the presence of additional splenocytes. Our results are consistent with others in that without additional antigen presenting cells, ES-HPs poorly stimulated CD4+ T cells. However, with additional splenocytes, ES-HPs were able to stimulate CD4+ T cells in our MLRs. This finding was also consistent between syngeneic and allogeneic settings.

We then performed a phagocytosis assay to determine the relationship between macrophages and ES-HPs. As predicted by our immunogenicity marker panel ES-HPs were preferentially phagocytosed compared to Lin- BM. This result was consistent with Balb/c and NSG macrophages, as well as 129 macrophages, although 129 macrophages phagocytosed fewer ES-HPs. Both ESCs and ES-HPs were phagocytosed at higher rates than Lin-BM. These results are consistent with Sionov et al. that found embryo-derived teratocarcinoma cells were phagocytosed live and subsequently destroyed by non-activated macrophages [216,217]. The same group also reported that the inner cell mass where ESCs are derived from was easily destroyed by macrophages, while the trophoblast actively repelled macrophages [162].

After determining that ES-HPs antigens could stimulate CD4+ T cells and that macrophages preferentially phagocytosed ES-HPs, we then combined these approaches and performed an antigen-presenting assay combining macrophages, ES-HPs, and CD4+ T cells. Macrophages were indeed able to present ES-HP antigens to T cells; furthermore, adding anti-MHC-II antibodies that blocked host Balb/c macrophages from presenting ES-HP peptides to CD4+ T cells. We conclude that ES-HP antigens are indirectly presented to CD4+ T cells in vitro. In Chapter 5, we will discuss our studies on the relationship between macrophages and ES-HP survival and engraftment in vivo.
Chapter 5. Functional Immunogenicity of ES-HPs in vivo

5.1 Introduction

The degree to which ESC and their derivatives recruit immune infiltrates after transplant is highly variable (Table 5.1). ESCs and their derivatives often disappear after transplant [78]. With ES-HPs, transplantation has been successful after BCR/ABL or HoxB4 transfection and after intra-femoral injection, whereas standard methods have been largely unsuccessful [78,81]. After transplant, macrophages are found in the majority of ESC and ESC-derived cellular grafts (Table 5.1). Based on the results of others, from our immunogenicity profiles, and the in vitro results where macrophages preferentially phagocytose ES-HPs, we hypothesized that host macrophages were a barrier to ES-HP transplantation.

In 1958 both macrophages and T cells were implicated in graft rejection, and since then, the field of transplantation has focused on the role of T cells in allograft rejection [218]. Today, the exact contributions of macrophages to rejection are unclear [219]. For example, infiltration of macrophages within transplanted tissue is associated with poor graft outcomes [219]. In rejecting tissues, 38-60% of infiltrating leukocytes were macrophages (CD68) [219]. Macrophages have been implicated as antigen-presenting cells, cross-presenting donor antigens to T cells [219]. They may also damage tissues directly through the production of reactive oxygen species [219]. Further depleting macrophages using clodronate-loaded liposomes can prevent rejection of allografts [219].

Macrophages are a barrier to hematopoietic cells in several contexts. Abe et al. found that depletion of macrophages increased engraftment of porcine hematopoietic cells in sublethally irradiated SCID mice [125]. CD47-deficient cells are readily cleared from circulation. Blazar et al., found that CD47-deficient bone marrow could not reconstitute CD47 expressing hosts [143]. Further, they showed that this was mediated by red pulp macrophages in the host [143]. Jaiswal et al., found that CD47-deficient hematopoietic cells are subject to phagocytosis by macrophages [195].

Based on our data on expression of markers of immunogenicity (Chapter 3) and our phagocytosis assays (Chapter 4), we wanted to test how ES-HPs would fare in an environment devoid of adaptive immunity (B, T) and innate (NK cells) [220,221]. For this we chose the NSG mouse as our recipient. To test the contribution of macrophages, we depleted them using clodronate loaded liposomes [222].
<table>
<thead>
<tr>
<th>Author</th>
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<th>CD4+</th>
<th>CD8+</th>
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<td>Formed follicle like structures + to +++</td>
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<td>- to ++</td>
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<td>Formed follicle like structures - to +++</td>
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<td>Lui et al., 2010 [160].</td>
<td>Rejecting EB differentiated for 14 days and transplanted in kidney capsule</td>
<td>Activated macrophages (F4/80+, MHC-II+)</td>
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<td>+++</td>
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<td>Alternatively activated macrophages (F4/80+, Mannose Receptor+)</td>
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<td>Zhao et al., 2011 [166]</td>
<td>129 ESC into B6 mice</td>
<td>Infiltrate</td>
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<td>Wu et al., 2008 [167]</td>
<td>ESF134 Undifferentiated (C57BL/6) into kidney capsule into allogeneic mice</td>
<td>CD11b infiltrate at day 5</td>
<td>Infiltrate</td>
<td>Infiltrate at day 10</td>
<td>Infiltrate at day 10</td>
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5.2 Materials and Methods

ESC Culture

Undifferentiated D3 ESCs (derived from 129 mice with an MHC haplotype H-2b) were purchased from ATCC (Manassas, VA, USA). ESCs were maintained and expanded on a mitomycin-C treated fibroblast STO (mSTOs) cell line also from ATCC in 6-well tissue culture treated plates. ESCs were cultured in DMEM media contained 15% ES-qualified fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA, USA), supplemented with 0.15mM monothioglycerol (Sigma-Aldrich, St. Louis, MO, USA), 1x Pen/Strep (Invitrogen) and 1000 U/ml LIF (Millipore, Billerica, MA, USA) to maintain ESCs in an undifferentiated and pluripotent state [13]. ESCs were passaged or fed daily. ESCs were passaged every 2 to 3 days by trypsinization (0.25% Trypsin-EDTA (Invitrogen)) of colonies and replating 0.5x10^6 ESC onto new mSTO cells in the presence of ESC cell media. Two days prior to differentiation cells were transferred to 0.1% gelatin coated plated to wean them from the feeder cells in IMDM (Invitrogen) media supplemented as described above [13]. Cells were incubated in a humidified CO₂ incubator at 37°C.

ESC Differentiation to ES-HPs

To differentiate cells using coculture on bone marrow stromal cells, OP9 cells (ATCC) were cultured to 80% confluency in alpha-MEM media (Invitrogen) containing 20% FBS (Atlanta Biologicals) and 1x Pen/Step. ESCs were seeded onto OP9 layers at 130,000 cells per plate in 150mm diameter tissue culture plates in the presence of the cytokines Flt3L and IL-7 (Peprotech) both at 5 ng/ml in 20 ml of media per plate. Cocultures were fed at D4 post plating, by adding 10 ml of new media as listed above plus cytokines at 10 ng/ml instead of 5 ng/ml. For cultures longer than 7 days, at D7 cells were harvested from coculture and filtered through 64-µm nylon mesh (Small Parts Inc.) and replated onto fresh OP9 monolayers, and fed again on D11.

Transplantation

The UC Merced Institutional Animal Care and Use Committee approved all animal procedures. NSG and 129 mice were purchased from the Jackson Laboratory. Mice were housed in specific pathogen free conditions. NSG mice received 200 rads of sub-lethal irradiation [221]. After a minimum of 4 hours, mice received 1 to 5x10^5 ES-HPs or 10x10^6 whole bone marrow cells or 5x10^4 lineage depleted bone marrow cells via retro-orbital injection. Both whole bone marrow and lin- BM are controls for transplants. Subsequently, mice received neomycin sulfate solution at 2 mg/ml for 2 weeks (Sigma).

Transplant Analysis
Transplants were analyzed at 17-34 days post-transplant. Spleens were weighted and then processed into single cell suspensions. For flow cytometry, a maximum of 1x10^6 live cells were aliquoted for staining. Fc receptors were blocked with 2.4G2 (anti-CD16/CD32) hybridoma supernatant for 30 minutes on ice. Cells were subsequently stained with specific antibody cocktails for 30 minutes on ice. Antibodies for the alleles CD45.1 (NSG) and CD45.2 (129) were used to assess donor chimerism in bone marrow, spleen and thymus. CD3, CD4, CD8, B220, IgM, Gr-1, CD11b, and F4/80 were used to confirm lineage development. To set compensation both single color and N-1 color controls were collected on the BD Aria II, cleaning between samples, and ran in order of least to most donor cells to decrease sample cross contamination.

**Macrophage Depletion with Clodronate Loaded Liposomes**

Macrophage depletion was achieved by treatment of mice with clodronate-loaded liposomes (CLL) or phosphate buffered saline (PBS) loaded liposomes (PLL) obtained through clodronateliposome.com. Mice were treated on day -3 with 0.04 mg per gram of mouse weight, and 0.02 mg per gram of mouse weight on day -3, -1, +5, +10, and +15, with D0 being the day mice received irradiation and transplant. Hematopoietic tissue with less than 5% hematopoietic content or less than 5,000 CD45+ cells was excluded from statistical analysis to ensure validity of results.

**Histology for F4/80+ Macrophages**

Histology was performed by freezing half the spleen in Optimal Cutting Temperature (OCT) Compound (Tissue-Tek) (the other portion was prepared for flow cytometry). 7 um sections were cut on a cryostat, flash fixed in acetone for 15 seconds and stored at -80C. At time of staining sections were fixed in 4% paraformaldehyde for 10 minutes. Sections were stained with F4/80 (clone BM8) or RatIg2a (Isotype control) (BioLegend) were added to section at 1:50. HistoMouse™-SP Broad Spectrum AEC Kit (Invitrogen) was used to develop signal.

**Statistical Analysis**

Two-tailed Student’s t-test assuming unequal variance between groups was performed on Graph Pad (Prism) and differences were considered statistically significant if p ≤ 0.05.
5.3 Results

To test how ES-HPs performed in an environment where macrophages were the only source of cells capable of mediating rejection, NSG mice were sublethally irradiated and transplanted with D16 CD41+ ES-HPs or 129 whole bone hematopoietic cells (BMCs). At D17-34 post-transplant the bone marrow, spleen, and thymus were analyzed for chimerism. Untransplanted NSG served as the positive control for CD45.1 (host) and the negative control for CD45.2 (donor), conversely WT (129) mice served as the positive control for CD45.2 and the negative control for CD45.1, while the BMT mice served as a control for radiation dose (Fig. 5.1). As expected, BMT mice were quickly reconstituted by donor BM (Fig. 5.1A, column 3). ES-HP transplanted mice showed low levels of reconstitution in comparison to BMT mice in the spleen and bone marrow (Fig. 5.1, column 2). Some, but not all ES-HP transplanted mice had a higher level of chimerism in the thymus compared to other hematopoietic organs and tissues (Fig 5.1 row 3). In the thymus, signs of T cell development into double positive and CD4+ cells were evident in 50% of recipients (Fig 5.2). Multi-lineage reconstitution was also seen in ES-HP recipients with B220+, CD19+ and IgM present in the spleen (Fig 5.3 bottom rows) as well as Gr-1+, CD11b+ monocytes (Fig. 5.3 top row).

Despite the low level of chimerism, we saw a dramatic increase in the size of the spleen of all ES-HP recipients at D17-34 post transplant (Fig. 5.4). I began weighing the spleens to have a more quantitative assessment and detected a statistically significant increase in spleen weight (Fig. 5.4B). The spleen weight in the ES-HP recipients was increased 3.7 fold compared to untransplanted NSG mice and 1.64 fold compared to BMT controls. While the weight and size of the BMT recipients also increased, this would be expected, both because the level of donor chimerism was high and because these recipients were now reconstituted with T and B cells, which are lacking in the NSG mice (Fig. 5.5 A). In comparison to both BMT and NSG untransplanted controls, more host cells were present in the ES-HP transplanted mice (Fig. 5.5 B). Both CD41+ and CD45+ Day 16 cells were more successful at reconstituting NSG mice than D7 cells (Fig. 5.5 A). This correlates with the level of CD47 that they expressed (Fig. 3.3). We began to look for which cells were responsible for this increase and since NSG mice lack B cells, T cells, and NK cells, with only myeloid lineages remaining, we examined the expression of Gr-1, CD11b, and F4/80 on host cells (Fig. 5.6). F4/80 is a marker for macrophages, and was the only population of cells that was increased (Fig. 5.6). In ES-HP recipients there was a 2.23 fold increase in host F4/80+ macrophages in the spleen compared to untransplanted NSG mice, and a 2.68 fold increase compared to BMT controls. This result was confirmed by immunohistochemistry on sections of ES-HP transplant recipients (Fig. 5.7). Not only were more of these macrophages present, these macrophages were also larger in size compared to untransplanted and BM controls (Fig. 5.8), suggesting that they had phagocytosed ES-HPs.
From the macrophage responses in the spleens and from our *in vitro* studies showing that ES-HPs were readily phagocytosed by NSG macrophages (Chapter 4), we hypothesized that ES-HPs were responsible for the inefficient reconstitution of ES-HPs *in vivo*. To test this hypothesis directly, we depleted macrophages using clodronate-loaded liposomes (CLL). We performed a titration to determine the necessary dose of CLL needed to deplete host splenic macrophages (Fig. 5.9). Depletion of macrophages using CLL showed a 6.4 fold increase in ES-HP chimerism in the spleen to statistically significant levels compared to PLL treated controls (Fig. 5.11). Additionally, the spleens remained much smaller in ES-HP recipients treated with CLL compared to those treated with PLL, but were much more similar in size in BMC control animals (Fig. 5.10). While in the BM there is not a statistically significant increase in donor chimerism compared to CLL plus ES-HPs treated mice, similarly there is an 1.67 fold increase in chimerism between mice that received PLL plus ES-HPs compared to CLL plus ES-HPs (Fig. 5.12). There was no statically significant difference in donor chimerism between CLL and PLL transplanted with either whole bone marrow or 50,000 lin-BM cells (data not shown). These data together support the hypothesis that host macrophages specifically block engraftment of ES-HPs and are responsible for the low level of engraftment achieved in ES-HP recipients.
Figure 5.1: ES-HPs are able to achieve low levels of chimerism in sublethally irradiated NSG mice.

Data shown is for live cells gated additionally by size using FSC and SSC. NSG mice were sublethally irradiated prior to transplantation. Chimerism is shown using antibodies for specific alleles CD45.1 (NSG-host) and CD45.2 (129-Donor). Untransplanted 129, and NSG mice were used as gating controls.
Figure 5.2: ES-HPs reconstitute the thymus of NSG mice.

Data shown is gated on FSC and SSC, and live cells using DAPI as a viability markers. NSG mice were sublethally irradiated prior to transplantation. **Left column shows** chimerism using antibodies for specific alleles CD45.1 (NSG-host) and CD45.2 (129-Donor). **Middle column is gated on CD45.2 (Donor) cells.** Staining for T cell populations using CD4 and CD8. **Right column shows** CD3 staining on CD4+ single positive T cells (Blue), CD4+ CD8+ double positive T cells (gray), and CD8+ single positive cells (Black).
Figure 5.3: ES-HPs are capable of multi-lineage development *in vivo*.

Data shown is gated live donor (CD45.2+) cells. **Top** row shows myeloid lineage cells using Gr-1 and CD11b. **Middle** rows show B cell populations using B220 and CD19. **Bottom** row blue lines show IgM expression on B220+CD19+ populations, black line shows IgM expression on B220-CD19- cells.
Figure 5.4: ES-HP Recipients have an increased spleen size and weight.

A. Picture of spleens of NSG, ES-HP recipients (CD41 and CD45), BMT, and 129 wt mice. B. Spleen weight in grams (Mean + SEM are shown). p<0.05 are considered significant.
Figure 5.5: ES-HP recipients have a small increase in donor cells and a large increase in host cells.

Absolute number of host and donor cells in spleens. Calculated by multiplying number of host and donor cells by the number of cells present in each spleen A. Shows the number of donor cells in the spleens B. Quantification of the number of host cells in each spleen. Mean + SEM is shown. p<0.05 is considered significant.
Figure 5.6: ES-HP recipients show increases in host F4/80+ macrophages, but not CD11b+ or Gr-1+ populations.

A. Shows the number of host GR-1+ cells in the spleens. B. Shows the number of host CD11b+ cells in each spleen. C. Shows the number of host F4/80+ macrophages in spleen. Mean + SEM is shown. p<0.05 is considered significant.
Figure 5.7: F4/80+ macrophages are increased in ES-HP recipients.

A. F4/80 immunohistochemistry developed with AEC taken at 40x magnification. Top row is taken at the edge of the spleens, middle row is taken at the middle of the spleen, and bottom row is the Isotype control taken at the edge of the spleen.

B. F4/80 immunohistochemistry of spleens from ES-HP recipients taken under oil immersion (100x).
Figure 5.8: F4/80+ macrophages have increased FSC-A (size) in ES-HP recipients.

A. MFI for FSC-A (size) on host F4/80+ cells.  
B. Histograms of FSC-A on host F4/80+ macrophages.  Blue shows ES-HP recipient, black shows BMT, and gray tinted region shows untransplanted NSG macrophages.
Figure 5.9: Dose titration of clodronate loaded liposomes measured by remaining F4/80+ macrophages.

Data shown is gated on FSC and SSC, and live cells using the viability marker DAPI. Macrophage depletion after clodronate loaded liposomes.
Figure 5.10: Clodronate liposome treatment decreases spleen size of ES-HP recipients.

A. Weight of spleens treated with clodronate loaded liposomes (CLL) or control phosphate loaded liposomes (PLL) at D17-20 post-transplant with ES-HPs, whole bone marrow, or lineage negative bone marrow. B. Pictures of spleens treated with CLL or PLL and whole bone marrow (BMT) or ES-HPs.
Figure 5.11: Clodronate liposome treatment increases chimerism in the spleen of ES-HP recipients.

A. Donor chimerism in clodronate loaded liposomes (CLL) or control phosphate loaded liposomes (PLL) at D17-20 post-transplant with ES-HPs. B. Flow cytometry plots of CD45.2 (y-axis) and CD45.1 (x-axis).
Figure 5.12: Levels of chimerism in the bone marrow of clodronate treated ES-HP recipients.

A. Donor chimerism in clodronate loaded liposomes (CLL) or control phosphate loaded liposomes (PLL) at D17-20 post-transplant with ES-HPs. B. Flow cytometry plots of CD45.2 (y-axis) and CD45.1 (x-axis).
5.4 Discussion

Our ES-HP recipients displayed low levels of chimerism in hematopoietic tissues. Although they displayed a low level of chimerism the spleen of ES-HP recipients the spleens had a dramatically increased size compared to both BM and untransplanted NSG mice. This increase could be accounted for by an increase in host F4/80+ macrophages in ES-HPs recipients. Interestingly, we did not see an increase of CD11b+ macrophages in the spleens of our ES-HP recipients. F4/80+, CD11b- macrophages are associated as being red pulp macrophages in the spleen [225]. These macrophages normally have roles in filtering the blood and removing damaged erythrocytes [225]. Interestingly, extra-medullary hematopoiesis is especially common in the red pulp of both fetal and neonatal mice, and this decreases in adult animals [226]. This suggests that there might be stage specific relationship between the red pulp macrophages and the maturation of hematopoietic stem and progenitor cells. It is possible that in addition to the phagocytosis of ES-HPs, ES-HPs may also have an additional defect preventing their migration to the bone marrow cavity [27,65].

Depletion of macrophages increased the chimerism that could be achieved by ES-HPs, while chimerism in whole bone marrow transplants, Lin-BM, and untransplanted controls recipients remained unchanged by depletion of macrophages. This suggests that NSG macrophages preferentially affect ES-HPs compared to BM.

Several other groups have noticed strong recruitment of macrophages after ESC transplant [163,169]. Further, GFP+ ESC cells up taken by macrophages and microglia could falsely be identified as cells that have engrafted if fluorescence alone was used as the primary means of identification [163,169].

Understanding the mechanisms that cause ES-HPs to disappear after transplant can assist in development of conditioning protocols relevant to their rejection. Our research revealed that macrophages play an important role in the rejection of ES-HPs. It will be important for the field of stem cell biology to identify the factors underlying macrophage recruitment and phagocytosis of ES-HPs, as well as ESCs.
Chapter 6. Synthesis

We showed that ES-HPs could be derived in vitro that resemble late embryonic to adult BM hematopoietic populations. Immunophenotyping revealed that these cells might be vulnerable to macrophage phagocytosis after transplantation. Later, we confirmed that ESC and ES-HPs were preferentially phagocytosed by macrophages in comparison to lin-BM and whole BMT, and ES-HP phagocytosis was increased in allogeneic settings. We designed an antigen-presenting assay and determined that F4/80+ macrophages were indeed capable of presenting ES-HPs to allogeneic T cells. Our in vivo results also revealed macrophage responses to ES-HPs, since host macrophages were observed in the enlarged spleens of ES-HP recipients after transplant compared to whole BM, and Lin-BM transplant recipients, as well as untransplanted controls in NSG mice which lack T, B and NK cells [221]. Treating mice with clodronate loaded liposomes (CLL) to deplete macrophages increased the chimerism in the spleen that could be achieved in ES-HP recipients, but did not change the level of chimerism in BMT or Lin-BM transplants. Together these results suggest that macrophages are a barrier to ES-HPs, but not to adult bone marrow populations.

There are some suggestions in the literature that point to macrophages as a barrier to ESC transplantation. At the time of implantation the blastocyst resist high levels of macrophage infiltration within the uterine microenvironment. Paradoxically, teratocarcinoma cells which share characteristics with the inner cell mass were highly susceptible to killing by macrophages [162,216,217]. To investigate this, Sionov et al. placed macrophages with trophoblast and the inner cell mass of E3.5 blastocyst. Macrophages were repelled from the trophoblast, whereas the inner cell mass was completely covered and destroyed by macrophages [162]. This suggests that the trophoblast may confer some level of protection for the developing embryo and that ESCs and their derivatives may be vulnerable to macrophage mediated destruction in vivo.

Additionally, several groups use GFP as the primary method for detecting ES-HPs after transplantation. Recently, Molcanyi et al. identified GFP+ ESCs after transplantation into the brain and realized that these cells had been taken up by phagocytosis [163,224]. Further, they realized the implantation of stem cells led to the recruitment of large numbers of macrophages that were not seen in the sham treated control group [223,224]. Using confocal microscopy they found that GFP+ ESCs had been phagocytosed by activated macrophages-microglia [224].

The developmental stage of cell may reflect the ability of the host to accept or reject ESCs and their derivatives. Tanaka et al. transplanted cynomolgus ESCs in utero into fetal sheep. Transplants were successful before
50 days of gestation, whereas if the cells were transplanted at 60 days of gestation the ESCs disappeared within two weeks [227]. They suggest that the absence to low levels of innate immunity before 50 days of gestation in sheep makes this time period more permissive for ESC transplantation [227]. This suggests that ES-HPs maybe suitable for in utero transplantation, but not into adult hosts without additional maturation to prevent recognition via the innate immune system.

Data from several labs have indicated that ES-HPs survive poorly after transplantation in vivo [1,65,77,78]. Consequently, the field has primarily turned to HoxB4 transfection to overcome the hurdles of developing ES-HPs. HoxB4 transfection of hematopoietic stem cells is such a robust inducer of leukemia, that it is used as a model for the disease [70,71]. Further, HoxB4 knockout mice only have mild hematopoietic defects [73]. To determine the HoxB4 in hematopoietic fates several groups have performed microarray and ChIP-ChIP studies. Lee et al. found that in the primitive hematopoietic cell line EML transfected with HoxB4, pathway analysis showed the most changes in genes related to developmental hematopoiesis, however their results also showed changes in immune-phagocytosis, inflammation, and innate inflammatory responses [228]. Oshima et al. found that CD47 is differentially upregulated in ES cells transfected with HoxB4. Further, it was revealed that CD47 is a direct target of HoxB4 through chromatin immunoprecipitation studies [205]. This suggests that while HoxB4 transfection changes hematopoietic development and cell cycle progression it may also upregulate macrophage inhibitory receptors, thereby preventing phagocytosis and macrophage activation.

We have established that macrophages respond to ES-HPs (Table 6.1). We hypothesize that macrophage recruitment and phagocytosis to ESC and ES-HPs may be related to three aspects of their maturation state: 1) secretion of products that recruit monocytes and macrophages, or induce differentiation or proliferation of macrophages such as IL-4, G-CSF, GM-CSF, or M-CSF [229], 2) expression of activating ligands [128], and 3) lack of expression of macrophage inhibitory ligands (Fig. 6.1). We have shown that at D16 ES-HPs have little to no expression of macrophage inhibitory ligands; at D7 no expression of macrophage inhibitory ligands CD47 and CD200 is seen (Figure 1.3 and Table 6.1). At D16 express MHC-I, which has also been characterized as a macrophage inhibitory ligands [127,225,230]; MHC-I expression could account for the differences seen in syngeneic and allogeneic macrophage responses to D16 ES-HPs (Table 6.1). This model is consistent with the split-tolerance found in chimeras with CD47-/- into CD47 +/+ recipients, in which tolerance is generated to all hematopoietic cells except for red blood cells. Since red blood cells lack MHC-I, this could account for the difference in macrophage tolerance, with CD47 being the dominant signal and MHC-I providing a minor inhibitory signal [144]. Although there are hints of MHC-I being an inhibitory ligand for macrophages since some Ly49 receptors are expressed on macrophages [127,225,230]; it has not been fully tested as a macrophage inhibitory ligand. The lack of macrophage inhibitory
ligands alone is unlikely to account for the full phenotype of macrophage recruitment and phagocytosis of ESCs and ES-HPs. Based on expression of inhibitory receptors alone, one would predict that ESCs would be phagocytosed at higher rates than ES-HPs, but this is not the case. This suggests that either ES-HPs have an activating signal not present on ESCs or that ESCs have an inhibitory signal not present on ES-HPs. However, in line with this model there is no difference between syngeneic and allogeneic responses to ESCs where no MHC-I is expressed therefore no difference should be noticed between syngeneic and allogeneic responses. Examining the stage specific activating and inhibitory signals might help elucidate the mechanisms through which ESCs and ES-HPs are phagocytosed by macrophages and when these cells could be transplanted successfully. It is also possible that the few ES-HPs and their CD45.2 progeny that exist after transplant have increased levels of macrophage inhibitory ligands in comparison to the cells that are removed by macrophages. Transplantation of ES-HPs with CD47 deficient stromal cells may also be a method to induce macrophage tolerance to ES-HPs [144].

In the course of research, what has emerged is the immune responses to ESC and their derivatives, such as ES-HPs are distinct from immune responses to adult bone marrow or lin-BM cells. Understanding the immune response to ES-HPs and their derivatives will allow for more successful translation to clinical therapies.
The mechanism behind macrophage recruitment and phagocytosis to ESC and ES-HPs may be related to their maturation state: 1) production of products that recruit monocytes-macrophages, induce differentiation or proliferation of macrophages, 2) expression of activating ligands, and 3) lack of expression of macrophage inhibitory ligands, such as CD47 and CD200.

Figure 6.1: Model of macrophage interactions with ES-HPs in the spleen of NSG mice.
### Table 6.1: Expression of inhibitory ligands compared to syngeneic and allogeneic macrophage responses

<table>
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<tr>
<th>Expression of Macrophage Inhibitory Molecules</th>
<th>Syngeneic (Predicted Macrophage Response/Experimental Observed Response)</th>
<th>Allogeneic (Predicted Macrophage Response/Experimental Observed Response)</th>
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<tr>
<td><a href="#">Image depicting BMC with High CD47 and High MHC-I</a></td>
<td>• High levels of inhibitory signals, no syngeneic macrophage response predicted • No syngeneic macrophage response observed <em>in vivo or in vitro</em></td>
<td>• High levels of inhibitory signals, no allogeneic macrophage response predicted • No allogeneic macrophage response observed <em>in vivo or in vitro</em></td>
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<tr>
<td><a href="#">Image depicting ES-HP D16 with Low CD47 and Moderate MHC-I</a></td>
<td>• Moderate to low levels of inhibitory signals, moderate syngeneic macrophage response predicted • Moderate syngeneic macrophage response observed <em>in vitro</em>, suggesting some level of inhibitory signal is delivered through MHC-I</td>
<td>• Low levels of inhibitory signals, high level of macrophage response predicted • High macrophage response observed <em>in vitro</em> and <em>in vivo</em>. Low numbers of cells observed after transplantation.</td>
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<tr>
<td><a href="#">Image depicting ES-HP D7</a></td>
<td>• No inhibitory signals, moderate to high syngeneic macrophage response predicted • Not tested in syngeneic setting</td>
<td>• No inhibitory signals, moderate to high allogeneic macrophage response predicted • High macrophage response observed <em>in vivo</em>, no cells could be found after transplantation</td>
</tr>
<tr>
<td><a href="#">Image depicting ESC</a></td>
<td>• No inhibitory signals, high syngeneic macrophage response predicted • Low levels of syngeneic responses seen <em>in vitro</em></td>
<td>• No inhibitory signals, high syngeneic macrophage response predicted • Low level of allogeneic responses seen <em>in vitro</em>, but no change in response between allogeneic and syngeneic macrophages</td>
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*Image placeholders: [BMC](#), [ES-HP D16](#), [ES-HP D7](#), [ESC](#)*
References:

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