# BONE MARROW-DERIVED HOST CELLS IN MURINE CARDIAC ALLOGRAFTS

by

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## ABSTRACT

Despite the success of current immunosuppressive agents in controlling acute rejection in the field of transplantation, chronic rejection continues to limit survival and quality of life in heart Transplant vascular disease (TVD) is a rapidly progressive form of transplant recipients. atherosclerosis that occurs in the vessels of solid organ transplants. The pathogenesis of TVD has a multifactorial basis arising from a complicated interplay between immunological and nonimmunological factors. Together, these factors result in endothelial cell (EC) damage and accumulation of modified smooth muscle cells (SMC) in the arterial walls of the transplanted heart. Recent reports have shown discordant data on the ability of host stem cells to migrate to sites of damage following cardiac transplantation and repopulate cells of the vessel wall. Many questions remain unanswered, such as, the relative contributions of different sources of host progenitor cells to vascular regeneration and whether these new cells subsequently alter vascular function and ameliorate the pathogenesis of TVD. As well, the effect of immunosuppressive therapy commonly used following cardiac transplantation and the effect of cytokine treatment with granulocyte-colony stimulating factor (G-CSF) on the host cell response to the allograft remains to be determined.

Therefore, the major focus of this dissertation is to investigate the contribution of host BMderived cells to the replacement of cells in the blood vessels of cardiac allografts. A murine heterotopic heart transplant model of TVD will be used to study the role of host BM-derived cells in the vasculature of cardiac allografts. The effect of immunosuppressive treatment with tacrolimus on the host BM-derived cell response to the allograft and vascular chimerism will be examined. As well, G-CSF-induced mobilization of host BM-derived cells will be used to determine whether increased circulating levels of host progenitor cells leads to altered rates of vascular chimerism in the vessels of the allografts following transplantation. Finally, the

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therapeutic potential of using autologous progenitor cells, injected following cardiac transplantation, will be examined to determine whether these injected cells are able to seed to damaged areas within the vasculature, contribute to cell chimerism and subsequently improve graft outcome.

Using BM-GFP transgenic mice as recipients of heterotopic heart transplants, we found that host BM-derived progenitor cells contributed to both EC and SMC replacement in transplanted hearts. The rate of re-endothelialization was found to diminish significantly from  $11.8\% \pm 2.5\%$ at 14 days post-transplant to  $4.0\% \pm 1.2\%$  (p≤0.05) 30 days post-transplant. Also, immunosuppressive treatment of cardiac allografts with tacrolimus was not found to affect the frequency of re-endothelialization or SMC replacement by host BM-derived cells.

G-CSF treatment of BM-derived progenitor cells *in vitro* was found to improve survival, proliferation and angiogenesis of the infused cells despite treatment with immunosuppressive agents. G-CSF pretreatment of BM-GFP transgenic recipient mice prior to heterotopic heart transplantation resulted in the same rate of re-endothelialization at 14 days post-transplant as non-pretreated allografts. However, at the 30 day post-transplant timepoint, there was a higher rate of re-endothelialization in G-CSF pretreated allografts ( $9.3\% \pm 2.2\%$ ) relative to non-pretreated allografts ( $3.4\% \pm 1.6\%$ ). In addition, G-CSF pretreated allografts demonstrated less intimal narrowing in vessels of the transplanted heart relative to vessels in non-pretreated control allografts.

In addition, there was no evidence of vascular chimerism by the lineage negative cells injected into hearts at both 14 and 30 days post-transplantation, with no evidence of a beneficial effect from the injection of these BM-derived progenitor cells on TVD progression. At both timepoints

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of 14 and 30 days post-transplantation, there was no observed improvement in either the percent luminal narrowing or the intima to media ratio in the transplanted hearts of allografts between the control injected versus the lin- cell injected groups. As well, repeated injections of the lineage negative cells following transplantation did not result in any change in graft outcome.

In summary, the results obtained in this work provide valuable insights into the contribution of host BM-derived progenitor cells to the replacement of cells within the damaged vasculature following cardiac transplantation. In particular, cytokine-induced mobilization of circulating host BM-derived progenitor cells with G-CSF was found to augment EC repopulation and ameliorate the development of allograft lesions. In addition, injected lineage negative, BM-derived progenitor cells were not found to seed to sites of damage in a manner similar to endogenous BM-derived progenitor cells and were not found to contribute to vascular cell replacement in transplanted hearts.

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# LIST OF ABBREVIATIONS

6-MP	6-mercaptopurine			
Abbreviation	Definition			
AOTF	Acousto-optic tunable filters			
APC	Antigen presenting cell			
АроЕ	Apolipoprotein E			
ATG	Antithymocyte globuline			
BM	Bone marrow			
Cgt	Ceramide galactosyltransferase			
CMV	Cytomegalovirus			
CSF3	Colony stimulating factor-3			
EC	Endothelial cell			
FGF	Fibroblast growth factor			
FITC	Fluorescein:isothiocyanate			
FKBP	FK binding protein			
GCs	Galactocerebrosides			
G-CSF	Granulocyte-colony stimulating factor			
G-CSFR	Granulocyte-colony stimulating factor receptor			
GFP	Green fluorescent protein			
H&E	Hematoxylin and eosin			
HLA	Human leukocyte antigen			
HSC	Hematopoietic stem cell			
ICAM-1	Intracellular adhesion molecule-1			
IGF-1	Insulin-like growth factor-1			
IL	Interleukin			
iNOS	Inducible nitric oxide synthase			
IVUS	Intravascular ultrasound			
LIF .	Leukemia inhibitory factor			
Lin-	Lineage negative			
MHC	Major histocompatibility complex			

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MI	Myocardial infarction
MMP-9	Matrix metalloproteinase-9
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium inner salt
NFAT	Nuclear factor of activated T-cells
NO	Nitric oxide
OCT	Optimum cutting temperature compound
PDGF	Platelet-derived growth factor
PECAM	Platelet/endothelial cell adhesion molecule-1
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
ROI	Region of interest
Sca-1	Stem cell antigen
SDF	Stromal-derived factor
SM	Smooth muscle
SMC	Smooth muscle cell
SP	Side population
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
Thy1	Membrane bound T-cell antigen
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
TVD	Transplant vascular disease
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
vWf	von Willebrand factor

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## **DEDICATION**

To my parents, Azar and Manoochehr Rezai, for their unconditional love and support and to my sister and best friend Kathy, your compassion and brilliance inspires me.

I love you.

## **CO-AUTHORSHIP STATEMENT**

The following dissertation includes chapters which are based on published manuscripts which contain small sections co-written by supporting authors. All of the data, methods, results and discussion provided in this thesis was written by myself, Nana Rezai, and only a few sentences in the Methods and Results sections of Chapters 3 and 4 were provided by co-authors. Therefore, I was responsible for the identification, design, and execution of the research experimental design. As well, I was responsible for the data analysis provided in all Results sections provided in this dissertation. Finally, in all published manuscripts, I was the principal author involved in the preparation and publication of the research.

## CHAPTER 1: INTRODUCTION TO TRANSPLANT VASCULAR DISEASE AND HOST BONE MARROW-DERIVED PROGENITOR CELLS

## **Based on the Manuscripts:**

- 1. <u>Rezai N</u>, Walinski H, Kerjner A, Bohunek L, Rossi FM, McManus BM, & Podor TJ. Methods for examining stem cells in post-ischemic and transplanted hearts. *Methods Mol Med*. 2005; 112:223-38.
- 2. Wong BW, Rahmani M, <u>Rezai N</u>, McManus BM. Progress in heart transplantation. *Cardiovasc Pathol.* 2005 Jul-Aug; 14(4):176-80.
- 3. <u>Rezai N</u>, Podor TJ, McManus BM. Bone marrow cells in the repair and modulation of heart and blood vessels: emerging opportunities in native and engineered tissue and biomechanical materials. *Artif Organs*. 2004 Feb; 28(2):142-51.

#### **1.1 TRANSPLANT VASCULAR DISEASE (TVD)**

Cardiac transplant vascular disease (TVD) is the major cause of graft failure in patients surviving more than one year post-transplantation. TVD is believed to manifest from a complicated interplay between immunological and non-immunological factors, resulting in vascular injury represented by the formation of concentric, inflamed, lipid-rich plaques [1-3]. The obstructive vascular lesions characteristic of cardiac TVD are thought to progress through repetitive endothelial injury followed by repair response. Lymphocytes and macrophages migrate to the subendothelial area via the activity of endothelial adhesion molecules and, in turn, stimulate various cytokines and growth factors, which cause progression of the disease [4,5]. Mounting evidence suggests endothelial cells (EC) and smooth muscle cells (SMC) in lesions of allograft vessels derive, in part, from progenitor cells arising from the host [4-9]. To date, there has been great discrepancy in the reported contribution of host cell replacement of vascular cells following cardiac transplantation. It is yet to be determined whether host cell chimerism in the transplanted heart is beneficial or detrimental to the graft.

## **1.1.1 HISTORY OF CARDIAC TRANSPLANTATION**

The first successful human orthotopic cardiac transplant was performed by Christiaan Barnard in South Africa in 1967 [10,11]. This feat was followed closely by the first successful heart transplant in North America performed at Stanford in 1968 [10,11]. In the decade following these breakthroughs, the success rate of this procedure remained extremely low, due largely to insufficient knowledge of immunosuppression and post-transplant care. It was not until the late 1970s to early 1980s when immunosuppressive agents became widely used and understood that cardiac transplantation became a genuine option for the treatment of end-stage cardiac failure [12-14]. To date, there have been more than 70,000 heart transplants performed world-wide (according to the ISHLT Registry 2005), and this procedure has become the most effective treatment for end stage cardiac failure [15,16]. In adult cardiac transplantation, the incidence of patient mortality resulting from acute organ rejection has reduced dramatically since the 1980s, and the current 1, 5, and 10 year average survival rates are approximately 86%, 60%, and 40% respectively [15]. Although acute rejection remains the main cause of mortality during the first year post-transplantation, TVD as the main expression of chronic organ rejection in patients greater than 1 year post-transplantation has come to the foreground, and unlike acute rejection, the incidence of this vascular disease has not diminished appreciably with modern advancements in immunosuppression and other therapeutic interventions.

#### **1.1.2 CHARACTERISTICS OF TVD**

Cardiac TVD is an accelerated form of atherosclerosis, which occurs in 30-60% of transplant recipients within the first five years post-transplantation [17]. Studies using intravascular ultrasound (IVUS) have revealed intimal thickening in 75% of cardiac allograft recipients by the end of the first year post-transplantation [18]. Unlike native atherosclerotic lesions, TVD plaques are generally concentric and involve all portions of coronary vessels [1]. It has been demonstrated that early intimal proliferation observed in TVD progresses with time and with subsequent increases in lipid deposits in the coronary vessel [3]. Atheromas and diffuse intracellular and extracellular accumulation of lipids in both intimal and medial walls are frequent occurrences [19] and the internal elastic lamina remains intact except for small breaks [3]. A time-dependent spectrum of histopathological changes has been described [20]. Early after transplantation, diffuse fibrous intimal thickening or a vasculitis predominates. Late after transplantation, focal atherosclerotic plaques, diffuse intimal thickening, or a mixture of both is observed [20]. The smaller branches are often occluded before the larger epicardial arteries resulting in small stellate infarcts [21]. Despite exuberant intimal proliferation, the media of the vessel is rarely thickened and sometimes becomes narrower than in normal conditions [22]. The

cellular infiltrate of intimal proliferative lesions consists of modified SMCs, macrophages/monocytes, and T lymphocytes [22]. Although the initiation of TVD is similar to atherosclerosis in that it involves endothelial injury and dysfunction, these events in TVD involve more definitive immune-mediated endothelial injury and dysfunction and the secretion of cytokines and growth factors from EC and lymphocytes.

#### **1.1.3 PATHOGENESIS OF TVD**

The pathogenesis of TVD involves both immune-mediated cytotoxicity of allograft vascular cells and cytokine- and/or growth factor-mediated expansion of the intima. Although the exact pathogenesis of TVD is unclear, it most likely involves a combination of immunologic [23] and nonimmunologic factors [24] (Table 1.1). ECs are the primary target of both humoral and cellmediated immunity that initiate the inflammatory response [25-28]. Repetitive injury of the endothelial barrier results in a response to injury mechanism leading to endothelial dysfunction and intimal hyperplasia. Important insults include ischemia/reperfusion, acute rejection episodes, T-cell activation, antibody deposition, and complement fixation [29]. The T-cell interaction with graft ECs initiates and sustains the chronic immune response to injury [23,25,26]. A number of accessory molecules known to be crucial for effective CD4 T-cell activation are expressed by allograft ECs [25,30,31]. Thus, both immune and non-immune processes are believed to contribute to TVD, but the relative contribution of each may vary among individual patients.

#### **1.1.4 IMMUNE RESPONSE IN TVD PATHOGENESIS**

The presence of inflammatory cells both in the neointima and adventitia suggests that immunemediated injury is a predominant cause of cardiac TVD [32]. Limitation of the proliferative vascular disease to the allograft arterial and venous tree [1], the often diffuse nature of allograft vascular involvement [3], the development of TVD in cardiac allografts of animal models with some histocompatibility mismatch [33,34], and the lack of lesion development in syngrafts all support the immunologic hypothesis of TVD development. Several experimental models suggest that immunologic mechanisms operating in a milieu of nonimmunologic risk factors constitute the principal stimuli that result in progressive myointimal hyperplasia [3,35,36]. The initial event in TVD is believed to be graft coronary endothelial injury. The endothelium is the major determinant of vessel wall function. It normally inhibits thrombus formation and leukocyte adhesion, regulates vasomotor function, and inhibits vascular SMC proliferation [37]. Damage to the endothelium could alter any or all of these functions, predisposing the artery to inflammation, thrombosis, vasoconstriction, and vascular SMC growth. After human cardiac transplantation, humoral or cellular responses to human leukocyte antigen (HLA) and vascular EC antigens are potential sources of endothelial damage. Immune recognition of foreign antigen occurs through the detection of processed antigens in the context of major histocompatibility (MHC) complexes. MHC molecules are cell surface proteins responsible for antigen binding and display. These MHC molecules are highly polymorphic, and in this way distinguish between self and foreign tissue [38,39]. There are two types of MHC molecules: MHC class I and MHC class II. MHC class I molecules are normally ubiquitously expressed by all nucleated cells and are recognized by T-cell receptors (TCR) on CD8+ T-cells. MHC class II molecules are recognized by TCR on CD4+ T-cells. In the presence of appropriate co-stimulatory molecules, CD4 lymphocyte-induced upregulation of MHC class II antigens on ECs elicits a cellular immune response [40]. Through the secretion of cytokines, CD4+ T-cells then support either a cellular cytotoxic immune response through the activation of CD8+ T-cells, or support an antibody-mediated response. Irrespective of the initial specific immune-mediated injury, the cascade of events that follows appears to be a physiologically nonspecific inflammatory response

[27].

Immune targeting of MHC mismatched allografts occurs through the recognition of donor antigen expressed with MHC on donor antigen presenting cells (APC), referred to as direct allorecognition, or of donor alloantigens expressed by recipient APC, referred to as indirect allorecognition. Direct allorecognition is the most rapid means of allograft recognition and is believed to account for the majority of cellular responses during acute allograft rejection [41]. Alternatively, indirect allorecognition is important in prolonged immune activation in transplanted organs [41]. Studies using animal models of transplantation have shown that transgenic mice which are incapable of direct antigen recognition and thus solely rely on indirect antigen recognition, are able to survive for over 100 days post-transplant, but still develop TVD [42]. These results suggest that indirect antigen recognition alone is enough for the development of TVD, although an additional contribution of direct antigen recognition to TVD is expected.

Minor histocompatibility antigens are normal cellular constituents of cells that are processed and displayed in the context of MHC molecules. In transplantation, allorecognition of these antigens during transplantation results in allelic variations in these minor histocompatibility antigens between individuals. As such, this process mediates rejection of allografts between different individuals that express identical MHC molecules [42]. The immune response towards minor histocompatibility antigens is capable of mediating acute organ rejection, although it is generally not as severe as in MHC mismatched transplants. In mouse cardiac allograft models, MHC mismatched animals generally survive 7 days post-transplant in the absence of immunosuppression. In minor histocompatibility mismatched models, survival is highly variable and depends on the animal strains utilized, with the average graft survival being 12-19 days post-transplant [42]. In addition, rejection in MHC mismatched models includes a combination of

antibody-mediated and cell-mediated cytotoxicity, while minor histocompatibility models of rejection rely more on cell-mediated cytotoxicity [43].

Once activated, T-cells release proinflammatory cytokines including interleukin (IL)-1, IL-2, tumor necrosis factor- $\alpha$  and interferon- $\gamma$ . In turn, expression of cell adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), is upregulated, allowing circulating leukocytes to adhere [44]. The involvement of adhesion molecules plays a crucial role in regulating the interaction of inflammatory cells with cells in the vascular wall because the adherence of leukocytes to vascular endothelium is a prerequisite for transmigration. Under normal circumstances, ECs constitutively express only MHC class I antigens (HLA-A and -B), but in the presence of interferon-y begin to express MHC class II antigens [37]. Activation of antigen-specific B cells by soluble MHC class II products and the presentation of multiple HLA allopeptides by self B cells to CD4 T-cells results in the production of immunoglobulin G anti-MHC class II antibodies. The presence of these antibodies has been associated both with episodes of recurrent high-grade cellular rejection and the development of TVD [45,46]. The intercellular network, via macrophages, T lymphocytes, ECs, and SMCs, generates a variety of stimulatory cytokines (IL-1, IL-2, IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )) and growth factors (platelet derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), and transforming growth factor-ß (TGF-  $\beta$ )) that promote the development of the chronic allograft lesion [27]. Thus, at the end of the "endothelial injury process" chronic inflammation elicits a repair response that causes the production of a connective tissue matrix [47] and the migration and proliferation of vascular wall SMCs that compromise the vascular lumen.

The impact of vasoactive mediators, apoptosis, and vascular remodeling on the development of TVD has been investigated recently. Dong et al. [48] demonstrated an association between Fasmediated apoptotic cytotoxicity and TVD in humans. During acute cardiac allograft rejection, nitric oxide is (NO) induced, influencing apoptosis [49] and thus mediating an acute deleterious effect. Endothelium-derived NO is the most potent endogenous vasodilator known, and some debate exists regarding its role in vascular injury [50]. Endothelium-derived NO is reported to inhibit platelet and leukocyte adherence to the vessel wall and suppresses the expression of adhesion molecules and chemokines regulating endothelial interaction with circulating blood elements [51,52]. Endothelium-derived NO also inhibits vascular SMC proliferation [53]. This is, in part, mediated by an effect of NO, an increase in vascular smooth muscle cell apoptosis [54]. In contrast, NO is a survival factor for endothelial cells [55]. These observations are consistent with the view that NO is an endogenous antiatherogenic molecule. The inducible form of NO synthase (iNOS) is expressed in the vessel wall of the aortic allograft. Inhibition of iNOS activity in the aortic allograft significantly increases intimal hyperplasia at 4 weeks [56]. Furthermore, early overexpression of iNOS by the use of ex vivo gene transfer completely prevents the development of structural changes in rejecting grafts [56]. In addition, structural changes are found to accelerate in iNOS-knockout mice [57]. The protective effects of iNOS in these studies may be due to an effect of NO, specifically, the inhibition of SMC proliferation and suppression of the adhesion of platelets and leukocytes to the endothelium [54,58]. Immunemediated upregulation of iNOS expression partially protects aortic allografts from arteriosclerosis [56]. Importantly, iNOS mRNA and protein are expressed in human arteries with TVD, where they are associated with extensive nitration of protein tyrosines, indicating that peroxynitrite plays a role in the development of TVD [59]. Furthermore, endothelin, the most important vasoconstrictor with mitogenic properties, has been suggested to be involved in the pathogenesis of TVD [60]. Studies in rat cardiac allografts demonstrate local upregulation of

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endothelin-1 suggesting an important role in TVD [61]. In addition, data from human studies suggest that myocardial endothelin expression is associated with coronary endothelial dysfunction following cardiac transplantation [62] and occurs in coronary arteries with TVD significantly more often than in normal vessels [63]. Beyond the clear role for immunological processes in the pathogenesis of TVD, non-immunological mechanisms are also believed to contribute to this type of vasculopathy.

#### **1.1.5 NON-IMMUNE RESPONSE IN TVD PATHOGENESIS**

Several nonimmunologic mechanisms also contribute to the progression of TVD (Table 1.1). These include both recipient characteristics (age, gender, obesity, hypertension, hyperlipidemia, insulin resistance, and cytomegalovirus infection) and donor characteristics (age, gender, preexisting coronary disease, and donor ischemic time) [64-66]. In a large cohort of patients, donor age (older donors), donor hypertension, and recipient or donor gender (male) predicted earlier onset of angiographic TVD [64]. However, conflicting results were reported in a multicenter study [67] indicating no apparent association between the progression of intimal thickening and multiple nonimmunologic factors, including donor and recipient characteristics (recipient/donor age and gender, gender mismatch, pretransplant diagnosis, ischemic time, post-transplant hypertension). In general, hyperlipidemia and insulin resistance are the most significant non-immunologic factors in cardiac TVD, occurring in 50%-80% of the heart transplant population [68].

Hyperlipidemia is commonly seen in cardiac transplant patients for several reasons. Many of these patients are hyperlipidemic before transplantation. In addition, the immunosuppressive therapy given to patients may result in or exacerbate pre-existing dyslipidemia. The frequent and diffuse accumulation of lipids in both the intimal and medial cell layers of cardiac allograft arteries indicates that the alloimmune environment may strongly promote lipid uptake [69]. The extent of lipid accumulation in these lesions is highly correlated with luminal narrowing [19]. Increased levels of circulating triglycerides and total cholesterol are highly associated with the development of TVD in humans and plasma levels of oxidized low density lipoproteins are correlated with increasing severity of TVD as assessed with angiography [70,71]. Finally, TVD is increased in apolipoprotein E (ApoE) knockout mice relative to nomocholesterolemic mice. Due to a null mutation in the ApoE gene, ApoE knockout mice contain extremely high levels of circulating lipids and are prone to the spontaneous development of atherosclerosis [72]. The augmented TVD in these mice suggests that hypercholesterolemia is important in the pathogenesis of this disease [72].

Ischemia-reperfusion injury is also believed to contribute to TVD in humans [73]. Despite advances in surgical techniques and tissue handling, ischemia of donor organs during harvesting and transportation is difficult to eliminate. Upon activation of the microvascular endothelium, oxygen free radicals are believed to lead to a subsequent activation of passing host leukocytes and macrophages [73]. Activated cells release oxygen radicals and other aggressive mediators, such as proteases and cytokines, which chemotactically attract host leukocytes. Thus, postischemic reperfusion injury represents the result of network interactions mediated by a large variety of oxidative molecules and aggressive mediators. A significant correlation has been shown between the extent of ischemic injury, as assessed histologically from biopsies, and the severity of TVD in humans [74]. It has also been reported that ischemia may increase the activation of and/or damage EC, thereby providing mechanisms of ischemia-mediated augmentation of TVD [75]. EC apoptosis has been observed after ischemia reperfusion injury in ex vivo animal models and is abundant following myocardial infarction, suggesting that inducer of EC Importantly, ischemia/reperfusion is an important death [76].

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ischemia/reperfusion-induced EC death has been found to precede myocyte death in *ex vivo* animal models of this disease, suggesting that this early event may be an important contributor to vascular pathologies [77]. Inhibition of apoptosis by overexpression of heme oxygenase-1 has been found to attenuate ischemia-reperfusion injury and prolong graft life [78]. As well, inhibition of ischemia-reperfusion-induced EC apoptosis has been found to prevent inflammation in the kidney of rats, suggesting a link between EC death and inflammation in this type of tissue damage [79].

## **1.1.6 ANIMAL MODELS OF CARDIAC TRANSPLANTATION**

Several animal models have been developed to investigate the pathogenesis of TVD. The heterotopic cardiac transplantation or orthotopic artery transplantation in rodents are the most commonly used allograft models. Heterotopic cardiac transplantation is performed by the interposition of a donor heart into a non-physiological position, normally located in the abdomen, in the recipient. Upon placement within the abdomen, the vena cava and aorta of the donor heart are anastomosed to the descending aorta and ascending vena cava, respectively [80,81]. There is limited blood flow into the ventricles of this transplanted heart in spite of maintained cardiac contractions. Although cardiac contractions are indicative of viable myocardium, there is limited blood flow into the ventricles of the transplanted heart. Therefore, the transplanted heart does not function under normal physiological load due to the limited blood volume within the ventricles [80,81]. However, in this model, the myocardium remains perfused with recipient blood since blood flows from the descending aorta, retrograde through the ostia and into the coronary circulation. Thus, the vasculature and myocardium are exposed to the host immune system in a similar manner to clinical heart transplantation. Although technically challenging, the heterotopic heart transplant model permits the evaluation of rejection and graft survival since cardiac contractions can be monitored by palpation in the abdomen [81,82].

The heterotopic cardiac transplant model normally relies on transplantation between different rat or mouse stains. This provides the advantage of being able to use different immunogenetic combinations in order to reflect the clinical scenario or to investigate specific pathogenic mechanisms. Often, completely MHC mismatched strains of mice, such as Balb-c donors and C57BL/6 recipients, are used to investigate acute rejection [83,84]. Due to a rapid immune attack from both cellular and humoral immune responses, these hearts are normally found to reject after 7-10 days post-transplantation. Minor histocompatibility antigen mismatched rats and mice are also often utilized to investigate TVD. Generally, the immune response generated in these animals is less severe than in complete MHC mismatched animals and depending on the type of minor histocompatibility antigen mismatch, these allografts can survive from as little as 12 days to as many as 260 days post-transplantation [33,34]. Single MHC mismatched animals can also be used in heterotopic heart allograft models. In these strain combinations, donors that contain certain coding polymorphisms in either MHC class I or class II molecules are transplanted into recipient mice, thus normally only one amino acid in the mismatched MHC molecule is distinct between the donor and recipient. Generally, these grafts are able to survive indefinitely due to the slight disparity in MHC molecules in this setting [85], and significant luminal narrowing has been found to develop as early as 30 days post-transplantation [86].

In order to more closely mimic the clinical manifestations of organ rejection, immunosuppressed rats and mice receiving heterotopic heart transplants have been utilized [87]. One of the most common immunosuppressive agents used in animal models of heart transplant rejection include treatment with CD4+ and CD8+ neutralizing antibodies. In order to prevent acute rejection, rat and mouse allografts are treated with neutralizing antibodies [88]. This type of immunosuppressive treatment prolongs graft life indefinitely while still allowing the

development of TVD in the experimental allograft model [88]. However, neutralizing antibodies have not been used extensively clinically and the pathology observed in this model may not be the closest representation of clinical disease in humans. In rat and mouse allograft models, immunosuppression with tacrolimus is also widely used and more closely resembles clinical heart transplantation.

## **1.2 IMMUNOSUPPRESSIVE TREATMENT**

#### **1.2.1 HISTORY AND ORIGINS OF IMMUNOSUPPRESSION**

The availability of cardiac transplantation as an established therapeutic option for select patients with end-stage heart disease is in large part attributable to the development of successful immunosuppressive regimens. Over the past 40 years, immunosuppressive drug regimens have evolved greatly and heart transplantation has been transformed into a routine clinical procedure. The breakthrough in chemical immunosuppression for transplantation came with the observation that 6-mercaptopurine (6-MP) could induce immunological unresponsiveness in rabbits to a foreign protein (human serum albumin), and later that it could prolong the survival of skin grafts in rabbits [89-91]. Following these observations Calne and co-workers demonstrated that 6-MP prolonged the survival of canine renal transplants, albeit with severe morbidity from the drug [92]. Around the same time Elion and Hitchings, working in the Burroughs Wellcome laboratories in New York, created a number of nucleotide analogues in the hope of finding novel chemotherapy agents for use in the treatment of leukemia [93,94]. Calne obtained some of these from Elion and Hitchings and tested their ability to prolong kidney allograft survival in the dog in Murray's laboratory in Boston. One of the compounds, BW57-322 (azathioprine), stood out in terms of efficacy and tolerability [95,96]. Azathioprine was much less toxic than 6-MP and afforded better prolongation of allograft survival. It rapidly moved into clinical use but it was

not potent enough to permit most recipients to keep their graft [97]. In France in the early 1950s, Rene Kuss had used corticosteroids to try to prolong kidney graft survival, but it was only when corticosteroids were combined with azathioprine by Starzl in the early 1960s that effective chemical immunosuppression became a reality [98].

Azathioprine and steroids remained the mainstay of immunosuppression for the next 25 years, as efforts were made to develop compounds that affected lymphocyte function. This regiment had low specificity and consequently led to nonspecific suppression of the host's immune response. The incidence of infection, including fungi, protozoa, and viruses was consequently increased [99]. Thereafter, their use evolved to a progressive narrowing of the target of immunosuppressive strategy, starting in the 1970s with antithymocyte globuline (ATG), prepared from the serum of horses or rabbits inoculated with human lymphocytes. It proved a valuable adjunct to steroids and azathioprine, and was used both for the treatment of rejection and as part of the initial immunosuppressive regimen [100].

#### **1.2.2 CYCLOSPORINE AND TACROLIMUS**

Prednisone and azathioprine, with or without ATG, were powerful enough to permit successful renal transplantation but both heart and liver transplantation struggled for success with the level of immunosuppression available and in the face of exposure to such a large dose of steroids. It took the discovery of cyclosporine in 1971 [101] for thoracic organ and liver transplantation to be truly successful. Cyclosporine was initially studied for its potential as an anti-fungal compound by the Sandoz laboratories in Basle, but when it was discovered to have potent anti-lymphocyte properties, its development was temporarily halted. Borel, a scientist at Sandoz, showed that cyclosporine permitted the survival of skin grafts in mice [101], and the next year it was shown to prolong the survival of kidney transplants in the dog [102,103]. Clinical trials began the following year in Cambridge, UK, and cyclosporine was shown to facilitate not only

kidney transplantation, but also transplantation of the pancreas and liver, and later the heart and lungs [104]. Cyclosporine was approved for clinical use in transplantation in 1983 by the United States Food and Drug Administration and revolutionized the management of post-transplant rejection [105].

In 1984, a substance was discovered in a soil sample taken at the foot of Mount Tsukuba which stands just outside Tokyo and given the name tacrolimus [106]. In spite of initial results suggesting the drug caused lethal vasculitis in a canine renal transplant model, Starzl later used tacrolimus in clinical liver transplantation and showed that it was not only potent, as predicted, but also devoid of the adverse effects seen in the dog [106]. Due to its perceived potency, tacrolimus was used initially as rescue therapy for patients with intractable rejection [107,108]. Further studies confirmed it to be of similar potency and with similar side effects to cyclosporine [109,110].

The calcineurin inhibitors, cyclosporine (Sandimmune<sup>®</sup>, Neoral<sup>®</sup>; Novartis, Basel, CH), a small fungal cyclic peptide, and tacrolimus, the microcline antibiotic from *Streptomyces tsukubaensis* [111], (Prograf<sup>®</sup>; Astellas Pharma Inc., Tokyo, Japan) have become the cornerstone of immunosuppressive therapy in solid organ transplantation. Each agent forms complexes with cytosolic proteins called immunophilins [112], cyclosporine binds to cyclophilin and tacrolimus binds to the 12 kDa FK506-binding protein (FKBP-12). This complex binds to calcineurin, a pivotal enzyme in T-cell IL-2 production [112] (Figure 1.1). Calcineurin functions by dephosphorylating the cytoplasmic subunit of nuclear factor of activated T-cells (NFAT-c), thus enabling its translocation to the nucleus. Here, NFAT-c complexes with the nuclear subunit NFAT-n and enhances binding of transcription factors to the promoter region of genes encoding for pro-inflammatory cytokines such as IL-2, IL-3, IL-4, interferon-  $\gamma$  (IFN- $\gamma$ ) and TNF- $\alpha$  [113]. These cytokines, specifically IL-2, induce the proliferation and/or activation of T-cells and other

immune system cells by autocrine and paracrine pathways [114]. Therefore, when the tacrolimus-FKBP or cyclosporine-cyclophilin complex binds to calcineurin, the resulting complex inhibits cytokine transcription by the CD4 cell. The end result of the blockade of cytokine production and cytokine receptor expression is the inhibition of T-cell proliferation and differentiation such that the various effector arms of the immune response are not activated.

#### **1.2.3 SIROLIMUS**

In the 1990s, antiproliferative immunosuppressants, including sirolimus, were introduced targeting downstream effects of the IL-2 receptor (Figure 1.1). Antiproliferative agents act by directly or indirectly inhibiting T-cell and B-cell proliferation. Sirolimus (rapamycin), a macrocyclic antibiotic and a naturally occurring fermentation product of the actinomycete *streptomyces hygroscopicus*, is structurally related to tacrolimus and forms a complex with FKBP, but its mechanism of immunosuppression differs. Sirolimus binds to a target downstream of the IL-2 receptor, the mammalian target of rapamycin (mTOR), a serine/threonine kinase involved in the phosphatidylinositol 3-kinase (P13K)/AKT (protein kinase B) signalling pathway [115]. Inhibition of mTOR has a profound effect on the cell signalling pathway required for cell cycle progression and cellular proliferation. The net effect is blockade of T-cell activation by preventing progression of the cell cycle from the G<sub>1</sub> to the S phase [116-118].

A range of different immunosuppressive agents, including those described above as well as others such as, mycophenolate mofetil and monoclonal antibodies, are now available for clinical use in solid organ transplantation. Largely due to the use of these therapies, the average occurrence of acute rejection episodes following cardiac transplantation is now lower, being estimated at approximately 1.25 episodes in the first year post-transplant, and 0.18, 0.13, and 0.02 episodes in years 2, 3, and 4, respectively [119].

## **1.3 DIAGNOSIS OF REJECTION**

The detection of allograft rejection is one of the most important and still evolving areas in cardiac transplantation. In the early years of transplantation, the successful identification and treatment of rejection was restricted due to technical limitations. Sakakibara and Konno introduced the biopsy catheter or "bioptome" in 1962 as a means of sampling endomyocardium, in contrast to previous epicardial and transmural sampling [120]. The benefit of this new biopsy catheter was adequate endomyocardial sampling, with fewer complications, which occurred via a transvascular approach under fluoroscopic guidance. In the early years following its invention, the endomyocardial biopsy found limited use worldwide until advances in cardiac transplantation necessitated a means to monitor graft rejection. In 1972, the Stanford group developed a new percutaneous, flexible biopsy forceps that could serially obtain right ventricular endomyocardial biopsies after transplantation [121-123]. With this new transvenous endomyocardial biopsy, the right ventricular heart biopsy procedure gained acceptance as a clinically useful interventional technique, and the use of cardiac biopsies to investigate other non-transplant related pathologies became more commonplace [121]. However, the diagnosis of TVD as an expression of chronic rejection remains difficult due to the concentric and diffuse nature of the disease [36]. The accelerated intimal proliferation seen in TVD has been found to affect the entire vascular tree of allografts [1], thereby limiting physical intervention by balloon angioplasty and other localized treatments to ameliorate luminal narrowing. The diffuse and concentric nature of TVD limits the accuracy of angiography, which provides luminographic representation of allograft arteries [124]. Despite the potential of angiography to under-diagnose TVD, a classification system has been developed out of Stanford University that recognizes that TVD can range from typical atherosclerosis to concentric arterial obliteration. With this system, type A lesions are focal and proximal, similar to coronary artery disease. Type B1 and B2 lesions consist of smooth narrowing with abrupt or smooth obliteration of the lumen, respectively. Type C lesions are severely narrowed and terminate abruptly [125]. Angiograms, as well, should be interpreted serially as there is a risk that new and concentric lesions may be missed on one-time angiograms. The diagnosis of vasculopathy is believed to be most sensitively made by IVUS, which permits the three dimensional, high-resolution visualization of intimal and other vascular lesions [126]. By allowing measurements of both actual lumen diameter and appearance of thickness of intima and media, IVUS gives much more information about onset, rate of progression, prognosis, and risk factors of TVD [124]. Thus, the value of lesion identification by IVUS is enhanced by the availability of information on lesion structure and composition. Although IVUS is more sensitive than coronary angiography in the detection of TVD, several problems still exist. First, complete evaluation of the coronary tree is not possible. Only the proximal large arteries can be imaged easily. Second, there is considerable cost involved with the procedure. Third, the procedure is time-consuming and technically challenging due to catheter size (most are just under 1 mm) [126]. Since angiography and IVUS are invasive tests, they pose increased risks for the patients. Noninvasive tests, however, have not been sensitive or specific enough to date to be reliable to screen for cardiac TVD. Noninvasive tests demonstrate low sensitivities and specificities in detection of TVD compared with coronary angiograms with the sensitivity and specificity of dipyridamole Thallium-201 single-photon emission computed tomography imaging reported as 21 and 80%, respectively [127].

With advances in immunosuppression and surgical techniques, the pathologies seen following transplantation have changed. Over the last 20 years, the rates of acute rejection and infection leading to graft failure have greatly declined owing to refined immunosuppressive drug regiments, better diagnosis of injury, and improved monitoring of immune status. As such,

chronic rejection and the events involved in the pathogenesis of cardiac TVD have become the focus of investigative and clinical attention.

## **1.4 ROLE OF HOST CELLS IN TVD**

Advances in the field of transplantation have shown that host or recipient cells may contribute to the replacement of cells in the arteries and myocardium of transplanted hearts. More specifically, the interaction between donor and host cells after organ transplantation has received great attention in an attempt to elucidate the pathogenesis of graft rejection [128-130]. More than 30 years ago, it was recognized that transplanted organs become genetic chimeras. In an initial study in 1969, karyotyping was used to demonstrate that reticuloendothelial cells in the transplanted liver are replaced with host cells [130]. A landmark study followed in 1992 wherein it was reported that donor cells of dendritic cell origin could be identified in multiple recipient organs, including blood, lymph nodes, skin, intestine, and heart, suggesting that the chimeric state in long-term organ transplant recipients, i.e., liver and kidney, was not restricted to the organ, but was in fact systemic [130-132]. Microchimerism specifically refers to the contribution of cells from at least two sources, namely host and donor [129]. Presently, the origin, fate and role of host progenitor cells in the transplanted human heart is still a matter of intense debate.

Recent studies have raised the possibility that undifferentiated progenitor cells may translocate from the host to the graft, contributing to ventricular remodeling [133-135]. However, the degree of cardiac chimerism is currently a matter of intensive discussion [136] since there are discrepancies in the reported rates of host cell chimerism in transplanted hearts [7,9,137]. Sexmismatch cardiac transplantation, in which male patients receive hearts from female donors and

vice versa, have provided the opportunity to investigate whether stem cells or tissue-specific progenitor cells migrate from the host to the graft. In these allografts, female hearts transplanted into male recipients are analyzed for cardiac chimerism by determining Y chromosome-positive cells from the host present in the donor heart [7,9]. Fluorescence *in situ* hybridization using Y chromosome-specific hybridization probes are used to detect host cell chimerism and can be correlated with immunohistochemistry of cardiac specific markers. Other detection systems have also been employed to study the role of host cells in allografts, including green fluorescent protein (GFP) expression [138] and *LacZ* expression from bone marrow (BM) chimeric *LacZ* transgenic mice [6,134,139,140]. Regardless of the method of detection, host cells have been identified within cardiac allografts following transplantation in several published reports.

These reported studies in transplanted hearts have sparked debate regarding the extent of EC and SMC repopulation by extra-cardiac, host-derived progenitor cells. Quaini *et al.* [7] studied the degree of chimerism in 8 sex-mismatched transplant recipients using fluorescent Y-chromosome in situ hybridization to detect cells from recipient origin. They counted the frequency of Y-positive vessels (defined as 30% or more Y-positive cells) and reported significant levels of chimerism in arterioles (21% to 50% extra-cardiac-derived ECs in vessels) and 18% of cardiomyocytes. Simper *et al.* [141] investigated the levels of circulating ECs in 5 female-to-male heart transplant patients demonstrating angiographic evidence for TVD, and compared EC chimerism between diseased and non-diseased segments of the coronary arteries. The authors reported EC chimerism in the diseased segments ranging from 1% to 24% while non-diseased regions of arteries showed only 0.2% EC chimerism. Glaser *et al.* [142] recently reported a range of 0.8% to 5.6% host-derived SMCs in coronary arteries from 6 male transplant recipients who received a female donor heart.
Finally, vascular cell repopulation in the transplanted human heart was also examined using sexmismatched biopsy samples by Murry and colleagues [8]. In this study, endomyocardial biopsies from 7 patients were stained for EC and SMC markers and the Y chromosome was identified with in situ hybridization. ECs showed the highest degree of chimerism, averaging  $24.3\% \pm 8.2\%$  from extra-cardiac or host sources. Vascular SMC chimerism was reported to be  $3.4\% \pm 1.8\%$ . All 3 cell types showed substantially higher chimerism in the same biopsy samples than previously observed for cardiomyocytes (0.04% +/- 0.05%) [9]. Analysis of serial endomyocardial biopsies also revealed that high levels of endothelial chimerism by host cells occurred as early as 1 month after transplantation (22% +/- 6.6%) with no significant increases even up to 10 years after cardiac transplantation. These results indicate that extra-cardiac host progenitor cells are capable of repopulating vascular cell types in the heart, but they do so with varying frequency. The results also suggest that the signals for progenitor cell recruitment occur early and could relate to injury during allograft harvest or transplantation. However, in all of the studies described above, the detection method and study design did not allow for the identification of the original source of the host cells contributing to vascular chimerism in the transplanted hearts. To date, the contribution of different sources of host progenitor cells in TVD, as well as the role of host-derived cells within the graft remains unclear and the physiological consequences of cardiac and vascular chimerism in transplanted hearts merits further investigation. Recently, interest has evolved in the contribution of BM-derived host cells to this observed phenomenon in allograft hearts.

#### **1.5 BONE MARROW (BM)**

#### **1.5.1 BM DEVELOPMENT, CONSTITUENCY, AND BIOLOGY**

Mammalian bone consists of bone cells at different developmental stages (including preosteoblasts, osteoblasts, and osteocytes), collagen fibrils, and mineral deposits such as calcium and phosphate [143,144] as well as more primitive cell types, such as mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) (Figure 1.2). Stem cells possess two characteristic features: first, they must be able to self-renew and, second, they must have the capacity for multilineage differentiation [145,146]. The BM contains stem cells as well as progenitor cells which are not able to self-renew indefinitely (Figure 1.1). The bone cavity is filled with soft BM and blood vessels, and the developing hematopoietic cells within the bone cavity are retained in the BM until they have matured and are released into the vascular system [143,147]. HSCs and their progeny are surrounded by stromal cells in BM. MSCs also reside in the bone cavity and are proposed to give rise to the majority of marrow stromal cell lineages, including chondrocytes, osteoblasts, fibroblasts, adipocytes, ECs, and myocytes, as demonstrated in vitro and partially in vivo [148-150]. In addition, ECs may also originate from hemangioblasts, progenitor cells that can give rise to both hematopoietic and ECs during embryonic stage [151-153]. Originally, cells within the BM were thought to function solely in the regeneration of cells within the marrow and circulating hematopoietic cells in the peripheral blood. However, work over the past decade suggests that cells from the BM have the potential to generate other cell types including skeletal muscle fibers [154-156], hepatocytes [157], ECs [134,158], neuronal cells [159,160] and cardiac muscle cells [134,155]. These studies have generated much excitement since the possibility is now raised that these BM-derived cells could be used as a source of tissue for clinical use in the repair and modulation of a variety of damaged or degenerated tissues. Among all adult stem cell

or progenitor cell populations, those arising from the BM have shown the greatest potential with respect to multilineage differentiation and functional engraftment into host animals.

#### **1.5.2 HEMATOPOIETIC STEM CELLS**

Progenitor cells are primitive BM cells that have the capacity to proliferate, migrate and differentiate into various mature cell types. The adult BM is constituted by two main categories of stem cells. One population, referred to as HSCs, has the ability to provide permanent longterm reconstitution of the entire hematopoietic system. HSCs have been characterized extensively in mice and humans by techniques including cell sorting and transplantation. Despite their rarity, as few as 1:10,000 BM cells, HSCs constantly replenish the differentiated cells of the peripheral blood through a population of committed progenitors arising from the BM [161,162]. In the mouse, HSCs can be highly enriched or purified by flow cytometry using a combination of cell surface markers, such as the stem cell antigen (Sca-1) [161,162], the tyrosine kinase receptor c-Kit [162,163], and low or negative levels of lineage markers (lin<sup>-</sup>) [162,164]. Human HSCs have also been isolated, primarily through their expression of the marker CD34 [165], lack of lineage markers, and low expression levels of the membrane bound T-cell antigen Thy1 [166]. HSCs can also be highly enriched using fluorescent vital dyes such as Hoechst 33342, since such dyes are actively effluxed from primitive cells in the HSC population. These isolated cells within the HSCs are referred to as 'side population' or 'SP' cells, coming off the main population [167,168].

### **1.5.3 MESENCHYMAL STEM CELLS**

A second population of primitive cells present in the BM are stromal in origin and are referred to as MSCs, capable of giving rise to non-hematopoietic cells. MSCs are believed to be the common precursors to differentiated cell lineages found in the BM, including adipocytes, osteoblasts, chondrocytes and hematopoiesis-supporting stroma [169]. The precise relationship between MSCs and HSCs remains unclear at this stage, however MSCs are thought to support HSCs by the production of crucial cytokines, such as IL-6 and leukocyte inhibitory factor (LIF), and extracellular matrix which both aid in HSC function in the BM microenvironment [169]. Currently, there is debate as to whether MSCs are true stem cells or instead, determined progenitors of connective tissues. This controversy exists in part due to the lack of suitable assays with which to accurately assess the specific properties of MSCs, particularly their capacity for self-renewal. In addition, it remains to be determined if marrow-derived MSCs and the MSC-like cells isolated from other tissues, including peripheral blood [170] and adipose tissue [171], are cells of a single type or different cell populations with a similar capacity to differentiate along multiple lineages. Although to date there has been no evidence that MSCs circulate in the blood system, putative MSC-like cells have been identified [170]. BM MSCs are characterized by the expression of CD29, CD44, and CD166, but they lack CD34, CD45, HLA-DR (hematopoietic stem cell markers), CD14, CD31, and CD11a [172-177]. MSCs<sup>-</sup> can currently be isolated based on their plastic adherent property, yet no unique antigenic marker is currently available for the purification of MSCs. Upon appropriate stimuli and in a suitable environment, BM MSCs have been found to exhibit transdifferentiation properties or plasticity [178].

#### **1.5.4 ORIGINS AND HISTORY**

HSCs were originally defined as tissue-specific stem cells with the capability of self-renewal and proliferation/differentiation into a variety of hematopoietic cell lineages [179]. However, this limited view of the plasticity of BM-derived stem cells has been challenged by experiments dating back to the 1960s, which demonstrated the capacity of BM cells to repopulate nonhematopoietic tissues, both *in vitro* and *in vivo*.

Research on the potential of progenitor/stem cells in the blood and BM began when the concept of blood forming cells was established in the late 19<sup>th</sup> century [180-182]. These ideas were explored further in the next few decades until 1896, when physicians administered BM orally to patients with leukemia, the first attempt at therapeutic use of BM [183]. Schretzenmayr performed the first BM cell transplantation in 1937 when he injected freshly aspirated BM intramuscularly to treat parasitic infection [184]. Studies continued along these lines when in 1939, BM was infused intravenously to correct primary BM disorders [185].

As a result of these early studies, interest in the therapeutic use of BM cells for treatment of diseases continued to rise, particularly following the Second World War when BM transplantation was developed as a potential therapy for otherwise lethal irradiation damage. Pioneering studies by Friedenstein *et al.* demonstrated both *in vitro* and *in vivo* that stem cells in the BM have the potential to give rise to cells of the connective tissues, such as chondrocytes and osteoblasts [186,187]. Despite tremendous progress in the field of BM transplantation during that era, the BM was generally thought of as a source of stem cells restricted functionally for the regeneration of cells within the marrow and for circulating hematopoietic cells in the peripheral blood. However, this tenet of BM biology has been revolutionized by several recent reports suggesting that adult BM contains heterogeneous progenitor/stem cell populations with the

potential to differentiate into non-hematopoietic cells in ectodermal, mesodermal and endodermal lineages.

### **1.5.5 BM-DERIVED PROGENITOR CELLS IN CARDIAC INJURY**

Since the groundbreaking 1998 report of Ferrari et al. [154] that cells from the BM have the ability to contribute to skeletal muscle regeneration in mice, multiple groups have studied the potential of marrow cells to effect myocardial and vascular regeneration. Recent observations in the adult heart have suggested that adult cardiac and noncardiac stem cells, such as those obtained from the BM, skeletal muscle or peripheral blood, may adopt a cardiomyocyte phenotype after undergoing natural migration or experimental transplantation into the heart This evidence indicates that the presence of such cells in the adult [134,135,138,188]. extracellular cardiac environment induces the maturation of cardiac phenotypes. Interest in the capacity of adult cardiac cells to regenerate was further instigated by a study in 2001 [133] in which the investigators proposed the controversial concept that, contrary to previous assumptions, stem cells directly delivered to infarcted hearts promote myocardial regeneration in a therapeutic mouse model. The study used a transgenic mouse model to identify the destiny of acutely implanted BM cells that were injected directly into the border region of an experimental myocardial infarction. At 9 days, newly formed myocardium was reported as occupying 68% of the treated portion of the ventricle. However, to date, other groups have not been able to consistently reproduce these findings. Several of these groups report no significant cardiac differentiation after HSC transplantation and suggest that cardiomyocyte regeneration by stem cells is a rare event [189,190].

Murry and colleagues [189] used a similar transgenic mouse line into which a nuclear-localized β-glycosidase reporter was inserted to monitor the differentiation of transplanted HSCs in models of myocardial infarction induced by ligation. In these settings, the transplanted Lin-, c-Kit+ HSCs did not show evidence of differentiation into cardiomyocytes. In a subsequent study with BM transplantation in lethally irradiated mice, the same authors found very infrequent new myocytes of donor origin in the peri-infarct area [189]. These findings are consistent with that of a previous study by Jackson and colleagues [134] who observed donor cardiomyocytes in 0.02% of the myofibers in the peri-infarct zone following similar BM transplantation, using side population HSCs, 60 minutes after coronary ligation. A more recent publication by Balsam et al. [190] showed that GFP+ HSCs injected into infarcts did not form cardiomyocytes, but instead Despite the absence of differentiated into blood cells, predominantly granulocytes. transdifferentiation in their study, these authors did note an improvement in ventricular function in the HSC-injected group. Alvarez-Dolado et al. [191] demonstrated that marrow-derived cells occasionally fused with cardiomyocytes in the absence of injury, giving rise to hybrid cells. Their frequency of fusion was found to be roughly comparable to the frequency of progenitorderived cardiomyocytes in both mouse and human transplantation studies, raising the possibility that fusion is the principal mechanism through which such cells arise. Thus, there appears to be a general consensus that endogenous, BM-derived progenitor cells can give rise to rare cardiomyocytes through mechanisms that, at least in part, involve cell fusion. Despite the intense debate regarding the potential of cardiomyocyte regeneration by stem cells, vascular repopulation in cardiac injury models has been a more consistent and frequent finding in recent studies involving various injury models.

# **1.5.6 BM-DERIVED PROGENITOR CELLS IN VASCULAR INJURY**

BM cells have been found to contribute to vascular remodeling in different vascular injury models. In one set of experiments, the BM of wild-type mice was replaced with that of LacZ-mice. It was found that transplanted LacZ BM cells had settled in BM, spleen, and thymus, whereas no LacZ-positive cells were detected in uninjured femoral arteries of BM-LacZ mice [139]. Four to eight weeks after BM transplantation, a large wire was inserted into the femoral artery of the BM-LacZ mice, leading to complete endothelial denudation and marked enlargement of the lumen [139]. One week after the injury, a significant amount of neointimal  $(63.0\% \pm 9.3\%)$  and medial cells  $(45.9\% \pm 6.9\%)$  were LacZ-positive, suggesting that BM cells may give rise to vascular cells following mechanical injury.

Other recent reports in vascular injury models [142,192-194] have also suggested that neointimal cells are heterogeneous and that SMC in vascular lesions are composed of cells of diverse origin. It has also been shown that the cellular constituents of a lesion differ depending on the type of vascular injury [195]. In this study, 3 distinct types of mechanical injuries were compared in the same mouse model in which BM had been reconstituted with that of GFP or LacZ mice. After wire-mediated endovascular injury, a significant number of the neointimal and medial cells were reported to be derived from BM. In contrast, BM cells were rarely detected in the lesion induced by perivascular cuff replacement. Only a few BM-derived cells could be detected in the neointima following ligation of the common carotid artery. These findings suggest that the mode of injury is crucial for the recruitment of BM-derived cells to tissue remodeling and that BM cells substantially contribute to lesion formation only when arteries are subjected to severe injuries.

### **1.5.7 BM-DERIVED PROGENITOR CELLS IN CARDIAC TVD**

The recent observations that adult BM cells can differentiate into vascular ECs [196], and both ECs and SMCs could be differentiated from the same stem cells [197] has aroused interest in whether the BM progenitor cells contribute to the replacement of damaged ECs and to the growth of SMCs observed in transplant atherosclerotic lesions. Shimizu et al.[6] and Sata et al.[139] have demonstrated that approximately 10% to 20% of  $\alpha$ -actin-positive cells in the neointimal lesions of allografts were colocalized with  $\beta$ -galactosidase-positively stained cells in a chimeric mouse expressing  $\beta$ -galactosidase in BM cells. On the basis of these findings, the authors concluded that host BM cells are a source of smooth muscle-like cells in transplant neointimal lesions. However, several other groups have argued that a large number of leukocytes infiltrate the vessel wall of allografts in the development of the disease, and they are in close contact with SMCs in the lesion. This argument suggests that it would make it difficult to distinguish whether the double-stained cells in tissue sections are from 1 cell or 2 adjacent cells. Using animal models for transplant atherosclerosis, Hu et al. [194] performed aortic allografts in 3 types of transgenic mice expressing β-galactosidase: (1) all tissues (ROSA26 [198]), (2) only SMCs (SM-LacZ [199]), and (3) apolipoprotein E (apoE)-knockout mice [200] carrying LacZ genes in SMCs (SM-LacZ/apoE knockout) [201]. Their results provide strong evidence that SMCs of neointimal and atherosclerotic lesions in allografts derived from host, but not originate from the BM, and suggest that non-BM-derived progenitor cells are a possible source of SMCs in atherosclerotic lesions. With respect to EC replacement by host BM-derived cells, only a few studies have been performed in allograft models and these have reported inconsistent results. To evaluate the potential source of the host-derived neointimal ECs in rat aortic allografts, Hillebrands et al. (2002) used confocal microscopy to identify MHC class I haplotype-specific antibodies and an EC marker. These experiments indicated that the host-derived ECs originated

predominantly from a non-BM source, whereas only few BM-derived neointimal ECs were detected (<5%) [202,203]. In contrast, Hu *et al.* [204] recently reported, using an aortic transplant model in transgenic mice, a higher percentage of host BM-derived ECs (approximately 35%) identified in the aortic allograft vessels. Taken together, the varying results from all of these studies highlight the critical need for further careful experiments in models reflective of true lesion formation that is observed clinically in vessels of cardiac allografts.

#### **1.6 G-CSF-INDUCED MOBILIZATION OF BM PROGENITOR CELLS**

The local BM microenvironment, referred to as the so-called stem cell niche consisting of fibroblasts, osteoblasts, and ECs, governs the maintenance and mobilization of BM progenitor cells [205-207]. Mechanistically, cytokines inducing mobilization interfere with the interactions between stem cells and BM stromal cells, which allow progenitor cells to disengage from the BM and to pass through the endothelium to enter the blood stream. Currently, granulocyte colony-stimulating factor (G-CSF) is the most commonly used agent to mobilize progenitor cells. G-CSF is a growth factor that has been used for enhancing the recovery of neutrophils after chemotherapy and the mobilization of hematopoietic progenitor populations from the BM into the blood [208]. Natural human G-CSF is a 25 kiloDalton secreted glycoprotein encoded by the colony stimulating factor-3 (CSF3) gene and a member of the cytokine class I superfamily, the product of a single locus on chromosome 17q21-22 [209] and structurally characterized by four antiparallel  $\alpha$ -helices [210]. It is produced mainly by hematopoietic cells, such as monocytes/macrophages, lymphocytes, fibroblasts, ECs, astrocytes and BM stromal cells. The cloning and characterization of recombinant human G-CSF took place between 1984 and 1986 [211]. G-CSF is known to support the proliferation, survival and differentiation of neutrophils in vitro and provides non-redundant signals for maintenance of steady-state neutrophil levels in

*vivo* [212-215]. Another major and initially unexpected benefit of G-CSF is its ability to induce the egress of hematopoietic progenitor cells from the BM into the peripheral blood. It was discovered in the first Phase I studies of G-CSF that the numbers of multiple lineages of progenitor cells (myeloid, erythroid, megakaryocytic) in the blood were elevated dramatically (about 100 fold) 4-7 days after the beginning of G-CSF treatment [216]. The mechanism by which G-CSF mobilizes these cells into the periphery is not fully understood, but is thought to invoke multiple effector pathways.

# **1.6.1 MECHANISMS OF G-CSF-INDUCED MOBILIZATION**

Serum concentrations of G-CSF are normally undetectable or detectable at very low levels in humans [217]. However, in response to infection, serum levels are markedly elevated and fall in parallel with recovery from infection [218]. To explore the mechanisms of G-CSF-induced mobilization, gene deletion studies in mice have been utilized. G-CSF-deficient mice and G-CSF receptor (G-CSFR)-deficient mice are characterized by a resting neutropenia, a markedly reduced capacity to mount a neutrophilic response to invoked bacterial and fungal infections, an increased mortality from these infections, a susceptibility to bacterial pneumonia and a propensity to develop amyloidosis with age [214,219,220]. Studies of wild-type versus G-CSFR-deficient chimeric mice demonstrated that expression of the G-CSFR on BM stromal cells is not required for stem cell mobilization by G-CSF [221]. The authors next examined stem cell mobilization in chimeras reconstituted with both wild-type and G-CSFR-deficient BM cells, and found that G-CSF treatment of these mixed chimeras resulted in the equal mobilization of both wild-type and G-CSFR-deficient stem cells. Collectively, these data provide definitive evidence that expression of the G-CSFR on stem cells is not required for their mobilization by G-CSF. This supports a model in which G-CSFR-dependent signals act *in trans* to mobilize stem cells from the BM [221] (Figure 1.3). In this model, the first step toward stem cell mobilization is the

activation of a subset of mature hematopoietic cells by the mobilizing stimulus (e.g. G-CSF). The second step is the generation of secondary signals by these activated cells that, in turn, leads to stem cell mobilization.

The cell types responsible for the generation of these signals have not been definitively identified, although neutrophils and monocytes are believed to be the candidates involved in the case of G-CSF. The nature of the secondary signals responsible for the actual mobilization of stem cells from the BM is also unclear. However, recent studies suggest some possibilities, including protease release by activated cells and modulation of stromal-derived factor (SDF)-1 expression in the BM.

A study by Levesque *et al.* [222] suggested that serine proteases may play a key role in stem cell mobilization. The authors demonstrated that during mobilization with G-CSF, VCAM-1 expression in the BM stroma is strongly reduced. They showed that proteases secreted by the expanding neutrophil mass within the BM, namely neutrophil elastase and cathepsin-G, are capable of proteolytic cleavage of VCAM-1 [222-226]. However, the function of these proteases has been challenged by other data, indicating that G-CSF-induced mobilization was normal in mice lacking virtually all neutrophil serine protease activity, even when combined with a broad metalloproteinase inhibitor [226]. This suggests that other proteases and/or other mechanisms are likely involved in this process. The role of matrix metalloproteinase 9 (MMP-9) in G-CSF-induced mobilization is controversial at this point [226-228]. This debate might be explained by the fact that MMP-9 plays an important role in growth factor-induced stem cell mobilization in wild-type animals, whereas in the knockout model, compensatory upregulation of enzymes with a similar activity profile to MMP-9 may mask the impact of MMP-9 deficiency.

SDF-1 is a member of the chemokine CXC subfamily originally isolated from murine BM stromal cells [229]. SDF-1 has a single open reading frame of 267 nucleotides encoding an 89amino acid polypeptide and expressed on stromal cells of various tissues. CXCR4, a 7transmembrane-spanning G protein-coupled receptor, is the only known receptor for SDF-1 [230] and is expressed on BM progenitor cells. The interaction of SDF-1/CXCR4 is reported to play an important physiological role during embryogenesis in hematopoiesis [231], vascular development, and cardiogenesis [232]. Studies of mice lacking SDF-1 or its receptor, CXCR-4 on stem cells have established that SDF-1 is necessary for the migration of stem cells from the fetal liver to the BM [231,233]. SDF-1/CXCR-4 interactions tightly regulate the homing and repopulation of human stem cells, SCID repopulating cells, from BM and blood and mobilized CD34+ enriched cells in transplanted severe combined immunodeficiency disorder/non-obese diabetic (SCID/NOD) mice [234]. In addition, efficient mobilization of murine stem cell and progenitor cells is observed when SDF-1 concentrations are elevated in the blood due to injections of SDF-1 [222]. Elevation of SDF-1 levels in the blood by administration of SDF-1 or by injection of an adenoviral vector expressing SDF-1 is associated with a significant mobilization of stem cells to the blood [235,236]. Collectively, these observations provide evidence which supports SDF-1 involvement in stem cell mobilization.

A recent study by Katayama *et al.* [237] has provided an alternate mechanism for G-CSFinduced mobilization of stem cells, suggesting that the sympathetic nervous system may regulate stem cell trafficking. In 2000, it was shown that the sulfated fucose polymer fucoidan causes rapid mobilization of HSCs [238,239]. In order to assess whether a very similar molecule synthesized by mammalian cells, a sulfated galactolipid called sulfatide, might contribute to the endogenous mobilization process, Katayama and colleagues studied mice deficient in the UDPgalactose:ceramide galactosyltransferase (Cgt). The enzyme Cgt is highly expressed in

oligodendrocytes and Schwann cells [240] and its products, collectively referred to as galactocerebrosides (GCs), are a major component of the myelin sheaths that facilitate the transmission of saltatory conduction. The authors founds that mice lacking Cgt exhibited severely impaired mobilization in response to G-CSF. Unexpectedly, they found that the mobilization defect in mice lacking Cgt is not due to the absence of BM sulfatide, but likely originates from altered neural influence on osteoblasts. Pharmacological or genetic ablation of adrenergic neutrotransmission indicated that norepinephrine signaling controls G-CSF-induced osteoblast suppression, bone SDF-1 down-regulation and progenitor cell mobilization. Further, administration of a beta-2 adrenergic agonist was found to enhance mobilization in both control and norepinephrine-deficient mice. Thus, these novel results suggest that signals emanating from the sympathetic nervous system regulate osteoblast function and control the attraction of stem cells to their niche.

# **1.6.2 G-CSF-INDUCED MOBILIZATION IN CARDIAC INJURY**

Recently, promising data in animal models of different cardiovascular disorders have shown evidence that administration of G-CSF reduces myocardial damage and mortality [241-247]. Cytokine-mediated recruitment of BM stem cells has been reported to improve cardiac dysfunction and reduce mortality after acute myocardial infarction (MI) in mice [248]. In this study, cytokine treatment was started before MI and was reported to result in myocardial regeneration characterized by dividing cardiac myocytes and the formation of vascular structures 27 days after acute MI. Another study involved treatment with G-CSF both before and after left anterior descending coronary artery ligation and found that G-CSF increased the survival rate after infarction [247]. The authors reported that G-CSF led to BM stem cell migration into the infarcted border area and subsequent regeneration of cardiomyocytes. Although the issue of cardiomyocyte transdifferentiation is a matter of intense debate, other groups who have not

found evidence of adult stem cell transdifferentiation into cardiac myocytes have still reported that G-CSF treatment of mice following MI or other cardiac injury leads to functional improvement [249,250]. G-CSF has been shown to improve short-term survival and functional outcome 14 days after MI [251,252] and Deindl *et al.* [244] have recently extended these findings indicating that G-CSF application post-MI leads to a reduced decline of the LV-wall at day 6 and day 30 after MI, reduces scar extension at day 30 and reduces number of animals developing ischemic related ventricular wall expansion. As well, they report that G-CSF administration after MI enhances arteriogenesis by increasing the availability of ICAM-1 mediating leukocyte adhesion. The authors also observed a better perfusion of the peri-infarct region mediated by an enhanced growth of collateral vessels and a reduced number of apoptotic ECs and cardiomyocytes in the infarct and peri-infarct area, which is supported by previous findings from Ohtsuka *et al.* [252] and Harada *et al.* [251].

Despite these findings, several recent studies have found no beneficial effect of G-CSF treatment following MI. Ripa *et al.* [253] performed a randomized, double-blind, placebo-controlled trail examining the effects of G-CSF given in the postinfarction period. The authors utilized the gold standard method to assess the effects of cytokine mobilization on the regional wall-motion parameters of thickness and thickening and in doing so, have generated a robust data set from the control group on which to base further studies. The results showed no difference in left ventricular systolic wall thickening between the placebo and treatment groups. Similarly, the global measurements of left ventricular ejection fraction showed similar degrees of change, with no significant differences seen between the treatment and control arms. Thus, the results of this study demonstrated neither myocardial regeneration or remodeling attenuation in G-CSF-treated patients. These data also correlate with another recent study that used G-CSF after MI [254]. In this study, G-CSF was used at the same dosing level, but for 1 less day (10 µg/kg for 5 instead of

6 days), also to no effect. The primary end point in the study was reduction of left ventricular size and the secondary end point of left ventricular ejection fraction assessment actually demonstrated a slightly greater (but not significant) increase in the placebo group compared with the treatment group. Furthermore, in two previous studies using G-CSF in chronic myocardial ischemia, there appeared to be a deterioration in regional measures of left ventricular function and perfusion in patients treated with G-CSF [255,256]. It is difficult to reconcile the reported differences in the effect of G-CSF treatment following cardiac injury. It could be speculated that this is attributable to differences in the study design, such as timing of G-CSF injection, and different methods of left ventricular function assessment.

# **1.6.3 G-CSF-INDUCED MOBILIZATION IN CARDIAC ALLOGRAFTS**

With respect to cardiac transplantation, it has been shown recently that G-CSF plays a beneficial role in graft survival. In a rat heterotopic heart transplant model, pretreatment of donors with G-CSF was found to facilitate heart allograft acceptance. This improvement was attributed to the induction of a type 2 immune response by G-CSF pretreatment increasing type-2 T helper cells [257], type-2 dendritic cells [258] and IL-10-producing monocytes [259], which down-regulate type 1 cells secreting rejection-associated cytokines such as IFN- $\gamma$ , IL-2, IL-12 and TNF- $\alpha$  [260]. In another recent study, G-CSF pretreatment of recipient rats prior to heterotopic heart transplantation was found to facilitate graft survival only in accordance with treatment of recipients with the immunosuppressive drug tacrolimus [261]. The results of this study indicated that the addition of pretransplant G-CSF treatment facilitates tacrolimus-induced graft acceptance by downregulating intragraft expression of the type-1 T helper cell-driving cytokine IL-12. Taken together, these studies indicate that G-CSF treatment of either the donor or recipients of cardiac allografts leads to improvements in graft survival longterm. However, unlike in other cardiac injury models such as MI, the contribution of chimerism events by host

BM-derived cells to regeneration of damaged cells in hearts of G-CSF-treated cardiac allografts has not yet been determined.

# **1.7 RELEVANCE OF CONTENTS IN INTRODUCTION TO THESIS RATIONALE**

As described above, the contribution of BM cells to vascular lesions has been investigated in animal models of cardiac transplantation. However, due to the discrepancies in the reported rates of vascular cell chimerism by host-derived cells, the contribution of BM-derived cells to this chimerism in the transplanted heart requires further, diligent analysis.

Therefore, it is the aim of this dissertation to examine the relative contribution of host BMderived cells to the total host cell repopulation of damaged cells within allograft hearts using a murine heterotopic cardiac transplant model. The effect of immunosuppression with tacrolimus, one of the most commonly used immunosuppressive drugs in transplantation, on the host BMderived chimerism frequency in allografts will also be examined. In addition, the effect of cytokine-driven mobilization of host BM progenitor cells by G-CSF will be determined both in vitro and in vivo. BM cell response to G-CSF treatment in the presence and absence of immunosuppressive treatment will be determined using functional assays to measure parameters such as proliferation, cell survival and angiogenesis. In murine cardiac allografts, the effect of G-CSF pretreatment of recipients on vascular cell repopulation or chimerism by host BMderived cells in allograft vessels and the subsequent effect on allograft function will be determined. Finally, the effectiveness of the use of autologous, exogenously-introduced BMderived progenitor cells for therapeutic application for vascular injury in cardiac allografts will Lineage negative BM-derived progenitor cells will be injected following be assessed. transplantation and their role and effect on graft function will be determined. The insights gained from the results of these studies will provide critical information in the field of cardiac

transplantation for future strategies using either endogenous or exogenous BM-derived

progenitor cells for the treatment and amelioration of cardiac TVD.

# Table 1.1 Non-immunologic risk factors in cardiac TVD

Donor and recipient characteristics (age, sex, history)Ischemia-reperfusionDyslipidemia/hyperglycemiaCytomegalovirus infectionObesity/hypertensionDrug toxicity

Figure 1.1



Figure 1.1. Identification of the sites of action of different immunosuppressive agents. MHC II-antigen complexes are responsible for initiating the activation of CD4 T-cells. These MHC-peptide complexes are recognized by the TCR, which consists of trans-membrane proteins associated with transduction with activation of second messengers. Downstream the cytoplasmic  $Ca^{2+}$  concentration increases due to an influx of extracellular  $Ca^{2+}$ . Calcineurin is subsequently activated and dephosphorylates NFAT, allowing its translocation into the nucleus. These nuclear factors facilitate IL-2 gene transcription. Interaction of IL-2 with its receptor, IL2-R, on the cell membrane surface induces cell proliferation and production of T-cell specific cytokines. Both cyclosporine and tacrolimus act by forming a complex with calcineurin, preventing the dephosphorylation of NFAT. Steroids act at the IL-2 gene transcription level while sirolimus targets downstream of the IL-2 receptor.





**Figure 1.2. General model for the development pattern of BM stem cells.** Mature cell development progresses from a candidate BM stem cell (HSC, MSC) which can undergo either self-renewal or differentiation into a multilineage committed progenitor cell. These cells then give rise to more differentiated, mature cell types.



**Figure 1.3. Potential mechanisms for G-CSF mobilization of BM-derived progenitor cells.** In this model, G-CSF-dependent signals act *in trans* to mobilize BM-derived progenitor cells. In the first step, G-CSF activates a target subset of mature hematopoietic cells. This leads to the generation of a variety of secondary signals causing changes in the BM microenvironment. Protease release by activated cells has been suggested to alter the BM microenvironment by degrading molecules with known roles in hematopoiesis. Modulation of SDF-1 expression in the BM is also believed to be a result of secondary signals from G-CSF stimulation. Signals from the sympathetic nervous system have also been found to affect osteoblast suppression and altered SDF-1 expression in the BM leading to progenitor cell mobilization into the blood.

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# CHAPTER 2: RATIONALE OF RESEARCH PROPOSAL, CENTRAL HYPOTHESIS, AND SPECIFIC AIMS

# **2.1 RATIONALE**

Transplant vascular disease (TVD) is a rapid and progressive form of atherosclerosis that occurs in the vessels of solid organ allografts and is the major cause of late graft failure. TVD is believed to manifest from a complicated interplay between immunological and nonimmunological factors, resulting in vascular injury represented by the formation of concentric, lipid-rich plaques. Mounting evidence suggests ECs and SMCs in lesions of allograft vessels derive, in part, from host BM-derived progenitor cells. Investigating the origin of cells that may contribute to the restoration and replacement of the injured vessel wall is crucial to determining the factors which initiate or ameliorate TVD. As such, the major focus of this dissertation is to investigate the contribution of host BM-derived cells to the replacement of cells in the blood vessels of cardiac allografts. As well, cytokine-induced mobilization of these host BM-derived cells will be used to examine whether increased circulating levels of host progenitor cells consequently leads to altered rates of vascular chimerism in the vessels of the allografts following transplantation. A murine heterotopic heart transplant model of TVD will be used to study the role of host BM-derived cells in the vasculature of cardiac allografts. These experiments provide valuable insight into understanding the role of the host BM-derived cells in the setting of vascular damage, such as that observed in cardiac TVD.

### **2.2 CENTRAL HYPOTHESIS**

Host BM-derived cells migrate, localize and contribute to the mesenchyme of transplanted hearts and their vessels by contributing to the replacement of EC and SMCs within the coronary arteries of allografts in response to injury.

# **2.3 SPECIFIC AIMS**

**Aim 1-** To characterize the abundance and localization of host BM-derived cells in cardiac allografts and to examine the effect of immunosuppression on host cell seeding to the transplanted heart.

**Aim 2-** To determine whether immunosuppressive drugs and G-CSF treatment affect BMderived progenitor cell survival *in vitro*.

**Aim 3-** To examine the effect of G-CSF-induced mobilization of BM progenitor cells on vascular chimerism in the transplanted heart *in vivo*.

Aim 4- To examine the effect of injections of autologous BM-derived progenitor cells on vascular cell replacement in cardiac allografts.

#### **2.4 SIGNIFICANCE**

In Specific Aim 1, the role of host BM-derived cells within transplanted hearts will be examined using a murine heterotopic cardiac transplant model. In these experiments, transgenic mice expressing GFP-positive BM cells will be utilized as allograft recipients of heart transplants and compared to those transplanted into wild type syngraft control animals and histological and imaging techniques will be used to analyze the fate of these host progenitor cells in the allograft vessels. In addition to providing a TVD model which allows the investigation of grafts demonstrating intimal narrowing, the murine heterotopic heart transplant model also provides a model in which the response of host BM-derived progenitor cells to both an injured (transplanted heart) and non injured organ (native heart) can be evaluated. Subsequent to these experiments, a group of the transplanted animals will be treated either with the immunosuppressive drug tacrolimus or left untreated in order to determine whether immunosuppressive therapy affects the contribution of host BM-derived cells to vascular cell replacement in allograft vessels. Both *in* 

*vitro* and *in vivo* experiments will be performed in Specific Aim 3 to determine the effect of the cytokine G-CSF on BM progenitor cell mobilization and its subsequent effect on vascular replacement by host BM-derived cells in cardiac allografts. Finally, in Specific Aim 4, the potential of autologous injections of BM-derived progenitor cells to contribute to vascular replacement in allograft vessels will be investigated using a mouse heterotopic cardiac transplant model.







**Figure 2.1 Overview of Aims.** To investigate the first Aim, which is to characterize the abundance and localization of host BM-derived cells in cardiac allografts and to examine the effect of immunosuppression on host cell seeding, a murine heterotopic heart allograft model will be used. Recipient mice demonstrate GFP-positive (green) BM cells and at 14 and 30 days post-transplantation, transplanted hearts of both allografts and control syngrafts will be examined to evaluate the frequency of host BM-derived cells within the vasculature of the transplanted heart which stained double-positive for EC (yellow cells) and SMC (red cells). The effect of immunosuppression with tacrolimus on this chimerism frequency will also be determined. In Aims 2 and 3, the effect of G-CSF-induced mobilization of BM cells on this chimerism frequency within the vasculature of transplanted hearts will be examined *in vitro* and *in vivo*. In Aim 4, the effect of exogenous injections of autologous lineage negative BM-derived cells (GFP-positive) on the replacement of vascular cells within the transplanted heart will be evaluated.

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# CHAPTER 3: BONE MARROW-DERIVED HOST CELLS IN MURINE CARDIAC ALLOGRAFTS

# **Objective:**

To characterize the abundance and localization of host BM-derived cells in cardiac allografts and to examine the effect of immunosuppression on host cell seeding to the transplanted heart.

# **Based on the Manuscript:**

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**<u>Rezai N</u>**, Corbel SY, Dabiri D, Kerjner A, Rossi FM, McManus BM, Podor TJ. Bone marrowderived recipient cells in murine transplanted hearts: potential roles and the effect of immunosuppression. *Lab Invest*. 2005 Aug; 85(8):982-91.

#### **3.1 RATIONALE**

Studies both support and refute the ability of host progenitor cells to home to sites of vascular injury and repopulate ECs and SMCs in hearts following cardiac transplantation. The purpose of the experiments in this chapter was to determine the contribution of BM-derived progenitor cells to the reported total host cell seeding to cardiac allografts, we used BM-GFP transgenic recipient mice for heterotopic heart transplants. In addition, to date, there have been no reports on the effect of immunosuppression following cardiac transplantation on the host progenitor cell homing to the allograft heart. Therefore, the experiments in this chapter also aim to determine whether immunosuppression with a common agent used following human transplantation affects the ability of host BM-derived cell seeding and chimerism in cardiac allografts.

# **3.2 INTRODUCTION**

Heart transplantation is a life-prolonging procedure that has become an accepted surgical treatment for patients with end-stage congestive heart failure. Despite improvements in outcomes over the last two decades, both acute and chronic rejection continue to limit survival and quality of life in heart transplant recipients [1]. TVD, as an expression of chronic rejection, is a rapidly progressive form of atherosclerosis that occurs in the vessels of all solid organ transplants, including the heart. Cardiac TVD is estimated to affect more than 40% of recipients who survive beyond four years after transplantation [1,2].

Recent studies have suggested that undifferentiated progenitor cells may emigrate from the recipient or host to the graft, contributing to vascular remodeling and possibly contributing to the atherosclerotic process of TVD [3-5]. However, the degree of host cell repopulation of cells of the vessel wall is currently a matter of intense debate [6] since there are discrepancies in reported rates of chimerism in damaged vessels and hearts [3-5,7-10]. Studies have reported that as

many as 21% to 50% of ECs in vessels of cardiac allografts are extracardiac-derived [9]. However, other studies have observed much lower frequencies of host cell repopulation within allograft hearts, such as Glaser *et al.* [11] who have reported approximately 0.8% to 5.6% of host-derived SMCs in coronary arteries from 6 male transplant recipients who received a female donor heart. These discrepancies may be attributed to confounding factors, including different assays and techniques used to detect cell differentiation or colocalization events, as well as variations in models used to examine chimerism rates.

To address the fate of endogenous host BM-derived progenitor cells in the vasculature of transplanted hearts, we performed heterotopic heart transplantation using chimeric recipient mice expressing GFP-positive BM in order to track the migration of the BM-derived host cells. Moreover, we measured the effect of immunosuppression with tacrolimus versus no immunosuppression on the frequency of host GFP-labeled BM-derived ECs and SMCs in the coronary arteries of the transplanted heart.

#### 3.3 MATERIALS & METHODS

#### 3.3.1 Generation of BM-chimeric mice

The study protocol was approved by the University of British Columbia Office of Research Services Committee on Animal Care. C57BL/6 (CD45.2) and C57BL/6 (GFP/CD45.1) mice were bred in-house and maintained in a pathogen-free environment. The C57BL/6 (GFP/CD45.1) mice express GFP driven by the CMV enhancer-chicken beta-actin hybrid promoter. They were a kind gift of Dr. I. Weissman [12]. Eight week old C57BL/6 (GFP/CD45.1) mice as donors and 8-10 week old C57BL/6 (CD45.2) mice as recipients.

#### 3.3.2 GFP cell reconstitution

BM was flushed out the femurs and the tibias of C57BL/6 (GFP/CD45.1) mice and single cell suspensions were made. Red cells were lysed, cells were enumerated and 5 x  $10^6$  cells were injected into the tail vein of lethally (950 rads) irradiated recipient C57BL/6 mice.

### 3.3.3 Peripheral blood analysis of BM-chimeric mice

At various time-points after transplantation, 150  $\mu$ L of peripheral blood were collected from the tails of recipient BM-chimeric mice. Reconstitution of the hematopoietic system was analyzed by flow cytometry on a FACScan (Becton Dickinson) using a protocol as described previously [7]. Mice with more than 80% of GFP-positive peripheral blood leukocytes were used as recipient mice for the heterotopic heart transplant experiments 2 months after BM transplantation.

# 3.3.4 Heterotopic cardiac transplantation

Heterotopic cardiac transplantation was performed as described [13]. Hearts of 10-week-old male 129SV/j mice were transplanted into the reconstituted GFP-BM chimeric C57BL/6 mice described above. Control syngrafts consisted of 10-week-old C57BL/6 donor hearts transplanted into C57BL/6GFP-BM chimeric mice. Animals were anesthetized with 4% halothane and anesthesia maintained with 1% to 2% halothane (Halocarbon Laboratories, River Edge, NJ). Donor mice were infused with heparinized saline and their hearts excised. The recipient's abdominal aorta and inferior vena cava were located and clamped. The donor's aorta and pulmonary artery were anastomosed to the recipient's abdominal aorta and inferior vena cava, respectively, in an end-to-side manner. One dose of buprenorphine (Buprenex Injectable; Reckitt and Colman Pharmaceuticals, Richmond, VA) (0.01 mg/kg intramuscular) was administered after surgery. Implantation was performed within 30 to 40 minutes of removal of the donor heart.

Allograft mice were treated with tacrolimus (Fujisawa, Osaka) administered intraperitoneally (i.p.) (6 mg/kg) daily for a period of 14 (n=5) and 30 (n=5) days post-transplantation. A subset of allograft mice did not receive any immunosuppressive therapy, and were treated with daily i.p. injections of saline (n=5) for a period of 14 days post-transplantation. Mice were euthanized at 14 and 30 days post-transplantation.

#### 3.3.5 Immunofluorescence and histochemical staining

Native and transplanted hearts were excised from the allograft and control syngraft groups and routinely processed for cryosections. The hearts and control tissues were perfusion fixed with 2% paraformaldehyde and then immersion fixed in 2% paraformaldehyde for 4 hours. Tissues were then immersed in sucrose gradient (5%-20%) overnight before being embedded in optimum cutting temperature compound (OCT) and paraffin. Frozen midventricular sections were incubated with 10% normal goat serum for 1 hour at room temperature prior to incubation with primary antibodies anti-smooth muscle (SM)  $\alpha$ -actin (DAKO, Carpinteria, California), anti-von Willebrand factor (vWf) (DAKO) and anti-CD45 (BD Pharmingen) overnight at +4°C. The slides were then washed with Tris-buffered saline (TBS, pH 7.4) followed by incubation in a 1:200 dilution of goat anti-rabbit Alexa 594-conjugated secondary antibody (Molecular Probes, Eugene, Oregon) was used for 45 minutes at room temperature. Slides were washed in TBS and nuclei were counterstained with 1  $\mu$ g/mL Hoechst 33342 (Molecular Probes) for 10 minutes and then washed again with TBS before being coverslipped. Slides were also cut and stained with hematoxylin and eosin and examined using light microscopy.

#### 3.3.6 Confocal microscopic analysis and quantitation of host BM-derived cells

All images were obtained using a Leica AOBS<sup>TM</sup> SP2 confocal microscope. Ten micron sections of the transplant and native hearts stained for vWf, SM  $\alpha$ -actin, and CD45 were analyzed. The

number of GFP-positive BM-derived host cells in the hearts was quantitated at 14 and 30 days post-transplantation in all allograft and syngraft groups. Briefly, 10 representative microscopic fields at 1.4 x 10<sup>-3</sup> mm<sup>3</sup> from the same anatomical location in each myocardial section were taken and Image-Pro® Plus software (MediaCybernetics®, San Diego, CA) was used to count the number of nuclei and nucleated GFP-positive host cells in the confocal optical sections. For vWf and SM a-actin quantitation, analysis for colocalization with BM-derived host GFP-positive cells was performed on volumes of 5.3 x  $10^{-4}$  mm<sup>3</sup> optical stacks of vessels, 60-200  $\mu$ m wide in diameter, found in representative midventricular sections from transplant and native hearts. Stacks were reconstructed using Volocity<sup>™</sup> (Improvision®, Boston, Mass) and Metamorph® image softwares (Universal Imaging Corporation<sup>™</sup>, Downingtown, PA). Two independent, blinded observers reviewed each reconstructed vessel. Nucleated host BM-derived GFP-positive cells seeded within the vessel walls were quantitated and the number of morphologically distinguishable cells which stained dual-positive for GFP and either vWf or SM  $\alpha$ -actin in the vessel wall were scored. Cells were considered endothelial in origin if they were positioned on the luminal side of the vessel, thin and elongate in shape, and demonstrated intracellular granular vWf staining. Cells were considered vascular SMC if they were in the subendothelium or media, were spindle shaped, blunt-ended, and exhibited intracellular SM  $\alpha$ -actin staining. Each chimerism count was expressed as a percentage of total EC or SMC, respectively.

In addition, to confirm the presence of GFP within individual cells separated from the autofluorescent background, spectral unmixing was used to identify the GFP emission signal within the autofluorescent environment of the myocardium. Spectral distribution of autofluorescence and GFP was determined with the Leica confocal microscope. A lambda stack (a series of x-y images that sample emission data from a series of small wavelength bands) was acquired in a given tissue area in order to record the whole emission signal for every single pixel

of the image [14]. The region of interest (ROI) function was used to determine the spectral signature of a selected area in the scanned image. This approach allowed accurate identification of true GFP+ host cells with an emission wavelength peak between 509-512nm as opposed to autofluorescent cells which generally express emission wavelengths with a wider peak ranging between 530-580nm [15]. The spectrally resolved images were recorded using the 488nm line of argon laser in the 500-600nm spectral range for fluorescence detection.

#### 3.3.7 Statistical analyses

Data for the quantitation of host cell seeding are given as mean  $\pm$  SEM. Comparison between groups was made using ANOVA and a post hoc Tukey's test was used to determine statistical differences between groups. Kappa values were calculated for interobserver variability. The frequency of vWf or SM  $\alpha$ -actin and GFP double-positive cells in the vessel wall are shown as mean  $\pm$  SEM of the total EC or SMC. A *p* value of less than 0.05 was considered significant.

#### 3.4 RESULTS

3.4.1 Host cell seeding to the transplanted heart and the effect of immunosuppression

We utilized chimeric GFP-BM recipient mice in a heterotopic heart transplant model to identify the extent of contribution of host BM-derived cells to the repopulation of damaged cells in the vessels of transplanted hearts. Our data demonstrate the presence of infiltrating BM-derived host cells in the transplant hearts of both immunosuppressed and non-immunosuppressed allograft mice at both 14 and 30 days post-transplantation (Figure 3.1). Host BM-derived cell seeding to the transplant heart was significantly greater (p<0.05) as compared with the native heart controls in allograft groups receiving immunosuppression at both 14 (transplant=24%  $\pm$  2.0%, native=5.2%  $\pm$ 1.0%) and 30 (transplant=23%  $\pm$  3.6%, native=4.3%  $\pm$  1.1%) days post-

transplantation (Figure 3.2). Syngraft controls showed minimal host BM-derived cell seeding to the transplant and native hearts at both 14 days (transplant= $2.7\% \pm 0.59\%$ , native= $3.5\% \pm 2.2\%$ ) and 30 days (transplant= $6.6\% \pm 0.46\%$ , native= $6.8\% \pm 4.0\%$ ) post-transplantation. At 14 days post-transplantation, the total BM-derived host cell seeding was found to be significantly higher (p<0.05) in the transplant hearts of non-immunosuppressed allografts (41% ± 2.1%) as compared with immunosuppressed allografts (24% ± 2.0%). Kappa values were 0.69 for interobserver agreement, p<0.01.

#### 3.4.2 CD45 expression of host BM-derived cells

Allograft and syngraft hearts were stained for the pan-leukocyte marker CD45 to identify the inflammatory cell proportion of the host GFP-positive BM-derived cells which seeded to the cardiac interstitium and vasculature. The results (Figure 3.3) indicate that in the transplant hearts, CD45 was co-expressed by a large proportion of the GFP-positive BM-derived host cells found in the perivascular space and cardiac interstitium in allograft hearts at 14 and 30 days post-transplantation, both with and without immunosuppression.

#### 3.4.3 EC/host BM-derived cell double positive cells in transplanted hearts

In all transplant hearts of allograft groups, we found GFP-vWf dual-positive cells in the endothelial lining of vessels (Figure 3.4 & 3.5). The frequency of GFP-vWf dual-positive cells out of the total EC within the vessel wall was  $11.8\% \pm 2.5\%$  and  $4.0\% \pm 1.2\%$  for 14 and 30 days post-transplantation, respectively (Figure 3.6a). The results indicate that the frequency of differentiation events for host BM-derived cells into an EC phenotype within the vessel wall of transplant hearts appears to be higher at 14 days post-transplantation than at 30 days (p=0.04). The frequency of GFP-vWf dual-positive cells was similar in the vessels of transplant hearts in both the non-immunosuppressed (9.4%  $\pm$  2.1%) and the immunosuppressed (11.8%  $\pm$  2.5%)

allograft groups at 14 days post-transplantation (kappa values were 0.71 for interobserver agreement, p<0.01). These findings suggest that 14 days of immunosuppressive therapy does not significantly affect the frequency of host BM-derived cell chimerism in vessels of transplanted hearts. Native hearts demonstrated a low level of GFP-vWf dual-positive cells only in the vessels of non-immunosuppressed allograft hearts, and no dual-positive cells were found in any other allograft or syngraft native hearts.

# 3.4.4 SMC/host BM-derived cell double-positive cells in transplanted hearts

Smooth muscle  $\alpha$ -actin was used as a phenotypic marker for vascular SMC in transplant and native hearts. The results (Figure 3.6b)) indicate that at 14 days post-transplantation, the frequency of smooth muscle  $\alpha$ -actin-GFP dual-positive cells out of the total number of SMC within the vessel wall is not significantly different in the transplanted hearts of the allograft groups (non-immunosuppressed= 0.9% ± 0.89% and immunosuppressed= 2.0% ± 2.0%). This suggests that the frequency of differentiation events into a SMC phenotype is not affected by immunosuppressive treatment with tacrolimus (Figure 3-6). Further, there appears to be no significant difference in the number of smooth muscle  $\alpha$ -actin-GFP dual-positive cells in transplanted hearts of immunosuppressed allografts at 14 and 30 days post-transplantation (2.0% ± 2.0% and 0.4% ± 0.4%, respectively). No vessels in the native heart controls contained smooth muscle  $\alpha$ -actin-GFP dual-positive cells.

#### **3-5. DISCUSSION**

TVD is one of the leading causes of graft failure and death after the first peri-operative year and is now the most important problem in clinical organ transplantation. The original concept of TVD and vascular remodeling assumed that vascular EC and SMC in TVD originate from graft tissue and are therefore donor-derived [1,16]. In recent years, several groups have reported data both supporting [9,17,18] and discounting [19,20] repopulation of graft vessels by host-derived cells after solid organ transplantation. However, several key questions remain unanswered, including: 1) whether there is significant repopulation of cells of the vessel wall with hostderived cells, and if so, what the origin of these cells is, 2) if the repopulation of vascular cells is either an early or late event, or a continuous process throughout TVD progression, and 3) what the effect of immunosuppression is on host cell repopulation of cells of the vessel wall of transplanted hearts. The possibilities for the origin of host cells recruited to damaged vessels include circulating vessel wall-derived adult EC, tissue resident progenitor cells, or host BMderived cells. In this study we investigate these questions by elucidating the contribution of host BM-derived cells in the repopulation of cells of the vessel wall at both an early (14 days) and late (30 days) time-point post-transplantation as well as examining the effect of immunosuppressive therapy with tacrolimus on the frequency of host BM-derived cell differentiation into vascular cell phenotypes.

The results of this study support the concept that host BM-derived cells migrate and localize to the transplanted heart as early as 14 days post-transplantation. Although these host BM-derived cells predominantly consisted of inflammatory cell subsets, we were particularly interested in examining the role of non-inflammatory host cell populations found to seed sites within vessels of the transplanted hearts. Our results, using quantitative image analysis, indicate that host BM-derived cells are recruited early to vessels of allograft transplant hearts where they participate in

the replacement of EC, as well as SMC at a very low frequency. EC replacement in transplanted hearts by host BM-derived cells at 14 days post-transplantation was significantly greater as compared with transplanted hearts at 30 days post-transplantation. It is well established that immune-mediated damage of donor ECs is believed to be an initiating event in TVD. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)-labeling in coronary arteries affected by TVD has revealed apoptotic cells in these lesions and electron microscopic documentation of denuded donor EC in animal models of this disease indicate that large scale unregulated damage is abundant in TVD [21,22]. The resultant damage likely contributes to TVD by increasing vascular permeability, fostering the infiltration of plasma proteins into damaged blood vessels and the myocardial interstitium. Consequently, this donor EC damage and cell death early post-transplantation may lead to the release of a variety of cytokines and other signals in the vessel wall which in turn lead to the homing of host BM-derived cells. The precise signals which attract these host BM-derived cells to the sites of vascular damage in TVD remain unclear. This early EC apoptosis in TVD may account for why there is a decrease in EC replacement in the vessels of transplant hearts from 14 to 30 days post-transplantation.

SMC chimerism was consistently at a low level over time (14 and 30 days post-transplantation) in transplanted hearts of allografts. As well, at the 14 day time-point, the results suggest that immunosuppression with tacrolimus does not significantly affect the EC and SMC replacement by host BM-derived cells in the transplanted heart of allografts. Moreover, the higher levels of host BM-derived cell chimerism found in transplant as compared to native control hearts supports the concept of preferential seeding of these cells to sites of vascular damage.

Uncovering the true extent of EC and SMC replacement by host cells in TVD is an important biological objective that has yet to be addressed adequately. In order to determine the precise

contribution of host BM-derived cells to EC and SMC replacement in TVD, it is vital to utilize and compare findings between the same models of vascular disease, i.e. cardiac allograft vasculopathy, as other models in which there is endothelial injury may lead to variable findings due to differences in pathogenetic mechanisms. Our study depends on a well-established heterotopic cardiac allograft model to study the role of host BM-derived cells in EC and SMC However, even in similar animal models of TVD, there remains large replacement. discrepancies and debate regarding the frequency of host cell replacement of cells of the vessel wall and particularly the contribution of BM-derived cells. Some studies report that almost all cells in the vessel wall are host-derived in models of aortic transplantation [3,18,23,24], vein grafting [25], and cardiac allografts [4,24], while other studies have reported contrary results suggesting host cells only contribute minimally, if at all, to vascular cell replacement in similar models of allograft vasculopathy. Attempts at explaining these discrepancies have speculated that immunosuppressants may potentially affect the pathogenesis of TVD, while others suggest that certain models may underestimate the potential contribution of host cells in vascular remodeling. Furthermore, the contribution of host cell chimerism in allografts may vary in different tissues as suggested by one study which demonstrates that host-derived ECs replaced donor endothelium in aortic but not cardiac allografts [24].

Recent studies also propose that imaging artifacts related to the lack of stringent methodological approaches may explain the major discrepancies in the reported contribution of host BM-derived cells as a major source of SMC in graft vasculopathy (5% [23]-82.5% [4]) [26]. We have utilized more rigorous imaging techniques and analytical strategies to confirm the specificity of the GFP spectral tracking and three-dimensional imaging than those employed before when investigators have reported both high [4] and low [23] rates of EC and SMC replacement by host BM-derived cells. Several studies have utilized both conventional light microcopy [3,10,11] and

confocal microscopy [4,9] to examine the fate of host cells within transplanted hearts. With respect to confocal microscopy, although this technique does allow the power to analyze thicker tissue sections as compared to conventional light microscopy, there is also a risk in relying solely on the color overlap of two fluorophores in a given thickness of tissue to determine the phenotype of cells of interest. Confocal microscopy increases the capability of distinguishing adjacent structures in thicker sections of tissue if it is utilized to its full potential. By obtaining optical sections of a given tissue, confocal microscopy enables users to produce threedimensional reconstructions and volume renderings in order to examine target cells in that given volume of tissue in a three-dimensional, more accurate view. In this study, we have used 10  $\mu$ m sections and obtained 0.4-0.5 µm optical sections of tissue stained for EC and SMC markers. These optical sections were then reconstructed to produce a volume rendering of each vessel and were examined by segmentation analysis of the 3 different color channels and by rotating the volume through 180° at increments of 1°. In this manner, we anticipate accurate determination of colocalization events between cellular markers in the exact three-dimensional plane (x, y and z planes) of tissue. In addition, a wavelength (lambda) scan of the GFP-expressing host BMderived cells was performed using a confocal laser scanning microscope to confirm the presence or absence of GFP within individual cells [14,27,28] (discussed further in Chapter 5). We believe the present observations bring us closer to identifying the true frequency of host BMderived cell chimerism in vessels of cardiac allografts.

Thus, although our results for the frequency of differentiation events into both an EC and SMC phenotype in murine heart allografts is consistent with some recent findings [11,29], we report lower rates of host BM-derived cell replacement of EC and SMC in cardiac allografts as compared to other studies which utilized confocal microscopic analysis [3,4,9]. As mentioned above, these lower rates may be attributed to our utilization of rigorous methods of image
analysis for all tissue sections, thereby enhancing our identification of true EC and SMC of host BM origin, and diminishing the chance of both falsely identifying inflammatory cells as EC or SMC in the vessel wall and falsely identifying subadjacent cells as colocalization events. Another factor which may contribute to our lower rates of host cell chimerism is our selective examination of only host BM-derived cell contributions to chimerism in the vessels of transplant hearts. Indeed, the BM is likely not the only source of cells that contribute to this phenomenon and numerous reports have in fact shown that vascular cells are heterogeneous and that both EC and SMC in vascular lesions are composed of cells of diverse origins [30,31]. In addition, there may be a population of radiation-resistant BM progenitor cells that also contribute to vascular remodeling, which would not be labeled with the GFP marker following BM reconstitution, and thus, would not be detected by our stringent image analysis technique.

To address the question of whether immunosuppression may affect the host cell repopulation of EC and SMC in TVD, we compared the effect of immunosuppressive treatment with tacrolimus versus no treatment on the rates of host BM-derived cell chimerism in the vessels of our heterotopic heart transplant model. Our findings confirm that even as early as 14 days post-transplantation, immunosuppression in a cardiac allograft model does not significantly affect the frequency of host BM-derived cell repopulation of EC and SMC. Hence, our data suggest that discrepancies in the reported rates of host cell chimerism at early time-points in vessels of transplanted hearts to date are not due to an effect of immunosuppressive treatment on progenitor cell populations in the BM.

To our knowledge, this study is among the first to examine the effect of immunosuppressive therapy on host BM-derived cell chimerism in the transplanted heart. Interestingly, we found that although immunosuppression with tacrolimus led to a significant decrease in the total

number of host BM-derived cells seeding to sites within the transplanted heart of allografts at 14 days post-transplantation, it did not appear to affect the frequency of host BM-derived cell repopulation of EC and SMC. One possible reason for this is that the time-point used in this study was not long enough to see differences in chimerism rates between the immunosuppressed versus the non-immunosuppressed allograft hearts. The murine heterotopic heart transplant model utilized in this study does not allow survival of the transplant heart past 14 days without immunosuppressive therapy, as the non-immunosuppressed transplant heart ceases to beat past this time-period, and it is possible that we would begin to see differences in rates of chimerism due to immunosuppression at the 30 day time-point. However, there is a possibility that a progenitor cell population within the BM is not affected by immunosuppressive therapy with tacrolimus, and that this treatment only influences the inflammatory cell response to the heart in allografts.

The findings reported in this chapter are novel in the field of cardiac TVD and the study of vascular repopulation events. This is the first report which demonstrates that immunosuppression with tacrolinus does not affect the frequency of repopulation of damaged vessels in the donor heart by host BM-derived cells in a murine model of TVD. We demonstrate that EC replacement is an early event, found to occur at 14 days post-transplantation, but at a rate which diminishes with time up to 30 days post-transplantation. We found that the frequency of host BM-derived cell chimerism events at an early time-point of 14 days post-transplantation, into both EC and SMC phenotypes, remains constant irrespective of immunosuppressive treatment with tacrolimus. These data suggest that precursor cells are recruited early following transplantation to areas of donor vascular dysfunction in a process of attempted repair in the context of ongoing donor-recipient alloimmune interactions. Since cardiac TVD is primarily a vascular disorder affecting both intramyocardial and epicardial

coronary arteries and veins [32,33] and is characterized predominantly by EC damage and intimal proliferation, the results of this manuscript focus specifically on the role and contribution of host BM-derived cells in the repopulation of EC and SMC in the vasculature of cardiac TVD. However, there is also considerable interest and continued controversy surrounding the issue of whether BM-derived cells can directly or indirectly facilitate functional cardiac cell regeneration. Although the murine heterotopic heart transplant model is not the optimal model of cardiomyocyte injury to evaluate the potential rates of host BM-derived cells differentiation into cardiomyocytes, we have conducted preliminary studies and our results using desmin immunolabeling indicate that this phenomenon occurs at a very low frequency in this model. Additional studies will also be required to determine the degree to which other, non-BM derived sources of host cell populations contribute to this replacement process and to identify the factors that modulate this process.

Figure 3.1



Figure 3.1 Host BM-derived cell seeding to transplanted hearts of allografts. Hematoxylin and eosin (H&E) staining of allograft transplant hearts at 14 days post-transplantation without tacrolimus (a), with tacrolimus (b) and 30 days post-transplantation with tacrolimus (c). Serial sections of transplant hearts were stained with Hoechst 33342 to identify nuclei (blue) and the endogenous green fluorescence of host GFP-positive BM-derived cells was examined (d-f). Scale bars =  $30 \mu m$ .

Figure 3.2



Figure 3.2 Quantification of host BM-derived GFP-positive cell seeding. Host GFPpositive BM-derived cells seeded within transplant and native hearts of all allograft and syngraft groups were quantified and expressed as a fraction of total cardiac nuclei and graphically represented as the mean  $\pm$  SEM from animals per group. \*Significant difference, p<0.05.

Figure 3.3



Figure 3.3 Hematopoietic phenotype of GFP-positive host BM-derived cells in transplanted hearts. Confocal micrographs of (a) non-immunosuppressed and (b) tacrolimus-treated allografts 14 days post-transplantation and (d) tacrolimus-treated allografts 30 days posttransplantation reveal a population of GFP-positive host BM-derived cells (green) which stain positively for CD45 (red). (c,e) Control syngraft transplant hearts at 14 and 30 days posttransplantation, respectively. (f) Higher magnification of GFP-positive host BM-derived cells demonstrating CD45-positivity on the cell surface. Nuclei (blue) stained with Hoechst 33342. Inset (a) shows control immunostain with specific antibody isotype control. Scale bars = (a-e)  $50 \mu m$ , (f-i) 10  $\mu m$ .

### Figure 3.4

Non-immunosuppressed 14 days post-transplantation



Immunosuppressed 14 days post-transplantation



Immunosuppressed 30 days post-transplantation



Figure 3.4 von Willebrand factor (vWf) staining of EC in transplanted hearts. Confocal micrographs of (a, f, h) allograft vessels. (b) Hoechst 33342 nuclear staining (blue), (c) GFP-positive host BM-derived cells (green), (d) vWf-positive EC (red), and (e, g, i) merged images at higher magnification show dual-positive granular staining for vWf within the GFP cytoplasm of some cells (arrows). Scale bars = (a) 40  $\mu$ m, (b-e, g,i) 10  $\mu$ m (f,h) 20  $\mu$ m.

Figure 3.5



Figure 3.5 Smooth muscle  $\alpha$ -actin staining in vessels of allografts. Confocal micrographs of (a) transplant heart 14 days post-transplantation. (b) Hoechst 33342 nuclear staining (blue), (c) GFP-positive host BM-derived cells (green), (d) smooth muscle  $\alpha$ -actin-stained SMC (red), and (e) merged at higher magnification reveal green/red dual-positive staining of rare host cells. Scale bars = (a-e) 10 µm.



Mouse group

Figure 3.6 Chimerism frequencies of host BM-derived cells in vessels of transplanted and native hearts. Graphical representation of the frequency of (a) ECs double-positive for the vWf marker and GFP expression of the host BM-derived cells and (b) SMCs double-positive for the SMC marker and GFP expression of the host BM-derived cells in vessels of allografts. Data is represented as the mean  $\pm$  SEM from animals per group at each timepoint of 14 and 30 days post-transplantation. \*Significant difference, p < 0.05.

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## CHAPTER 4: EFFECTS OF GRANULOCYTE-COLONY STIMULATING FACTOR ON BONE MARROW-DERIVED PROGENITOR CELLS IN MURINE CARDIAC TRANSPLANTATION

## **Objective:**

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To determine whether immunosuppressive drugs and G-CSF treatment affect progenitor cell survival *in vitro* and to examine the effect of G-CSF-induced mobilization of host BM-derived progenitor cells on vascular chimerism in the transplanted heart *in vivo*.

# Based on the Manuscript:

<u>**Rezai N**</u>, Deisher TA, Wang X, Corbel SY, Leung J, Kerjner A, Rossi FM, Podor TJ and McManus BM. Effects of granulocyte-colony stimulating factor on bone marrow-derived progenitor cells in murine cardiac transplantation. (Submitted, *J Heart Lung Transplant, September 2006*)

#### 4.1 RATIONALE

In the previous Chapter, we utilized GFP-BM chimeric mice as recipients of heterotopic heart transplants to examine the role of host BM-derived progenitor cells following cardiac transplantation. The results indicated that host BM-derived cells seed to the areas within the damaged transplanted heart and contribute to the replacement of EC and SMCs within the vessels of the allograft at both 14 and 30 days post-transplantation, irrespective of immunosuppression with tacrolimus. The frequency of EC replacement by host BM-derived cells was found to diminish from the early timepoint of 14 days post-transplantation to the later timepoint of 30 days post-transplantation in transplanted hearts. The cytokine G-CSF has been shown to mobilize progenitor cells from the BM into the circulation and in different models of cardiac injury, including MI, G-CSF has been found to improve cardiac function and reduce damage. This improvement in function following G-CSF treatment has been attributed in many studies to the chimerism potential of mobilized progenitor cells and their contribution to the replacement of damaged myocardial and vascular cells in the heart. Recently, in cardiac transplantation studies have shown an improvement of graft survival following G-CSF pretreatment of either donors or recipients of heart allografts. However, the contribution of host progenitor cell chimerism into vascular phenotypes to this reported improvement in graft function and survival has not been examined to date. Therefore, it is the objective of this Chapter to investigate the effect of G-CSF pretreatment of recipients of cardiac transplants on vascular cell repopulation or chimerism by host BM-derived cells in allograft vessels and the subsequent effect on allograft function.

#### 4.2 INTRODUCTION

BM progenitor cells into the circulation of patients in response to cytokine or chemokine stimulation was first documented in the late 1970s and early 1980s [1]. Since then, this process,

referred to as mobilization, has been shown to be promoted clinically and in experimental animal models by several agents, including the most commonly used cytokine, granulocyte-colony stimulating factor (G-CSF) [2-8]. Mobilization of BM progenitor cells by G-CSF is achieved by the disruption of the homing mechanisms of stem cells in the BM and by selective mobilization of BM HSCs into the blood after M phase of the cell cycle [9]. In animal models, G-CSF-induced BM mobilization resulted in improved function after both myocardial ischemia [10] and hindlimb ischemia [11]. Additionally, injection of isolated G-CSF-mobilized adult human CD34+ cells stimulated neoangiogenesis in the infarct vascular bed [12]. In transplantation, it has been suggested that G-CSF-induced mobilization of BM cells leads to improved outcomes following heart transplantation [13-15]. Using experimental models of cardiac transplantation, these studies have demonstrated that G-CSF pretreatment of both donors and/or recipients of heart transplants lead to improved graft outcome post-transplantation [13-15]. However, to date, the contribution of vascular chimerism by host BM-derived cells to this observed improvement in graft outcome following G-CSF treatment in cardiac allografts has yet to be examined.

Several immunosuppressants are currently being used in transplantation medicine, including tacrolimus and rapamycin. Despite studies which have demonstrated an inhibitory effect of rapamycin treatment *in vitro* on the proliferative ability of BM cells, the effects of immunosuppressive drugs on progenitor cell populations in the BM are relatively unknown. As well, *in vivo* G-CSF pretreatment of donors or recipients of cardiac allografts has been found to lead to improved function following transplantation. However, the basis of this improvement in graft function has not been determined. The aim of this study is to examine the effect of immunosuppressive therapy and G-CSF treatment on *in vitro* BM progenitor cell survival and to determine the therapeutic potential of G-CSF-induced mobilization of host BM-derived progenitor cells on reendothelialization of damaged blood vessels characteristic of cardiac TVD.

#### 4.3 METHODS

#### 4.3.1 Blood and BM-derived progenitor cell characterization

Total mononuclear cells were isolated from human blood by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). Heparinized blood (30-50 mL) was diluted one to one with phosphate buffered saline (PBS) with 10% fetal bovine serum, (FBS; Hyclone, Logan, UT) and overlayed onto an equivalent volume of Histopaque-1077. Cells were centrifuged for 30 minutes at room temperature at 400 x g. After centrifugation, the opaque interface was carefully aspirated and transferred into a clean conical centrifuge tube. Mononuclear cells were isolated and washed three times with PBS with 10% FBS, each time with centrifugation at 250 x g for 10 minutes, and resuspended in endothelial basal medium (EBM-2; Clonetics-Cambrex, Guelph, ON) supplemented with EGM SingleQuots and 5% FBS. 1 x 10<sup>6</sup> cells were seeded per well in 24-well fibronectin-coated plates and exposed to increasing doses of immunosuppressive agents, tacrolimus (Fujisawa, Osaka) (0, 0.001, 0.01, 0.1 and 1  $\mu$ g/mL) in the presence and absence of G-CSF (Amgen, Thousand Oaks, CA, 20 ng/mL) and incubated in a 5% CO<sub>2</sub> incubator at 37°C. After 4 days in culture, nonadherent cells were removed, fresh media was applied and the culture was maintained through day 7 with media changes every 2 days thereafter. Adherent mononuclear cells were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyaninelabeled acetylated low density lipoprotein (DiI-ac-LDL; 2.4 µg/mL at 37°C for 1 hour; Molecular Probes), fixed with 2% formaldehyde for 10 minutes, and then stained with FITC-labeled Ulex europaeus agglutinin I (UEA-1 lectin; 10 mg/mL for 1 hour; Sigma). Double-positive cells were counted in 4 randomly selected high-power fields by 2 independent investigators.

Whole BM was harvested from C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) by flushing femurs and tibiae with cold PBS/2% FBS solution and plated at a density of 1 x  $10^6$  cells in 24-well fibronectin-coated dishes and maintained in EBM-2 media supplemented with

EGM SingleQuots and 5% FBS as described above in the presence and absence of G-CSF (20 ng/mL). BM cell plates were exposed to increasing doses of immunosuppressive agents, tacrolimus and sirolimus (Wyeth-Ayerst Laboratories, Madison, NJ) (0, 0.001, 0.01, 0.1 and 1  $\mu$ g/mL). After 4 days in culture, nonadherent cells were removed, fresh media was applied and the culture was maintained through day 7 with media changes every 2 days thereafter. Adherent BM cells were incubated with DiI-ac-LDL (2.4  $\mu$ g/mL at 37°C for 1 hour) and washed 3 times with PBS. DiI-ac-LDL-positive cells were counted in 4 randomly selected high-power fields in each well by 2 independent investigators.

#### 4.3.2 Apoptosis assay

Quantitative determination of the percentage of progenitor cells undergoing apoptosis with tacrolimus and G-CSF treatment *in vitro* was determined using an Annexin V apoptosis detection kit (BD) according to the manufacturer's protocol. Briefly, cultured BM-derived progenitor cells were treated with tacrolimus and G-CSF as described above and at day 7, harvested by digestion with 0.25% trypsin. After the recommended washing steps,  $1 \times 10^6$  cells were incubated for 15 minutes with fluorescein isothiocyanate (FITC)-conjugated Annexin V and/or propidium iodide (PI) in binding buffer in the dark. Unlabelled cells, cells stained with PI only, cells stained with Annexin V only, and cells stained with Annexin V and PI were immediately analyzed by flow cytometry. Each experiment was carried out in triplicate and repeated twice to ensure reproducibility.

#### 4.3.3 BM-derived cell proliferation

The effect of G-CSF and immunosuppressive treatment on BM-derived progenitor cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. After being cultured for 7 days, BM-

derived progenitor cells were digested with 0.25% trypsin and then cultured at the same concentration  $(10^5-10^6 \text{ cells/well})$  in serum-free medium in a 96-well culture plate (200 µL per well). G-CSF was added in increasing concentrations of 5, 10, 20 and 50 ng/mL while tacrolimus was added in separate wells in increasing concentration (0.001, 0.01, 0.1 and 1µg/mL). A subset of the tacrolimus-treated wells were also pretreated with 20 ng/mL G-CSF. Each concentration included 6 wells and the serum-free medium served as control. After being cultured for 24 hours, the cells were supplemented with 20 µL of MTS (Promega, Madison, WI, USA) and incubated for another 2 hours before the OD value was measured at 490 nm.

#### 4.3.4 Angiogenesis assay

An *in vitro* angiogenesis kit (Chemicon, Temecula, CA, USA) was used to assess the effect of G-CSF and tacrolimus treatment of BM-derived progenitor cells on tubule formation. ECMatrix<sup>TM</sup> solution was thawed on ice overnight, then mixed with 10 X ECMatrix<sup>TM</sup> Diluent and placed in a 96-well tissue culture plate at 37°C for 1 hour to allow the matrix solution to solidify. Cultured BM progenitors were harvested as described above and replated (20,000 cells per well) on top of the solidified matrix solution. Cells were grown with tacrolimus in the presence and absence of G-CSF and incubated at 37°C for 24 hours. Tubule formation was inspected under an inverted light microscope at X200 magnification. Tubule formation was defined as a structure exhibiting a length greater than 4 times its width. Five independent fields were assessed for each well, and the average number of tubules/X200 field was determined.

#### 4.3.5 Flow cytometric identification of neutrophils and progenitor cells

C57BL/6 male mice, 8 weeks of age, were treated subcutaneously with recombinant human G-CSF (Filgrastim) at concentrations of 50, 200, and 500  $\mu$ g/kg for 5 days. Blood was drawn by retro-orbital bleed into heparinized microcapillary tubes and then circulating blood cells were

analyzed on a Bayer Advia 120. BM was isolated and FACS staining of lineage negative (lin-), cKit positive (cKit+) and Sca-1 positive (Sca-1+) cells was carried out using the rat anti-mouse lin- PE-Cy7-cocktail (CD3e, CD11b, CD45R/B220, Ly-6G and Ly-6C (Gr-1), TER-119/erythroid cells), rat anti-mouse c-Kit- FITC and rat anti-mouse Sca-1-PE antibodies, along with their corresponding isotype-matched PE-Cy7, FITC or PE-conjugated rat immunoglobulin (BD Pharmingen, San Diego, CA) for 20 minutes at 4°C. Flow cytometric analysis was performed with a flow cytometer (FACScan; Becton Dickinson, Biosciences) and Cell Quest Software. Blood from control and G-CSF treatment mice was also cultured on fibronectin-coated plates as described above. On day 7, cells were stained with DiI-ac-LDL and positive cells were counted in 4 randomly selected high-power fields by 2 independent investigators.

#### 4.3.6 Generation of BM-chimeric mice

All experiments were approved by the University of British Columbia Animal Care Committee. BM-GFP chimeric C57BL/6mice were generated as described previously[16] and the reconstitution of the hematopoietic system was analyzed by flow cytometry as previously described[17].

#### 4.3.7 Heterotopic cardiac transplantation

Heterotopic cardiac transplantation was performed as described[16,18]. Briefly, hearts of 10week-old male 129SV/j mice were transplanted into the reconstituted GFP-BM chimeric C57BL/6 mice described above. A subset of the GFP-BM C57BL/6 recipient mice received daily injections of human recombinant G-CSF (500 µg/kg/day) for 8 consecutive days prior to undergoing heart transplantation surgery. Control syngrafts consisted of 10-week-old C57BL/6 donor hearts transplanted into C57BL/6GFP-BM chimeric mice (five transplants were performed for each group at each timepoint). Animals were anesthetized with 4% halothane and anesthesia maintained with 1% to 2% halothane (Halocarbon Laboratories, River Edge, NJ). Donor mice were infused with heparinized saline and their hearts excised. The recipient's abdominal aorta and inferior vena cava were located and clamped. The donor's aorta and pulmonary artery were anastomosed to the recipient's abdominal aorta and inferior vena cava, respectively, in an end-to-side manner. One dose of buprenorphine (Buprenex Injectable; Reckitt and Colman Pharmaceuticals, Richmond, VA) (0.01 mg/kg intramuscular) was administered after surgery. Implantation was performed within 30 to 40 minutes of removal of the donor heart. Allograft mice were treated with tacrolimus administered intraperitoneally (6 mg/kg) daily following heart transplantation. Mice were euthanized at 14 and 30 days post-transplantation.

#### 4.3.8 Tissue harvesting and morphometry

At 14 and 30 days post-transplantation, mice were anesthetized by injection with ketamine/xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The native and transplanted hearts were perfused with sterile saline at 2 mL/minute followed by 4% paraformaldehyde (PFA) (Fisher Scientific, Fairlawn, NJ) at the same flow rate. Hearts were then rapidly excised and immersion-fixed in 4% PFA for 4 hours followed by immersion in 5-20% sucrose gradient. Ventricular transverse-sections were then both OCT-frozen or paraffinembedded. Sections were cut serially and stained with hematoxylin and eosin (H&E) and Movat's pentachrome. To evaluate TVD, all visible medium to large coronary arteries from five transplanted hearts per group were photographed at X400 magnification using a Spot digital camera (n=48 arteries for control non-G-CSF treated recipients and n=36 arteries for G-CSF treated recipients). *Image-Pro® Plus* (MediaCybernetics®, San Diego, CA) was used to quantitate intimal and luminal areas and percent luminal narrowing was calculated using the following formula described by Armstrong *et al.* [19]: % Luminal narrowing = Intimal area/(Intimal area + Lumen area)

4.3.9 Immunofluorescence staining and confocal microscopic analysis and quantitation

Immunofluorescence staining was performed on OCT-frozen midventricular sections as previously described [16]. Briefly, sections were incubated with primary antibodies to von Willebrand factor (vWf) (DAKO) and anti-smooth muscle (SM) a-actin (DAKO, Carpinteria, California). Alexa 594-conjugated secondary antibody was used and nuclei were counterstained with Hoechst 33342 (Molecular Probes, Eugene, Oregon). Images were obtained using a Leica AOBS<sup>™</sup> SP2 confocal microscope and analyzed as previously described [16]. The number of GFP-positive host-derived BM cells in the hearts was quantitated at 14 and 30 days posttransplantation in allograft and syngraft groups. Image-Pro® Plus software was used to count the total number of nuclei and the number of nucleated GFP-positive host cells in the confocal optical sections. For vWf and SM  $\alpha$ -actin quantitation, colocalization with GFP-positive hostderived cells was determined on vessels 60-200 µm in diameter found in representative midventricular sections from transplanted and native hearts. Stacks were reconstructed using Volocity<sup>™</sup> (Improvision<sup>®</sup>, Boston, Mass) and Metamorph<sup>®</sup> image softwares (Universal Imaging Corporation<sup>TM</sup>, Downingtown, PA). Two independent, blinded observers reviewed each reconstructed vessel. Nucleated GFP-positive host-derived cells found within the vessel walls were quantitated and the number of morphologically distinguishable cells which stained dual-positive for GFP and either vWf or SM  $\alpha$ -actin in the vessel wall were scored. Cells were considered endothelial in origin if they were positioned on the luminal side of the vessel, thin and elongate in shape, and demonstrated intracellular granular vWf staining. Cells were considered vascular SMC if they were in the subendothelium or media, were spindle shaped, blunt-ended, and exhibited intracellular SM  $\alpha$ -actin staining. Each chimerism count was expressed as a percentage of total EC or SMC, respectively.

#### 4.3.10 Statistical analyses

Comparisons of neutrophil levels between *in vivo* G-CSF doses were made using ANOVA and a post hoc Tukey's test. Unpaired Student's t-test with Welch's correction was used for comparison of vehicle and 500  $\mu$ g/kg G-CSF-induced increases in circulating leukocytes and hematopoietic progenitor cells. All other comparisons between groups were made using ANOVA and a post hoc Tukey's test to determine statistical differences between groups. Kappa values were calculated for interobserver variability. The frequency of vWf-GFP double-positive cells in the vessel wall are shown as mean  $\pm$  SEM of the total EC number. A *p* value of less than 0.05 was considered significant.

#### 4.4 RESULTS

4.4.1 The *in vitro* effects of G-CSF treatment on blood and BM-derived progenitor cells

Mouse BM progenitor cells were cultured separately with increasing doses of 2 different immunosuppressive agents, tacrolimus and sirolimus. The observed effect with either tacrolimus or sirolimus is presented in Figure 1. Cultured cells staining positive for DiI-ac-LDL were analyzed (Figure 4-1a) and the progenitor cell count was found to decrease in a dose-dependent manner with both drugs, with sirolimus having a more significant effect at all drug doses (Figure 4-1b,  $p\leq0.05$ ). With tacrolimus treatment, the level of double-positive cells decreased significantly from 100% at baseline control levels to  $52.8\% \pm 4.3\%$  at 0.001 µg/mL,  $41.4\% \pm 4.2\%$  at 0.01 µg/mL,  $34.6\% \pm 4.2\%$  at 0.1 µg/mL, and  $30.3\% \pm 4.7\%$  at 1 µg/mL ( $p\leq0.05$ ). With sirolimus treatment, the level of baseline control levels (100%) to  $36.6\% \pm 6.5\%$  at 0.001 µg/mL,  $21.4\% \pm 5.9\%$  at 0.01 µg/mL,  $15.9\% \pm 4.2\%$  at 0.1 µg/mL, and  $13.3\% \pm 4.1\%$  at 1 µg/mL ( $p\leq0.05$ ).

Human mononuclear blood cells and mouse BM cells were also separately pretreated with 20 ng/mL of human recombinant G-CSF, followed by treatment with an immunosuppressive agent after G-CSF treatment. Figure 4-1c and 4-1d illustrates that in both mouse BM and human blood mononuclear cells, respectively, G-CSF treatment induced significant increased baseline levels of progenitor cells staining positive for the cell markers DiI-ac-LDL and UEA-1 lectin. In mouse BM, the baseline counts of cells pretreated with G-CSF and stained DiI-ac-LDL-positive were expressed as a percentage of the control, non-treated cell count (100% at baseline) and was found to significantly increase to  $128.0\% \pm 4.0\%$  (p $\leq 0.05$ , Fig. 4-1c). In the group of cells which were not pretreated with G-CSF and only treated with increasing dose of tacrolimus, the cell counts were found to decrease with increasing drug dose (52.8%  $\pm$  4.3% at 0.001 µg/mL, 41.4%

± 4.2% at 0.01 µg/mL, 34.6% ± 4.2% at 0.1 µg/mL, and 30.3% ± 4.7% at 1 µg/mL). In the G-CSF pretreated cultures, the cell count expressed as a percentage of the baseline control maintained significantly higher levels of these cells at all drug doses as compared to the cells not pretreated with G-CSF (86.0% ± 7.1% at 0.001 µg/mL, 79.2% ± 5.6% at 0.01 µg/mL, 78.8% ± 6.4% at 0.1 µg/mL, and 75.6% ± 6.4% at 1 µg/mL). In the cultured human mononuclear cells, a similar pattern was observed in non-pretreated and G-CSF pretreated cells staining double-positive for DiI-ac-LDL and lectin (Fig. 4-1d). In the non-pretreated control group, the cell counts, expressed as a percentage of the baseline control counts at 0 treatment (100%), were found to decrease with increasing drug dose (49.1% ± 6.0% at 0.001 µg/mL, 34.2% ± 3.1% at 0.01 µg/mL, 28.4% ± 7.1% at 0.1 µg/mL, and 22.0% ± 5.2% at 1 µg/mL, kappa values were 0.67 for interobserver agreement, p<0.01). In the G-CSF pretreated cells, there was a significant increase in the baseline double-positive cell counts with G-CSF treatment (no tacrolimus treatment) expressed as a percentage of the control cell not pretreated with G-CSF (145.0 ± 4.5%, p≤0.05).

4.4.2 Contribution of apoptosis to the observed reduction in progenitor cell number with tacrolimus and G-CSF

The percentage of BM-derived progenitor cells undergoing apoptosis following treatment with tacrolimus and G-CSF was assessed using Annexin V-PI flow cytometry (Figure 4-2 a,b). In the tacrolimus treated progenitor cells, the rate of apoptosis increased with increasing drug dose (Figure 4-2b), ranging from  $3.16\% \pm 0.502\%$  at baseline to  $33.0\% \pm 1.26\%$  at the highest dose of 1 µg/mL tacrolimus. G-CSF pretreated cells demonstrated a significantly reduced frequency of apoptosis at each given dose of tacrolimus. The percentage of G-CSF pretreated cells which were also treated with 0.001 µg/mL tacrolimus was found to be  $9.15\% \pm 0.70\%$  as compared to non-pretreated cells as the same dose of tacrolimus which was  $12.0\% \pm 0.78\%$  (p $\leq 0.05$ ). G-CSF

pretreated cells did not show an increase in the percent apoptosis at the 0.01 µg/mL dose tacrolimus (9.66%  $\pm$  1.08%), although non-pretreated cells at that same dose showed a significant increase in apoptosis (16.51%  $\pm$  0.94%, p≤0.05). The percentage of cells undergoing apoptosis in G-CSF pretreated cells did increase at the two highest doses of tacrolimus, 0.1 and 1 µg/mL, but were still significantly lower at each dose when compared to the non-pretreated progenitor cells.

4.4.3 G-CSF and immunosuppressive treatment *in vitro* effect on BM-derived progenitor cell proliferation

The effect of G-CSF and tacrolimus on BM-derived progenitor cell proliferation was assayed using an MTS assay (Figure 4-3). G-CSF dose-dependently increased progenitor cell proliferative activity, reaching maximal at 20 ng/mL (Figure 3a, p $\leq$ 0.05). Tacrolimus treatment of cells led to a dose-dependent decrease in progenitor cell proliferation (Figure 4-2b, p $\leq$ 0.01). However, cells which were pretreated with 20 ng/mL G-CSF demonstrated significantly higher proliferative activity at the same dose of tacrolimus (0.001, 0.01 and 0.1 µg/mL) (Figure 4-3b, p $\leq$ 0.05).

4.4.4 G-CSF and immunosuppressive treatment effect on BM-derived progenitor cell *in vitro* angiogenesis

The *in vitro* angiogenesis assay was used in this study to investigate the functional ability of BMderived progenitor cells to participate in angiogenesis. The response of the progenitor cells to tacrolimus and G-CSF pretreatment is depicted in Figure 4-4. Tubules were counted in all wells (Figure 4-4a) and the results demonstrate tubule numbers decrease in a dose-dependent manner to tacrolimus concentrations at 24 hours incubation (Figure 4-4b,  $p \le 0.0001$ , kappa values were 0.67 for interobserver agreement, p<0.01). However, pretreatment of cells with G-CSF led to significantly higher tubule counts at the same dose of tacrolimus ( $p \le 0.05$ ).

4.4.5 G-CSF mobilization of neutrophils and BM-derived progenitor cells in vivo Recombinant human G-CSF does not mobilize BM progenitor cells or enhance circulating neutrophil numbers in C57BL/6 mice as robustly as it is reported to in AKR or DBA/2 mice [20]. Therefore, we determined the ability of 5 days of repeated G-CSF dosing to increase circulating neutrophils (Figure 4-5) and progenitor cells (Figure 4-6) in C57BL/6mice. Circulating neutrophils were not increased in response to 50 µg/kg G-CSF daily doses, however, at 200 µg/kg, G-CSF increased circulating neutrophils 1.7 fold (Figure 4-5a) and absolute neutrophil numbers by 4.0 fold (Figure 4-5b). Neutrophil numbers were maximally increased with a dose of 500 µg/kg (4.7 fold circulating and 13.6 fold absolute neutrophil number). We subsequently determined the ability of 500 µg/kg G-CSF dosing for 5 days to also increase hematopoietic progenitor cell mobilization into the circulation by measuring the number of CFU-GEMM colonies formed per microliter of circulating blood. In this experiment, 500 µg/kg G-CSF increased circulating neutrophils 3.0 (Figure 4-5c) and 7.0 fold for absolute neutrophil numbers (Figure 4-5d). Flow cytometry was performed using BM samples from control and G-CSF treated mice to assess progenitor cell levels. Figure 4-6a shows the density plots of the flow cytometric analysis of the BM cells with forward (SSC) and side light scatter (FSC). The gate was set on the mononuclear/lymphocyte fraction (i) and the lin+ and lin- fractions were separated from the gated BM population (ii). The lin- population was then further sorted to quantitate the percent of the gated lin- cells which stained double-positive for cKit and Sca-1 (top right quadrant) (iii). Figure 4-6b graphically represents the flow cytometry data of the lin-/cKit+/Sca-1+ populations in the control and G-CSF-treated mice, demonstrating that G-CSF treated mice show a 8.3 fold increase in BM lin-/cKit+/Sca-1+ cells. Cultured blood progenitor cells staining positive for DiI-ac-LDL are shown in Figure 4-6c revealing a 2.7 fold increase in the number of double-positive progenitor cells in the G-CSF treated mice as compared to control mice ( $p\leq0.05$ ). Similarly, hematopoietic colony formation was assessed by using the CFU-GEMM (colony-forming unit–granulocyte, erythrocyte, monocyte) assay. Colony formation per microliter whole blood was increased by 500 µg/kg G-CSF by 60.5 fold over vehicle (Figure 4-6d).

#### 4.4.6 TVD morphometry

Ventricular cross-sections were assessed for intimal thickening at 14 and 30 days posttransplantation. At 14 days post-transplant, there was no significant difference in the degree of intimal narrowing in transplanted hearts between G-CSF pretreated and control non-treated recipients of cardiac allografts (Figure 4-7). However, the extent of intimal thickening of allograft arteries was significantly increased at the 30 days post-transplant in the control nonpretreated group as compared to the G-CSF pretreated group at that timepoint. In G-CSF pretreated recipients, there was an average of  $23.0\% \pm 2.3\%$  luminal narrowing of coronary arteries in cardiac allografts as compared to  $29.4\% \pm 3.0\%$  luminal narrowing in control nonpretreated recipients (Figure 4-7, p≤0.05). There was no luminal narrowing in syngraft controls.

4.4.7 The effect of G-CSF pretreatment of mouse heterotopic heart transplant recipients on total host BM-derived cell seeding to the transplanted heart

In order to determine the contribution of host-derived BM cells to the repopulation of damaged vessels in transplanted hearts, and to determine the effect of recipient G-CSF pretreatment on the regenerative capacity of the BM-derived cells, we utilized a murine heterotopic heart transplant model in BM-GFP chimeric recipient mice. We have previously demonstrated that host-derived BM cells migrate and seed significantly more to the damaged transplanted heart of allografts as

compared to the native control heart or to syngraft controls at 14 and 30-days posttransplantation [16]. As shown in Figure 4-8a, G-CSF pretreated recipients, which were also immunosuppressed with tacrolimus, have significantly greater rates of host-derived GFP+ BM cells seeding to the transplanted heart (40.9%  $\pm$  7.1%) as compared to the transplanted hearts of non-treated, immunosuppressed allografts at 14 days post-transplantation (23.0%  $\pm$  2.3%). The host-derived GFP+ BM cell seeding frequency (Figure 4-8b) in the transplanted allograft hearts was found to decrease in the G-CSF pretreated group (27.5%  $\pm$  7.6%) to similar levels as that observed in the transplanted hearts of non-treated allografts by 30 days post-transplantation (24.8%  $\pm$  2.7%). The seeding rate of host-derived GFP+ BM cells was not significantly different in G-CSF pretreated allograft native hearts nor in either the transplanted or native hearts of the syngraft control groups.

# 4.4.8 Vascular cell regeneration in G-CSF pretreated recipient mice in the heterotopic heart transplant model

The frequency of EC and vascular SMC chimerism events with GFP-positive host-derived BM cells was quantitated in vessels of transplanted and native hearts of G-CSF pretreated and control groups. At 14 days post-transplantation, the frequency of host-derived BM cell colocalization events with the EC-specific marker vWf (Figure 4-9) did not significantly differ between the G-CSF pretreated ( $10.1\% \pm 3.5\%$ ) and the control group ( $9.3\% \pm 2.4\%$ ). The control non-treated allografts also demonstrated a decrease in the frequency of host-derived-ECs between 14 and 30 days after heterotopic transplantation. In contrast, in G-CSF pretreated allografts at 30 days post-transplant, the frequency of host-derived-ECs found in the transplanted hearts remained elevated. At 30 days after transplantation, host-derived GFP+ BM cells colocalized with an EC-specific marker at a significantly higher frequency of  $9.3\% \pm 1.9\%$  in the vessels of G-CSF-pretreated recipients as compared to non-treated recipients ( $3.4\% \pm 1.6\%$ ;  $p \le 0.05$ ). Vascular

SMC repopulation by host-derived GFP+ BM cells was not significantly different at either 14 or 30 days post-transplantation, comparing G-CSF pretreated recipients to non-pretreated recipients. Native hearts demonstrated a negligible level of GFP-vWf and SMC double-positive cells in the vessels of G-CSF pretreated allografts.

#### 4-5. **DISCUSSION**

Recent reports have demonstrated that G-CSF treatment increases the abundance of circulating progenitor cells expressing the endothelial lineage phenotype [4,12] and accelerates the rate of reendothelialization in some models of vascular injury [3,4,7]. In this study, we demonstrate for the first time that G-CSF pretreatment of cardiac allograft recipients leads to no change in vascular chimerism at an early 14 day timepoint post-transplant, however G-CSF pretreated recipients demonstrate elevated EC replacement by host-derived cells at a later 30 day posttransplant timepoint, with no effect on vascular SMC repopulation in vivo. These findings may have therapeutic implications in that they suggest that the improvement in allograft function observed by others, [13,21] as a result of G-CSF pretreatment of transplant recipients, may be a consequence of the effect of maintained higher progenitor cell levels in the recipient, subsequently leading to enhanced EC repopulation by 30 days post-transplant. In addition, to date, several published reports suggest an improvement in vascular function and reendothelialization in vascular injury models, however they did not specifically examine the source or nature of cells contributing to the observed phenomena. Our findings demonstrate the effect of G-CSF treatment on BM-derived cells both in vitro and in vivo and the subsequent contribution of elevated numbers of the host circulating BM-derived progenitor cells to EC replacement in the allograft recipient.

The *in vitro* findings in this study suggest that BM-derived progenitor cell numbers are reduced with increasing treatment of immunosuppressive drugs tacrolimus and sirolimus. Both of these immunosuppressive agents form a complex with the FKBP, a complex which binds to calcineurine, a pivotal enzyme in T-cell IL-2 production [22]. Tacrolimus binding to the FKBP complex results in an inhibition of cytokine transcription by the CD4 cell. Blockade of cytokine production and cytokine receptor expression inhibits T-cell proliferation and differentiation.

Sirolimus, a macrocyclic antibiotic, is structurally related to tacrolimus and forms a complex with FKBP, but its mechanism of immunosuppression differs. Sirolimus binds to a target downstream IL-2 receptor, the mammalian target of rapamycin (mTOR), and inhibits T cell cycling at the G1 to S phase [23-25]. Therefore, through varying mechanisms, these immunosuppressive drugs lead to an inhibition in BM-derived cell proliferation and differentiation. However in this study, BM-derived cells pretreated with G-CSF were found to be more resistant to immunosuppressive treatment. One possible explanation for this effect is that G-CSF is known to mobilize BM HSCs after M phase of the cell cycle [9], and therefore, the observed difference in response of BM progenitor cells to immunosuppressive treatment in the presence and absence of G-CSF may be a consequence of the failure of these immunosuppressive agents to block proliferation once resting cells have entered the cell cycle.

Previously published studies have demonstrated that G-CSF pretreatment of either donors or recipients has been shown to lead to improved cardiac function [13,21]. In a rat heterotopic heart transplant model, pretreatment of donors with G-CSF was found to facilitate heart allograft acceptance. This improvement was attributed to the induction of a type 2 immune response by G-CSF pretreatment increasing type-2 T helper cells [21], type-2 dendritic cells [26] and IL-10-producing monocytes [27], which downregulate type 1 cells secreting rejection-associated cytokines such as IFN- $\gamma$ , IL-2, IL-12 and TNF- $\alpha$  [15]. In another study, G-CSF pretreatment of recipient rats prior to heterotopic heart transplantation was found to facilitate graft survival when treatment was with the immunosuppressive drug tacrolimus [13]. Despite these findings, to date, the effect of G-CSF treatment and its particular impact on host BM-derived progenitor cell contributions to vascular repair in cardiac allografts has not been examined.

The precise mechanisms involved in the G-CSF-induced mobilization and homing of BMderived progenitor cells remains unclear. Although our study did not specifically examine the mechanisms governing the mobilization, homing and differentiation of BM-derived progenitor cells in the allograft model, other groups have demonstrated that G-CSF induces both proliferation and the release of neutrophil proteases, such as elastase, cathepsin G and MMPs, which participate in cell egress by degrading retention signals, including VCAM-1 and SDF-1, and by remodeling the extracellular matrix. Recent published reports have suggested an essential role for the chemokine SDF-1 and its receptor CXCR4 in mobilization of BM-derived progenitor cells into the circulation and in directing them to sites of injury in tissues [28-32]. A role for SDF-1 in hematopoietic stem cell (HSC)/EPC recruitment from BM to peripheral blood has been proposed, based on evidence that G-CSF-mediated HSC/EPC mobilization causes an imbalance between the expression of BM SDF-1 and CXCR4 in HSCs [29]. Wright et al. [33] have also demonstrated that purified adult murine hematopoietic stem cells migrate to SDF-1 and not to any other known chemokine, thus revealing a central role for SDF-1/CXCR4 interactions in adult murine BM mobilization. In transplantation, a recent study in human heart transplant biopsy specimens revealed increased SDF-1 mRNA and protein expression in the transplanted hearts as compared to the control group [34]. These findings suggest that increased expression of SDF-1 at sites of damage may act as a signal triggering CXCR4-expressing host BM-derived progenitor cells, which have been mobilized by G-CSF to migrate through the circulation towards the SDF-1 gradient and subsequently, to home to sites of injury in the transplanted heart.

Another potential mechanism for the observed relative increase in EC repopulation rates in vessels of G-CSF pretreated allografts as compared to non-pretreated allografts at the later timepoint of 30 days post-transplant is that G-CSF pretreatment of recipients may alter the ability of BM-derived progenitor cells to survive in the circulation. This possibility is supported by a

study which demonstrated that, in the absence of G-CSF, a significantly greater proportion of marrow myeloid progenitors and granulocytes were found to be undergoing spontaneous apoptosis. Other studies have published findings which suggest that G-CSF acts as an anti-apoptotic factor leading to an improvement in survival of BM progenitor cells [35,36].

In conclusion, the present study demonstrates that G-CSF pretreatment of cardiac allograft recipient mice led to higher EC replacement by host BM-derived cells in arteries of transplanted hearts at 30 days post-transplantation as compared to non-pretreated allografts. No difference was seen in the rate of EC replacement by host BM-derived cells at the earlier 14 day posttransplant timepoint between G-CSF pretreated and non-pretreated recipients. The rate of vascular SMC repopulation by host BM-derived cells was found to remain the same at all timepoints following transplantation irrespective of G-CSF pretreatment of the allograft Daily high dose G-CSF treatment was found to lead to significantly elevated recipient. circulating progenitor cells levels in vivo. G-CSF treatment was also found to lead to higher BM-derived progenitor cell survival in vitro, even in the presence of immunosuppressive treatment, which alone was found to have a dose-dependent negative effect on progenitor cell survival. These findings suggest that pretreatment of cardiac allograft recipients with G-CSF prior to transplantation may be a potential therapeutic means of reendothelialization of injured blood vessels characteristic of chronic cardiac transplant vascular disease.

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Figure 4.1 In vitro effects of G-CSF and immunosuppressive treatment on blood and BM-

derived progenitor cells. (a) Cultured human blood and mouse BM progenitor cells were stained for DiI-ac-LDL (red) and UEA-1 lectin (green). In blood mononuclear cultures, double-positive cells (yellow) were counted, while DiI-ac-LDL-positive cells were counted for mouse BM cultures. (b) Treatment of BM progenitor cultures with tacrolimus and sirolimus induced a dose-dependent decrease in cell counts. (c) G-CSF treatment led to improved baseline cell levels as well as significantly higher cell counts at all drug doses with tacrolimus. (d) G-CSF pretreatment of blood mononuclear cell cultures led to significantly higher baseline cell levels and led to a sustained higher cell count at all drug doses. \*Significant difference, p≤0.05 between wells within each drug dose, #p≤0.05 for each dose compared to the lowest dose of 0.001 µg/mL. Data presented as mean ± SEM.



Figure 4.2 Effect of tacrolimus and G-CSF treatment on frequency of apoptotic events in BM-derived progenitor cells. (a) Flow cytometric analysis of control untreated BM-derived progenitor cells stained for PI and Annexin V. (b) Progenitor cells were found to shift into the Annexin V quadrant with increasing tacrolimus. (c) Graphic representation of apoptosis frequency with increasing tacrolimus treatment in the presence or absence of G-CSF. Data presented as mean  $\pm$  SEM, \*p≤0.05 between G-CSF treated and non-treated samples at each dose.
Figure 4-3.



Figure 4.3 The 3- (4,5,dimethylthiazol-2-yl) -5- (3-carboxymethoxy-phenyl) -2- (4sulfophenyl)-2H tetrazolium (MTS) assay of BM-derived progenitor cell proliferative response to G-CSF and tacrolimus. (a) Progenitor cells showed a dose-dependent increase in proliferative activity with increasing G-CSF dose, reaching maximal at 20 ng/mL. (b) Tacrolimus treatment of progenitor cells led to a decrease in progenitor cell proliferation, with significantly lower levels at the highest two doses of 0.1 and 1  $\mu$ g/mL. Cells pretreated with 20 ng/mL G-CSF demonstrated significantly higher proliferative activity at the same dose of tacrolimus. Data presented as mean  $\pm$  standard deviation (SD), n=6, \*p≤0.05, #p≤0.01 vs control.

Figure 4-4.



Figure 4.4 In vitro angiogenesis assay measurement of the effect of immunosuppression and G-CSF on progenitor cell tubule formation. (a) Typical vascular tubes could be seen in some fields. (b) Concentration-dependent effect of tacrolimus could be seen on progenitor cell angiogenesis. G-CSF pretreatment of progenitor cells prior to tacrolimus led to sustained tubule formation and significantly higher tubule counts at all immunosuppressive drug doses. Data presented as mean  $\pm$  standard deviation (SD), n=6, \*p≤0.05 non-GCSF treated vs G-CSF treated cells at each dose of tacrolimus, #p≤0.0001 vs non-treated control.

Figure 4.5



Figure 4.5 In vivo effects of G-CSF treatment on white blood cell counts. Increased G-CSF dosing led to augmented circulating (a) and absolute (b) neutrophil levels in mice. The 500  $\mu$ g/kg/day dose led to a significant increase in both circulating (c) and absolute (d) neutrophil levels. Data presented as mean ± SEM, \*p≤0.05.

Figure 4-6.



Figure 4-6. In vivo effects of G-CSF treatment on progenitor cell levels. (a-i) Density plot of the flow cytometric analysis of mouse BM of G-CSF treated and control non-treated mice with forward (SSC) and side light scatter (FSC). Lin- cells were separated (a-ii) and the population of cKit+/Sca-1+ cells in the lin- population was determined (a-iii). (b) The percentage of gated BM cells identified as lin-/cKit+/Sca-1+ was significantly higher in the G-CSF treated group as compared to non-treated BM. (c) Progenitor cells staining positive for DiI-ac-LDL were found to be significantly higher in blood from G-CSF treated mice than non-treated. (d) 5 days of repeated G-CSF dosing at 500  $\mu$ g/kg led to increased hematopoietic progenitor cell mobilization by 60.5 fold over vehicle (CFU-GEMM), \*p ≤ 0.05. Data presented as mean ± SEM.

Figure 4-7.

a.

b.



Figure 4.7 TVD in allograft arteries in G-CSF pretreated and control non-treated recipients. (a) Ventricular transverse sections from transplanted hearts 30 days post-transplantation from control non-treated (i) and G-CSF pretreated (ii) recipient mice (magnification, X400). (b) Luminal narrowing in allograft coronary arteries was quantitated in control and G-CSF pretreated allografts at 14 and 30 days post-transplant. There is a significant reduction in the extent of TVD in allograft coronary arteries from G-CSF pretreated recipients as compared to control counterparts. Data presented as mean  $\pm$  standard deviation (SD), \*p≤0.05.

Figure 4-8.



Figure 4-8. Quantification of host BM-derived GFP-positive cell seeding. GFP-positive host BM-derived cells seeded within transplanted and native hearts of all allograft and syngraft groups at both 14 days (a) and 30 days post-transplantation (b) were quantified and expressed as a fraction of total cardiac nuclei and graphically represented as mean  $\pm$  standard error of the mean (SEM), \*p≤0.05.

Figure 4-9.



Mouse Transplant Groups

Figure 4-9. vWf staining of EC in G-CSF pretreated transplanted hearts. (a) Representative confocal micrographs of allograft transplant vessel demonstrating vWf-positive EC showing dual-staining for GFP (circle). Nuclei are blue with Hoechst 33342, green represents GFP-positive host BM-derived cells, and red identifies vWf-positive EC. The XZ and YZ images represent orthogonal 3-dimensional views which confirm the EC/host bone marrowderived cell positivity in all planes. Scale bar 30  $\mu$ m. (b) Quantitation of vWf and GFP doublepositive cells expressed as a percentage of total ECs in the allograft and syngraft hearts at both 14 and 30 days post-transplant. Data presented as mean  $\pm$  SEM, \* p<0.05

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#### **CHAPTER 5: EXOGENOUS LINEAGE NEGATIVE** BONE **CELLS** NOT **PROGENITOR MARROW-DERIVED** DO REPLACEMENT AND **CONTRIBUTE** TO VASCULAR **FUNCTION MURINE CARDIAC** • IN **IMPROVED TRANSPLANTS**

### **Objective:**

.To examine the effect of injections of autologous bone marrow-derived progenitor cells on vascular cell replacement in cardiac allografts.

### **5.1 RATIONALE**

The results from Chapters 3 and 4 in this dissertation have demonstrated that endogenous host BM-derived cells have chimerism potential and contribute to the replacement of ECs and SMCs in vessels of cardiac allografts following transplantation at both 14 and 30 days posttransplantation. G-CSF mobilization of transplant recipient mice was found to lead to elevated EC replacement or chimerism by host BM-derived progenitor cells at the later 30 day posttransplantation timepoint as compared to control non-pretreated recipients. Recently, autologous progenitor cells have been used in both animal and human studies as a potential cellular therapeutic strategy for cardiovascular injuries, such as MI, in hopes of improving function and survival. Evidence for the potential of transplanted BM-derived progenitor cells in new blood vessel formation and revascularization emerged from studies, in peripheral ischemia and myocardial ischemia models, that demonstrated the formation of capillary-like structures from isolated HSCs or *ex vivo* expanded EPCs following injection. These autologous progenitor cells are isolated from adults and exogenously re-introduced into the circulation following tissue injury. To date, there has been a discrepancy in the reported safety and outcome following the injection of these progenitor cells to the heart, highlighting the need for further long-term followup studies.

With respect to cardiac transplantation, there has only been one recent study to date which has attempted to examine the therapeutic potential and efficacy of using these adult progenitor cells in a cardiac allograft model. Inoue *et al.* [1] isolated MSCs from the BM of rats and injected the cells following heterotopic heart transplantation at different doses into the transplanted heart. Despite the observed promising *in vitro* capabilities of MSCs, the authors found that injection of MSCs in their rat model resulted in accelerated graft rejection *in vivo* instead of the expected prolonging of allograft survival. This study, along with others from different models of cardiac

injury, stresses the need and importance of further examination into the effect and outcome resulting from the transplantation of adult progenitor cells for cellular therapy. Thus, the aim of this Chapter is to investigate the effect of exogenous injections of autologous host BM-derived progenitor cells on graft function and potential vascular chimerism events in the transplanted hearts of mice following heterotopic cardiac transplantation.

### **5.3 INTRODUCTION**

Progenitor cells within the BM have been suggested to be involved in the regeneration of damaged cells under conditions of tissue injury. With the identification of BM-derived progenitor cells as important players in adult vascular chimerism, several studies have attempted to utilize these progenitor cells to restore blood flow and reduce damage in ischemic tissues. Recent studies both support and refute the ability of BM-derived progenitor cells to contribute to this phenomenon [2-6]. Neverthéless, these BM-derived progenitor cells represent an exciting new avenue for potential therapeutic coronary angiogenesis and repair in experimental models and more recently in humans. Intravenously administered progenitor cells have been shown to be attracted to sites of cerebral injury [7], hindlimb ischemia [8-10], pulmonary hypertension [11,12], and myocardial ischemia [13-15]. These findings suggest that exogenously, injected progenitor cells have the ability to sense injured tissues similar to the seeding observed with endogenous progenitor cells.

In various models of cardiovascular damage, reports indicate that exogenously transplanted BMderived progenitor cells may be important in future cell-based therapies aimed at repairing injured cells and enhancing neovascularization within the heart following damage, ultimately leading to improvement in cardiac function. Following cardiac MI, injection of HSCs reported to lead to the regeneration of cardiomyocytes, ECs and SMCs [16-19] as well as a subsequent improvement in function attributed to the transplanted cells and their regenerative capacity. However, in contrast to these positive reports, studies have also demonstrated that the differentiation of these transplanted progenitor cells is extremely rare even in the microenvironment of the injured heart [20,21]. In fact, a recent report by Inoue et al.[1] in a cardiac transplantation model has shown evidence that exogenously isolated MSCs led to accelerated graft rejection following injection into the transplanted heart at different doses. These discrepancies highlight the need for additional studies focused on examining the potential of these transplanted BM-derived progenitor cells for cell-based therapies under different experimental settings.

We have previously demonstrated that host BM-derived progenitor cells contribute to vascular EC and SMC replacement in a murine heterotopic cardiac allograft model [22]. However, the contribution of exogenous BM-derived progenitor cells to vascular chimerism in a mouse cardiac transplant model has yet to be examined. Therefore, the aim of this study was to determine whether exogenously injecting autologous, lineage negative BM-derived progenitor cells following cardiac transplantation results in the differentiation of these cells into vascular phenotypes in the new environment of the transplanted heart and if so, whether this leads to an improvement in cardiac TVD and graft outcome following transplantation. In addition, the efficacy of using repeated administration of the injected progenitor cells following cardiac transplantation.

### **5.3 METHODS**

### 5.3.1 Lineage negative cell isolation

Whole BM was harvested from C57BL/6-GFP mice (Jackson Laboratories, Bar Harbor, Maine) by flushing femurs and tibiae with cold phosphate buffered saline (PSB)/2% fetal bovine serum (FBS; Hyclone, Logan, UT) solution. The BM cell solution was resuspended within the range of 2-8 X 10<sup>7</sup> cells/mL in the recommended medium (PBS + 2% FBS). Cells were incubated at 4°C with rat serum for 15 minutes following by a subsequent incubation with the lineage antibody cocktail (CD5 (Ly-1), erythroid cells (TER119), CD45R (B220), Ly-6G (Gr-1), and CD11b (Mac-1). Following the recommended washing steps, the cell solution was then incubated at 4°C for 15 minutes with a biotin-labeled tetrameric antibody complex and magnetic colloid beads were added prior to running the samples on an autoMACS<sup>TM</sup> (Miltenyi Biotec, Auburn, CA). 450,000 lin- cells were collected per run and resuspended in 100 μL of saline immediately prior to injection.

### 5.3.2 Heterotopic cardiac transplantation

Heterotopic cardiac transplantation was performed as described [22]. Briefly, hearts of 10-weekold male 129SV/j mice (Jackson Laboratories) were transplanted into male C57BL/6 mice. Control syngrafts consisted of 8-10-week-old C57BL/6 donor hearts transplanted into C57BL/6 mice (four transplants were performed for each group at each timepoint). Animals were anesthetized with 4% halothane and anesthesia maintained with 1% to 2% halothane (Halocarbon Laboratories, River Edge, NJ). Donor mice were infused with heparinized saline and their hearts excised. The recipient's abdominal aorta and inferior vena cava were located and clamped. The donor's aorta and pulmonary artery were anastomosed to the recipient's abdominal aorta and inferior vena cava, respectively, in an end-to-side manner. One dose of Buprenorphrine (Buprenex Injectable; Reckitt and Colman Pharmaceuticals, Richmond, VA) (0.01 mg/kg

intramuscular) was administered after surgery. Implantation was performed within 30 to 40 minutes of removal of the donor heart. Immediately after the surgical implantation, the prepared GFP-lin- cell suspension was injected directly into the aortic appendage. Control allografts and syngrafts were injected with saline at the same time during surgery. Each allograft and syngraft group was maintained to 14 and 30 days post-transplantation. There was also a separate GFP-lin- injected group which was maintained until 30 days post-transplant, but which also received an additional injection of cells 14 days following the day of surgery into the penal vein of mice. Allograft mice were treated with tacrolimus (Fujisawa, Osaka) administered intraperitoneally (6 mg/kg) daily following heart transplantation.

### 5.3.3 Tissue harvesting and morphometry

At 14 and 30 days post-transplantation, mice were anesthetized by injection with ketamine/xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The native and transplanted hearts were perfused with sterile saline at 2 mL/minute followed by 4% paraformaldehyde (PFA) (Fisher Scientific, Fairlawn, NJ) at the same flow rate. Hearts were then rapidly excised and immersion-fixed in 4% PFA for 4 hours followed by immersion in 5-20% sucrose gradient. Ventricular transverse-sections were then both OCT-frozen or paraffinembedded. Sections were cut serially and stained with hematoxylin and eosin (H&E) and Movat's pentachrome. To evaluate TVD, all visible medium to large coronary arteries from five transplanted hearts per group were photographed at X400 magnification using a Spot digital camera (n=29 arteries for control 14 day group, n=26 for injected 14 day group, n=32 for control 30 day group, n=28 for 30 day injected group, and n=30 arteries for 30 day double-injected allografts). *Image-Pro® Plus* (MediaCybernetics®, San Diego, CA) was used to quantitate intimal, medial and luminal areas. Intima to media ratios were calculated as well as the percent

luminal narrowing, using the following formula described by Armstrong *et al.*[23]: % Luminal narrowing = Intimal area/(Intimal area + Lumen area)

# 5.3.4 Immunofluorescence staining and confocal microscopic analysis and quantitation

Immunofluorescence staining was performed on OCT-frozen midventricular sections as previously described [22]. Briefly, sections were incubated primary antibodies, anti- von Willebrand factor (DAKO, Carpinteria, California), anti-CD45 (BD Pharmingen) and antidesmin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) Alexa 594-conjugated secondary antibody was used and nuclei were counterstained with Hoechst 33342 (Molecular Probes, Eugene, Oregon). Images were obtained using a Leica AOBS<sup>™</sup> SP2 confocal microscope. The number of injected GFP-positive lin- cells was quantitated at 14 and 30 days post-transplantation in allograft and syngraft groups. The total volume of tissue examined in the transplanted hearts of all allograft and syngraft groups equaled 2.052 mm<sup>3</sup> of reconstructed optical stacks. For vWf and desmin quantitation, colocalization with GFP-positive lin- cells was determined in representative midventricular sections from transplanted and native hearts. Stacks were reconstructed using Volocity<sup>TM</sup> (Improvision<sup>®</sup>, Boston, Mass) and Metamorph<sup>®</sup> image softwares (Universal Imaging Corporation<sup>™</sup>, Downingtown, PA). Two independent, blinded observers reviewed each reconstructed image. The number of GFP-positive lin- cells in each tissue volume was scored as well as the number of morphologically distinguishable lin- cells which also stained double-positive for GFP and either vWf, desmin or CD45.

Spectral unmixing was used to identify the GFP emission signal within the autofluorescent environment of the myocardium. Spectral distribution of autofluorescence and GFP was determined with a Leica AOBS<sup>TM</sup> SP2 confocal microscope (Leica Microsystems, Richmond

Hill, ON). A lambda stack was acquired in order to record the whole emission signal for every single pixel of an image, then the ROI function was used to determine the spectral signature of a selected area in the scanned image. The spectrally resolved images were recorded using the 488nm line of argon laser in the 500-600nm spectral range for fluorescence detection. To avoid the alteration of the autofluorescence spectral shape by photobleaching resulting from consecutive scans, spectral data presented here were always recorded from the first scan of each tissue segment.

### 5.3.5 Statistical analyses

Results are represented as mean  $\pm$  SEM. Comparisons for morphometric studies were made using ANOVA and a post hoc Tukey's test was used to determine statistical differences between groups. A *p* value of less than 0.05 was considered significant.

### 5.4 RESULTS

### **5.4.1 TVD morphometry**

Ventricular cross-sections of transplanted hearts at 14 and 30 days post-transplantation were assessed for intimal thickening by measuring both the intima to media ratio as well as the percent intimal narrowing in vessels of transplanted hearts (Figure 5.1a). At 14 days post-transplant, there was no significant difference in the degree of intimal narrowing as well as the intima to media ratio in transplanted hearts between control injected and lin- cell injected allografts (Figure 5-1b). However, both intimal thickening and the intima to media ratio of allograft arteries in control injected allografts significantly increased in the 30 day post-transplant group (% intimal narrowing =  $29.37\% \pm 3.0\%$ ; intima to media ratio= $0.57 \pm 0.18$ ) relative to the 14 day post-transplant group (% intimal narrowing =  $19.82\% \pm 2.75\%$ ; intima to media ratio= $0.30 \pm 100\%$ 

0.05,  $p \le 0.05$ ). There was so significant difference in either the intima to media ratio or percent luminal narrowing between all injected groups at the 30 day post-transplant timepoint. There was no luminal narrowing in syngraft controls.

### 5.4.2 Presence of GFP+ lin- cells in allografts following cardiac transplantation

The GFP emission of lin- cells believed to be identified in the allograft and syngraft hearts was confirmed using spectral unmixing. To identify the GFP emission signal within the autofluorescent environment of the myocardium, a lambda stack, a series of x-y images that sample emission data from a series of small wavelength bands, was acquired in order to record the whole emission signal for every single pixel of an image (Figure 5.2a). The ROI function was then used to determine the spectral signature of a selected area of the scanned image (Figure 5.2b). Using this technique, we were able to accurately identify true GFP+ lin- cells which demonstrated an emission wavelength peak between 509-512nm (Figure 5.2c, arrows) as opposed to autofluorescent cells which generally express emission wavelengths with a wider peak ranging between 530-580nm[24] within the tissue segments.

Allografts and syngrafts were analyzed to evaluate the presence of GFP+ lin- injected BMderived progenitor cells in hearts following transplantation. The total numbers of GFP+ lin- cells found in the volume of tissue examined in each tissue is represented in Table 5.1. At 14 days post-transplant, there was a larger number of GFP+ lin- cells identified in the given volume of tissue examined (159 cells found/2.05 mm<sup>3</sup>) than in the syngraft group at the same timepoint (24 cells found/2.05 mm<sup>3</sup>). At 30 days post-transplant, there was a decrease in the number of GFP+ lin- cells which were found in the same volume of tissue in the transplanted hearts of both injected groups, the first being injected only at the time of surgery and the second group receiving two injections, at surgery and another injection intravenously at 14 days posttransplant. The low numbers of injected lin- cells found in the transplanted hearts suggests that over time, most of the cells in each group examined either die or migrate elsewhere. As expected, no GFP+ cells were identified in the control saline injected allografts.

We then proceeded to examine the organs in all allograft and syngraft groups, including the liver, spleen, native heart, lungs, and kidneys at both 14 and 30 days post-transplantation. At both timepoints following transplantation, GFP+ lin- cells were found in the spleen and lungs of both allografts and syngrafts, however, at a higher frequency than was found in the same given volume of tissue as examined in transplanted hearts.

### 5.4.3 Fate of injected GFP+ lin- cells in transplanted hearts

Transplanted hearts of lin- injected allografts and syngrafts were stained with EC, inflammatory cell and muscle markers. Although there were GFP+ lin- cells found in the transplanted hearts, none of these were found to co-express with the EC-specific marker (Figure 5.3). GFP+ lin-cells also did not stain with antibodies against desmin. The lack of both EC and muscle-specific markers in these injected lin- cells suggests that the lin- cells have instead differentiated into alternate cell types. The pan-hematopoietic marker CD45 was co-expressed by many of the GFP+ lin- cells in all the injected allograft and syngraft groups at 14 and 30 days post-transplantation (Figure 5.3).

### 5.5 **DISCUSSION**

Recently, endogenous BM-derived progenitor cells have been reported to have the ability to migrate to sites of injury in the heart following injury in different cardiovascular models, including cardiac transplantation [16,25]. To date, in other models of vascular injury, the regenerative potential of BM-derived progenitor cells has been attempted to be used as a means of cellular therapy following tissue injury. Injections of autologous, exogenously isolated progenitor cells have been described to result in improved cardiac function due to revascularization and repair of cells within the damaged heart [13,19,26]. The aim of this study was to determine whether the injection of autologous, lineage negative BM-derived progenitor cells following cardiac transplantation leads to the differentiation of these cells into vascular phenotypes within the transplanted heart and if so, whether this leads to an improvement in TVD The results demonstrated no evidence of vascular or outcome following transplantation. myocardial chimerism by the lin- cell injected hearts at both 14 and 30 days post-transplantation. We also found no evidence of a beneficial effect of these injected BM-derived progenitor cells on TVD severity. At both timepoints of 14 and 30 days post-transplantation, there was no observed improvement in either the percent intimal narrowing or the intimal to medial ratio in the transplanted hearts of allografts between the control injected versus the lin- cell injected groups. As well, repeated injection of the lin- cells following transplantation did not result in any change in graft outcome.

The transplantation of progenitor cells has been shown to significantly improve blood flow recovery and capillary density in several models of vascular injury. Kalka *et al.* [10] demonstrated that *ex vivo* expanded human endothelial progenitor cells (EPCs) could be used to promote neovascularization of ischemic hindlimbs in athymic nude mice. Mice receiving a single injection of 500,000 EPCs had increased capillary density and significantly improved

blood flow in the ischemic limb following transplantation. Subsequent studies have also shown that autologous BM cell injections in a rat ischemic hindlimb model and BM mononuclear cell transplantation in a rabbit model of hindlimb ischemia resulted in improved collateral vessel formation and blood perfusion in the ischemic limb[8,9].

Just as progenitor cell transplantation restored blood flow to ischemic hindlimbs, BM-derived progenitor cell transplantation after MI has also been reported to induce neovascularization. Kawamoto et al. [15] demonstrated that transplanted, ex vivo expanded EPCs had a favorable impact on the preservation of left ventricular function. Labeled EPCs were injected intravenously following induction of myocardial ischemia and the injected cells were shown to accumulate in the ischemic area and to participate in myocardial neovascularization. Echocardiography also revealed ventricular dimensions and scarring were significantly smaller and fractional shortening that was significantly greater in the EPC transplant group. Similarly, Kocher et al. [13] reported that transplantation of G-CSF mobilized CD34+ cells containing both HSCs and EPCs led to improved myocardial function, prevented cardiomyocyte apoptosis, and limited myocardial remodeling. As well, neovascularization was observed both within the myocardial infarct and along the infarct border. A subsequent study using a swine MI model also showed that implantation of BM-derived mononuclear cells improved regional cardiac function [14]. Three weeks following transplantation, regional blood flow, capillary density and the number of visible collateral vessels were significantly higher in transplant recipients as compared to controls. The beneficial effects were speculated to be achieved due to the potential of the transplanted cells to become incorporated into new vessels.

Despite these promising results, a study by Nagaya *et al.* [12] have called into question the true potential of cell based therapies for vascular regeneration and functional improvement. In a

model of pulmonary hypertension, the authors transfected EPCs with the vasodilator gene adrenomedullin (AM) and subsequently injected either AM-transduced EPCs or control EPCs into rats intravenously. The results indicated that transplantation of EPCs alone modestly attenuated pulmonary hypertension (16% decrease in pulmonary vascular resistance) while transplantation of AM-transduced EPCs markedly attenuated pulmonary hypertension in rats (39% decrease in pulmonary vascular resistance) and resulted in a significantly higher survival rate than those given culture medium or EPCs alone. Therefore, this report suggests that the majority of the benefit observed following transplantation of exogenous BM-derived progenitor cells is derived from the 'gene' rather than the 'cell therapy'. These findings raise the possibility that exogenously transplanted progenitor cells may best serve as excellent vehicles for gene delivery to injured tissues.

The results in this Chapter also suggest that transplantation of exogenous BM-derived progenitor cells may not be an adequate means of cell therapy following cardiac transplantation. Although the results contrast some other recently published studies in different models which report extensive vascular regeneration following direct injection of lin-/cKit+ cells into infarcted hearts [13,19,26] our findings are, however, more in line with recent reports by Murry *et al.* [21] and Balsam *et al.* [20], in which they used similar techniques to look at the fate of injected stem cells in infarcted hearts. Their results also found no evidence of transdifferentiation into vascular and cardiomyocyte phenotypes. To our knowledge, this is the first study to date which looks at the role of injected progenitor cells in the setting of the allograft heart and which examines the effect of these cells on the outcome and severity of TVD following transplantation. The data presented here collectively suggests that direct injection of BM-derived lin- progenitor cells into the circulation following heterotopic heart transplantation in mice does not result in *de novo* vascular or tissue regeneration. In addition, TVD outcome was not found to be altered in the allograft

hearts treated at different timepoints following transplantation, suggesting that injection of autologous progenitor cells does not affect TVD development or severity.

There are several factors which may speculatively account for our inability to identify any true vascular chimerism capacity in the injected progenitor cells following cardiac transplantation and discrepancy in the reported rates of transplanted cell seeding within injured hearts following injection. One possibility is that the intrinsic autofluorescence of the myocardium complicates the accurate identification of the injected cells. In this study, to ensure the accurate identification of injected GFP+ cells in the heart, spectral unmixing was used to identify the GFP emission signal within the autofluorescent environment of the myocardium. The challenge in multicolour imaging in the heart is the ability to separate the true fluorescence signal from the background autofluorescence. A lambda stack is a stack of x-y images that sample emission data from a series of small wavelength bands [27-29]. Lambda stacks can be considered in much the same way as a time series or x-dimension series. The x-y data can be viewed along the wavelength axis to determine how the intensity of pixels in the image change because of the amount of signal at different emission bands [28]. Therefore, the emission spectra of a particular dye can be revealed by plotting the pixel intensity versus the center wavelength of each emission band. For confocal microscopy, acousto-optic tunable filters (AOTF) have been used to generate lambda stacks [24,30]. The tunable filter is inserted in from of the photomultiplier tube (PMT) and selects a wavelength for that image. The AOTFs are tuned by changing the frequency of the resonating crystal, thus, x-y scans can be made across a series of wavelengths generating a lambda stack. Thus to identify real GFP+ cells within the heart tissue, a lambda stack was acquired in order to record the whole emission signal for every single pixel of an image, then the ROI function was used to determine the spectral signature of a selected area of the scanned

image to ensure that the green emission was true GFP spectral emission as opposed to autofluorescence.

Another plausible explanation for the lack of effect resulting from injection of cells following cardiac transplantation is that the numbers of cells used for injection was too low for detection 14 to 30 days following injection. However, the numbers and resuspension volumes used in this study are in line with those used in other published reports in which there was an observed vascular benefit in cardiac injury models following cell injection. Kalka et al. [10] used 500,000 cells for injection and found evidence of neovascularization and improved functional outcome. Iwaguro et al. [31] performed dose-dependent transplantation experiments to determine the minimum number of progenitor cells that are required to achieve a magnitude of therapeutic neovascularization following hindlimb ischemia. The results indicated that as little as 450,000 cells resuspended in 100-200 µL of solution led to improvement in vascular function following injection. These results are in line with the methodological strategy which was employed in our study, although discrepancies based on different models used may also contribute to slight variances. As well, in our study, we isolated and injected the total lineage negative BM cell population at each timepoint and there is a wide variety of different cells types that have been used in the studies published to date which may account for discrepancies in final results following cell therapy.

These findings suggest highlight the importance of using caution in the use of autologous BMderived progenitor cells for exogenous injections into the heart and vasculature. Currently strategies which employ progenitor cell transplantation in certain models of tissue injury and report improvements in organ function may not generate the same outcome in other models of injury. Thus, strategies enhancing the function of transplanted progenitor cells may not prove to be beneficial in all population groups and may even have detrimental effects in certain conditions. Indeed, recently studies have also reported adverse effects following adult stem cell transplantation, with one study [32] reporting the development of unexpected severe intramyocardial calcification following direct transplantation of syngenic unselected BM cells into the infarcted myocardium. Therefore, further extensive studies are required to verify the efficacy of using different types of adult BM-derived progenitor cells for use in the long-term repair of damaged cells within the heart.

Figure 5.1



Figure 5.1 TVD in allograft arteries in injected and control transplant recipients. (a) Ventricular transverse sections from transplanted hearts 14 days ((i) 14 day control non-injected, (ii) 14 day lin- injected) and 30 days post-transplant ((iii) 30 day control non-injection, (iv) 30 day lin- injected) stained with Movat's pentachrome. (b) Intima to media ratios reveal a significant difference between non-injected control allografts at 14 days post-transplants as compared to control counterparts at 30 days post-transplant. However, no significant difference was found in ratios between injected allografts at either 14 or 30 days post-transplantation. (c) Luminal narrowing in allograft coronary arteries was quantitated in control and lin- injected transplanted hearts at 14 and 30 days post-transplant. There was no significant difference in the extent of TVD in allograft coronary arteries from lin- injected transplants as compared to noninjected control counterparts. Data presented as mean  $\pm$  SEM. \*p≤0.05. Scale bar = 30 microns.

### Figure 5.2

a.



Emission wavelength (nm)

**Figure 5.2 Spectral unmixing of GFP from background autofluorescence.** (a) Confocal micrographs of potential GFP+ lin- cells in allograft tissues were obtained using the 488 nm excitation laser line. (b) ROIs were taken of different green emitting regions within the micrographs and lambda scans of all ROIs were obtained and graphically represented (c) to identify emission profiles of the green signals within each selected ROI. White arrows indicate the two ROIs which were found in this representative confocal micrograph to have emission wavelengths which represent GFP emission as compared to the remainder of the selected ROIs which had emission wavelengths indicating autofluorescence.

Figure 5.3



**Figure 5.3 Fate of injected GFP+ lin- BM-derived cells in cardiac allografts.** Representative confocal micrographs of transplanted hearts. Nuclei are blue with Hoechst 33342, lin- BM cells are GFP+ (green) and Alexa 594 was used to identify the tissue-specific markers (red). Lincells injected immediately following cardiac transplantation in a heterotopic murine heart transplant model do not adopt cardiac or endothelial phenotypes. Injected lin- cells were found to co-express the CD45 marker in transplanted hearts. Scale bar = 20 microns

Original # of GFP+ cells injected	Volume of tissue examined	#GFP+ cells in transplant	#GFP+ cells in organs
450,000	2.05 mm <sup>3</sup>	0.	0
450,000	2.05 mm <sup>3</sup>	159	176
450,000	2.05 mm <sup>3</sup>	24	119
450,000	2.05 mm <sup>3</sup>	.0	0
450,000	2.05 mm <sup>3</sup>	41	112
450,000 ays	2.05 mm <sup>3</sup>	35	153
450,000	2.05 mm <sup>3</sup>	21	131
	Original # of GFP+ cells injected 450,000 450,000 450,000 450,000 450,000 450,000 ays	Original # of GFP+ cells injected Volume of tissue examined   450,000 2.05 mm <sup>3</sup>	Original # of GFP+ cells injectedVolume of tissue examined#GFP+ cells in transplant450,000 $2.05 \text{ mm}^3$ 0450,000 $2.05 \text{ mm}^3$ 159450,000 $2.05 \text{ mm}^3$ 24450,000 $2.05 \text{ mm}^3$ 0450,000 $2.05 \text{ mm}^3$ 41450,000 $2.05 \text{ mm}^3$ 35450,000 $2.05 \text{ mm}^3$ 21

## Table 5-1. Estimated number of GFP+ cells present in transplanted hearts

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# CHAPTER 6: SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

### 6.1 SUMMARY

TVD is a rapid and progressive form of atherosclerosis that occurs in the vessels of solid organ allografts and is the major cause of late graft loss and patient death. The pathogenesis of TVD is believed to involve both immune and non-immune factors involving EC damage and vascular SMC accumulation within vessels of allografts. Recent studies in cardiac transplantation suggest that ECs, as well as both intimal and medial SMCs in TVD lesions, are derived, in part, from host BM cells. However, there has been discordant data regarding the incidence and significance of host cell chimerism after transplantation. The studies described in this dissertation focus on investigating the contribution of host BM-derived progenitor cells to the restoration and replacement of the injured endothelium and remodeling within the vessel wall which are characteristic of cardiac TVD. In addition, the effect of common immunosuppression regiments and cytokine mobilization of BM cells on the observed host-derived vascular chimerism events following heart transplantation was examined. Finally, the potential of using exogenously-isolated, autologous BM-derived cells for injection into the vasculature of the heart following transplantation as a means of vascular therapy will be assessed.

Using BM-GFP transgenic mice as recipients of heterotopic heart transplants, we found that host BM-derived progenitor cells contributed to both EC and SMC replacement. The rate of reendothelialization was found to diminish from  $11.8\% \pm 2.5\%$  at 14 days post-transplant to 4.0%  $\pm 1.2\%$  30 days post-transplant. Also, immunosuppressive treatment of cardiac allografts with tacrolimus did not appear to affect the frequency of re-endothelialization or SMC replacement by host BM-derived cells.

G-CSF treatment of BM-derived progenitor cells *in vitro* was found to improve survival, proliferation and angiogenesis function of the cells despite treatment with immunosuppressive agents. G-CSF pretreatment of BM-GFP transgenic recipient mice prior to heterotopic heart transplantation resulted in the same rate of re-endothelialization at 14 days post-transplant as non-pretreated allografts. However, at the 30 day post-transplant timepoint, there was a higher rate of re-endothelialization in G-CSF pretreated allografts ( $9.3\% \pm 2.2\%$ ) relative to nonpretreated allografts ( $3.4\% \pm 1.6\%$ ). In addition, G-CSF pretreated allografts demonstrated less intimal narrowing in vessels of the transplanted heart relative to those in non-pretreated control allografts.

To investigate the effect of injections of autologous BM-derived lin- cells in transplanted hearts, heterotopic heart transplants were performed and GFP-positive lin- cells were injected immediately after surgery into the aortic appendage. Hearts were analyzed 14 and 30 days post-transplantation and spectral unmixing was used to identify the true GFP+ signal of the lin- cells in allografts. Although GFP+ lin- cells were identified in transplanted hearts of allografts at both 14 and 30 days post-transplantation, no lin- cells were found to stain double-positive for both vascular cell and cardiomyocyte markers. The pan-hematopoietic marker CD45 was co-expressed by many of the GFP+ lin- cells in all the injected allograft and syngraft groups at 14 and 30 days post-transplantation. The synopsis of the experimental findings and specific aims of this study are presented in Figure 6-1.

## 6.2 CONCLUSION

The theory that adult progenitor cells have an intrinsic regenerative capacity in the heart has not yet been fully proven with regards to its extent and relevance, but it has stimulated the imagination of many investigators. The results of this dissertation indicate that the contribution of host BM-derived cells to EC and SMC chimerism in the murine heterotopic heart transplant model occurs as early as 14 days post-transplantation with EC chimerism occurring at a higher rate than SMC chimerism. While the frequency of SMC chimerism was found to remain constant from the 14 day- to the 30 day post-transplant timepoint, the rate of host BM-derived EC chimerism was found to diminish in vessels of the transplanted hearts by 30 day posttransplant. However, G-CSF mobilization of host BM cells in recipients of cardiac transplants was found to elevate EC chimerism by host-BM-derived cells at the 30 day post-transplant timepoint to a rate that was similar to the 14 day post-transplant timepoint. In addition, intimal narrowing in transplant vessels was found to improve in G-CSF pretreated allograft recipients at the 30 day post-transplant timepoint. Although immunosuppressive treatment in vitro reduced BM-derived progenitor cell proliferation and function, in vivo, tacrolimus treatment was not found to affect the ability of host BM-derived cells to contribute to vascular replacement in transplanted hearts. This may potentially reflect the inability of immunosuppressive agents to affect BM-derived progenitor cells once these cells have already been mobilized out of the BM and localized within sites of damage. Despite the ability of these endogenous host BM-derived cells to migrate to and contribute to the replacement of ECs and SMCs following cardiac transplantation, exogenously isolated and injected BM-derived lineage negative progenitor cells did not seed to sites of damage within vessels of transplanted hearts following injection. The inability of autologous, transplanted BM-derived progenitor cells to migrate to and repopulate damaged vessels of allografts suggests that these injected cells are lacking key migratory signals

which help attract endogenous BM-derived cells to the injurious sites within the heart following damage.

Therapeutically, alterations in the frequency of host BM-derived cell EC chimerism may serve as a future means of attenuating vascular remodeling which is characteristic of cardiac TVD. The findings arising from this dissertation suggest that EC chimerism frequencies may be best controlled through alterations in the levels of endogenous progenitor cells mobilized from within the BM in recipients of cardiac transplants, rather than transplantation of exogenously isolated, autologous progenitor cells.

## 6.3 FUTURE DIRECTIONS

Here, we have provided evidence that host BM-derived progenitor cells contribute to the replacement of ECs and SMCs in the vasculature of transplanted hearts. Although the above studies indicate that host progenitor cells derived from the BM have the potential to regenerate vascular cells within the heart following cardiac transplantation, there are many questions remaining with regard to their future therapeutic use. First, there is still a void in clarifying the mechanisms involved in the observed cellular differentiation within the heart. Thus, further experiments that dissect the molecular mechanisms by which these BM-derived progenitors are recruited and differentiate at the site of injury are warranted. Several different mechanisms of stem cell recruitment have been postulated [1-4]. In adulthood, hematopoiesis is restricted to the extravascular compartment of the BM by a single layer of BM ECs that form specialized vessels termed sinusoids [1,5]. Therefore, to enter or exit the hematopoietic compartment, stem cells must first recognize or be recognized by the surface of the BM EC. The initial phase involved in the homing or mobilization of stem cells is believed to be mediated through selectins, such as Eselectin, followed by firm adhesion mediated by VCAM-1/very late antigen-4 (VLA4) and ICAM-1/LFA1 ligand pairs, and finally by interaction with interjunctional adhesion molecules such as platelet/EC adhesion molecule-1 (PECAM) [6-9]. The SDF-1/CXCR4 interactions and signaling have been implicated as a principal axis regulating retention, migration, and mobilization of stem cells during steady-state homeostasis and injury, as described in Chapter 4.

Recently, the role of angiogenic factors in stem cell recruitment to sites of injury have been examined. Under physiological conditions, only a small amount of soluble bioavailable vascular endothelial growth factor (VEGF) is detected in the peripheral circulation [10-12]. However, vascular trauma results in plasma elevation of VEGF, which promotes recruitment of ECs to the site of injury. Tissue injury induced by MI or TVD results in up-regulation of angiogenic

factors, including VEGF, which in turn promote mobilization and recruitment of stem cells to sites of injury [12]. Our laboratory, along with others, has previously shown that VEGF is expressed in the coronary arteries following cardiac transplantation [13,14]. Hence, upregulation of VEGF in the damaged blood vessels of allografts may be a key signal in recruitment of BM-derived host cells to the sites of damage. Understanding the kinetics and the exact mechanism involved in the mobilization of stem cells within the BM allows the potential for the future development of strategies in which a large number of stem cells can be recruited to damaged areas within the heart for potential therapeutic treatment. Considering the complex interactions between cytokines, selectins and growth factors which are involved in BM progenitor cell mobilization, it is possible that exogenous and endogenous progenitor cells require different mechanistic signals to be recruited to sites of injury. As such, there is also a need to decipher the differences in the recruitment mechanisms for endogenous versus exogenous stem cells in the heart.

Despite the vigorous imaging and analysis techniques employed in these studies in order to determine the precise contribution of host BM-derived progenitor cells to potential vascular chimerism events in cardiac allografts, the contribution of cell fusion to this observed phenomenon is unclear. Earlier studies which utilized genetic markers of dyes to track cells *in vivo* indicated that transplanted cells could differentiate into multiple phenotypes within the heart and thereby contribute to myocardial regeneration [15-17]. However, other studies have suggested that phenotypic changes of stem cells may occur as a consequence of cell fusion rather than transdifferentiation [18,19]. Both *in vitro* coculture [18,19] and *in vivo* cell transplantation [20,21] experiments using BM-derived stem cells showed that cell fusion is responsible for a certain percentage of phenotypic changes. For example, using a Cre/lox recombination system to identify transplanted cells indicated that after BM transplantation, BM-derived cells fusion.

*vivo* with hepatocytes in the liver, with Purkinje neurons in the brain and with cardiac muscle in the heart, resulting in the formation of multinucleated cells [21]. Another study using a Cre/lox donor/recipient pair suggested that both fusion and transdifferentiation are equally responsible for the phenotypic changes of cardiac progenitor cells into myocytes after experimental MI [20]. These studies have raised the fundamental question of whether fusion between stem cells and cells of other organs has a physiological role in the development or maintenance of these organs. Future experiments are needed to address the contribution of fusion in BM-derived progenitor cell transplantation to cardiac regeneration, and the fate of the fused versus transdifferentiated cells in different animal models of cardiac diseases, including cardiac TVD.

Not all the BM-derived progenitor cells possess the same therapeutic capacity and efficacy. Therefore, studies are also needed to compare the therapeutic effects of different types of progenitor cells in cardiac TVD. As well, although the findings presented in this dissertation did not find a therapeutic benefit of autologous injections of BM-derived progenitor cells in a murine model of cardiac TVD, it is necessary to investigate whether the therapeutic effects are mediated through direct differentiation of progenitor cells or their humoral effects on remaining endogenous cells. Notwithstanding the plethora of questions remaining, host progenitor cells derived from the BM offer some exciting possibilities for the future therapy and research of cardiac TVD. Through further studies aimed at deciphering the precise mechanisms involved in the recruitment and mobilization of host BM-derived progenitor cells, future therapies for cardiac TVD could employ strategies for targeting these endogenous host BM-derived progenitor cells for damaged areas within the vasculature of the transplanted heart in hopes of ameliorating the progression of TVD.



**Figure 6.1 Summary of Results in Each Aim.** The results from Aim 1 indicated that host BMderived cells (GFP-positive, green) seeded to sites within vessels of transplanted hearts and led to replacement of EC and SMC at both 14 and 30 days post-transplantation, irrespective of immunosuppressive treatment with tacrolimus. EC replacement by host BM-derived cells was found to occur at a frequency which diminished from 14 to 30 days post-transplantation, while the frequency of SMC replacement was constant between these timepoints. In Aims 2 and 3, G-CSF pretreatment of recipients mice in the heterotopic heart allograft model was found to lead to no change in the frequency of SMC replacement by host BM-derived cells, but EC replacement was higher at 30 days post-transplantation in the G-CSF pretreated group as compared to controls. Exogenous injections of lineage negative (GFP-positive) cells following cardiac transplantation in Aim 4 was not found to lead to any vascular regeneration by the injected autologous cells at either 14 or 30 days post-transplantation.

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