ISOLATION OF ANGIOGENIC AND ARTERIOGENIC SUBPOPULATIONS OF PERIPHERAL BLOOD CD31^{+} CELLS

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Brandon A. L. Johnson

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ISOLATION OF ANGIOGENIC AND ARTERIOGENIC SUBPOPULATIONS OF PERIPHERAL BLOOD CD31^{+} CELLS

Approved by:

Dr. Young-Sup Yoon, Advisor
Division of Cardiology
Emory University School of Medicine

Dr. Andrés García
School of Mechanical Engineering
Georgia Institute of Technology

Dr. Changwon Park
Department of Pediatrics
Emory University School of Medicine

Dr. Ho-Wook Jun
School of Engineering
University of Alabama at Birmingham

Dr. Manu Platt
Department of Biomedical Engineering
Georgia Institute of Technology & Emory University

Date Approved: January 11, 2019

To Henry I. Johnson; Renita F. Cotton; Arthur M. and Shawn Johnson; and all my friends and family for all their love and support.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABI	ankle-brachial Index
ANGPT1	angiopoietin 1
ANGPT4	angiopoietin 4
BM	bone marrow
CCL2	monocyte chemoattractant protein 1
CDH5	vascular endothelial cadherin
CLDN11	claudin 11
CLI	critical limb ischemia
CM	conditioned medium
CM-DiI	chloromethylated 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
CMKLR1	chemerin chemokine-like receptor 1
CVD	cardiovascular disease
CXCL12	stromal cell derived factor 1
CXCR4	chemokine receptor 4
DAPI	diamidino-2-phenylindole
EGR3	early growth response 3
EPC	endothelial progenitor cell
FACS	flow assisted cell sorting
FGF2	fibroblast growth factor 2
FGFR1	fibroblast growth factor receptor 1
FITC	fluorescein isothiocyanate
FLT4	vascular endothelial growth factor receptor 4

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
HGF	hepatocyte growth factor
HLI	hindlimb ischemia
HSPC	hematopoietic stem and progenitor cell
HUVEC	human umbilical vein endothelial cell
IC	intermittent claudication
ICAM1	Intercellular adhesion molecule 1
ICC	immunocytochemistry
IFNG	interferon gamma
IGF1	insulin-like growth factor 1
IHC	immunohistochemistry
IL1	interleukin 1
ITGB2	integrin subunit beta 2
JUP	junction plakoglobin
LDPI	laser Doppler perfusion imaging
MNC	mononuclear cell
MSC	mesenchymal stem cell
PAD	peripheral artery disease
PB	peripheral blood
PCA	principle component analysis
PDE3B	phosphodiesterase 3B
PDGFB	platelet derived growth factor beta
PE	phycoerythrin
PML	promyelocytic leukemia

qRT-PCR	quantitative, reverse-transcription polymerase chain reaction
RAP1A	ras-related protein krev-1
RNA-Seq	ribonucleic acid sequencing
RPLP0	human large ribosomal protein P0
ROBO4	roundabout guidance receptor 4
SHC1	src homology 2 domain containing transforming protein 1
SMC	smooth muscle cell
$TCPO_2$	transcutaneous pressure of oxygen
TGFB	transforming growth factor type beta
TNF	tumor necrosis factor alpha
UNC5B	unc-5 netrin receptor B
VCAM	vascular cell adhesion molecule
VCL	vinculin
VEGFA	vascular endothelial growth factor alpha
ZNF304	zinc finger protein 304

SUMMARY

Cardiovascular disease (CVD) is the leading cause of death in the world and is predicted to remain so. Vascular growth and regeneration is a critical process necessary to the successful recovery of ischemic cardiovascular injuries and diseases. Unfortunately, postnatal vascular growth is difficult to achieve especially in the case of geriatric individuals who are likely to suffer from multiple cardiovascular complications. Therefore, the goal of this dissertation is to use markers CD14 and CD31 to isolate a cell population directly from peripheral blood capable of inducing vascular growth and repair.

We first sought to identify the lineage composition and expression profiles of isolated cells. To do this, we used flow cytometry do determine that the $CD31^{+}CD14^{+}$ population is enriched with injury repair blood cell lineages. Analysis of RNA expression revealed upregulation of hallmark angiogenic factors in $CD31^{+}CD14^{+}$ cells and upregulated arteriogenic expression in $CD31^{+}CD14^{-}$ cells. Further in vitro assays using conditioned medium displayed that paracrine factors secreted from $CD31^{+}CD14^{+}$ cells increased endothelial function and migration. We further determined the in vivo effectiveness of both PB- $CD31^{+}CD14^{+}$ cells and PB- $CD31^{+}CD14^{-}$ cells in a murine model of critical limb ischemia (CLI). Our results indicate that both the PB- $CD31^{+}CD14^{-}$ cell and the PB- $CD31^{+}CD14^{+}$ cell therapies strongly induce reperfusion in mouse hindlimbs when compared to all other therapies. Furthermore, PB- $CD31^{+}CD14^{+}$, - $CD31^{+}CD14^{-}$ and $–CD31^{+}$ cell treatments are all significant to PBS at 14 days. Further histological analysis of tissues shows PB- $CD31^{+}CD14^{+}$ cells rapidly increased capillary formation at early time

points and have limited vasculogenic potential while $CD31^{+}CD14^{-}$ cells increased SMC coverage of perfused vessels.

Our research confirms the highly angiogenic potential of PB- $CD31^{+}CD14^{+}$ cells. Furthermore, this research is the first to identify a cell population in PB-$CD31^{+}CD14^{-}$ cells with strong therapeutic capability that circumvents the angiogenic process, primarily functioning through arteriogenesis. These insights are significant for the further development of effective cell therapies for cardiovascular disease.

CHAPTER 1 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the world and accounted for $329.7 billion (14%) of global total health expenditures in 2014[1]. The AHA estimates that the total costs of CVD will reach $1.1 trillion by 2035[1]. Despite significant reductions in risk factors and improvements in the management of CVD, there remains a dearth of adequate treatments, particularly pertaining to diseases such a peripheral artery disease (PAD)[2].

Motivation

PAD, a state of obstructed blood flow in any arteries excluding the coronary and intracranial vessels, afflicts approximately 12 million people in the US and > 200 million people worldwide[3,4]. The majority of those suffering from PAD are asymptomatic and as disease progresses, patients may eventually develop critical limb ischemia (CLI) which has a 1-year amputation and mortality rate of 20% and 40%, respectively, for individuals without revascularization options[5-7].

PAD prevalence and incidence are both positively correlated with age, increasing to 10% by age 60 and 20% by age 80[7,8]. As the global life expectancy continues to increase, it is likely that PAD will become significantly more common in the future[7]. As such, it is imperative to continue to develop new and effective treatments for the management and repair of PAD.

Specific Aims

It has been previously demonstrated that bone marrow (BM) and PB derived $CD31^+$ cells are highly angiogenic and capable of inducing neovascularization in murine models of hindlimb ischemia[9-11]. However, given that $CD31^+$ cells are heterogeneous, the question remains as to whether this population may be further enriched for the angiogenic, arteriogenic and/or vasculogenic cell fraction. The central hypothesis of this dissertation is that PB-$CD31^+$ cells may be concentrated into a more effective therapy using the distinguishing marker CD14. The objective of this dissertation will be completed by testing the central hypothesis using the following aims.

Specific Aim 1

Characterize the expression of PB- $CD31^+CD14^{+/-}$ cells and determine their neovasculogenic potential in vitro.

Our hypothesis is that the $CD14^+$ fraction of PB-$CD31^+$ cells will retain the angiogenic phenotype. In this aim, we use FACS and flow cytometry sorting to isolate and identify our cell populations. We then used robust expression profiling tools such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) and RNA-Sequencing (RNA-Seq) to determine gene expression and functional characteristics. The effects of each cell fraction on endothelial cells is then evaluated using functional and characteristic assays on HUVECs cultured in conditioned medium.

Specific Aim 2

Evaluate the therapeutic effects of PB- $CD31^+CD14^{+/-}$ cells in vivo using a murine model of critical limb ischemia.

We hypothesize that the $CD31^{+}CD14^{+}$ fraction will strongly induce vascular growth through induction of angiogenesis. To test this hypothesis, a well-established hindlimb ischemia model was utilized to study the time course of limb perfusion in BALB/c nude mice. Laser Doppler perfusion imaging (LDPI) and histology were used to evaluate vascular growth and repair.

CHAPTER 2 LITERATURE REVIEW

Peripheral Artery Disease

Cardiovascular disease (CVD) is the leading cause of death in the world with Peripheral artery disease (PAD) attributing to 3.1% of fatal CVDs[1]. PAD occurs when there is an obstruction of blood flow in any arteries excluding the coronary and intracranial vessels and afflicts approximately 12 million people in the US and > 200 million people worldwide[3,4]. The majority of those suffering from PAD are asymptomatic and as disease progresses, patients may eventually develop critical limb ischemia (CLI) which has 1-year amputation and mortality rates of 20% and 40%, respectively, for individuals without revascularization options[6,7]. Among CLI patients that undergo treatment, only 25% will see their symptoms resolved after a year[12]. Unfortunately, intermittent claudication, which is the typical diagnostic method for detection of PAD, is absent in nearly 4/5th's of patients[5,13]. PAD is also known to become progressively more prevalent with age with incidence rates increasing sharply around 65 years of age[14]. Furthermore, due to the increasing life expectancies, it is expected that the number of PAD patients will continue to increase[4,15].

Risk Factors and diagnosis

Risk factors for PAD include hypertension, dyslipidemia, hyperhomocysteinemia, obesity, alcohol consumption, smoking, age, poor diet and lack of exercise[1,7,14]. Genetic factors such as gender and race are also important risk factors for PAD; for instance, increased incidence of PAD is found specifically in the African American and Hispanic

American populations when compared to the entire American populace[1,16]. PAD also tends to present with other comorbidities like diabetes mellitus, chronic kidney disease and hypercoagulable states.

PAD is diagnosed using the ankle brachial index (ABI). This is measured by using a continuous-wave Doppler device and dividing the higher of the 2 systolic pressures at the dorsalis pedis and posterior tibial arteries in the feet and legs by the higher of the systolic pressures in the brachial artery of each arm. A normal ABI registers between 1.00 and 1.40. An ABI $\leq$ 0.90 indicates 90% sensitivity and 95% specificity for PAD[13]. An ABI between 0.90 and 1.00, while not considered PAD, is correlated with a 10% - 20% increase in the rate of a cardiovascular event[13]. Abnormal ABI is also a strong indicator of coronary or cerebrovascular disease[17,18]. In fact, PAD is a risk factor for other cardiovascular diseases[1,19]. Unfortunately, nearly 50% of PAD patients are asymptomatic[20]. As a result, ABI measurement is recommended for all patients $\geq$ 65 years old or $\geq$ 50 years with a history of diabetes or smoking[21-23]. Although less precise, pedal pulse palpation is a secondary diagnostic tool and is sometimes used as a preliminary screening for PAD[24].

Pathophysiology

PAD has been classified in numerous ways since the 1950's beginning with the four Fontaine classifications (Table 1)[25]. However, given the variety of PAD presentation, these classifications were revised to delineate disease progression and classification based on symptomology[26], logistic angiography[27,28] and morphology[29]. Presently, The American College of Cardiology/American Heart Association Practice Guidelines defines the presentation of PAD using four categories: asymptomatic, claudication, CLI and acute limb

ischemia (ALI)[21]. This current classification is a comprehensive international collaboration effort known as the Trans-Atlantic Inter-Society Consensus Document II (TASC II). Using this system, asymptomatic patients are individuals whose ankle brachial index is consistently below 0.90 but do not express other symptoms. As disease progresses, patients may begin to experience intermittent claudication or pain from physical exertion that typically subsides with resting, thus entering the claudication category[12]. CLI patients will report chronic ischemic rest pain, that will often present with ulcers and gangrene. ALI indicates a sudden and rapid decrease in limb perfusion that often threatens the viability of the limb. ALI may occur due to a local thrombosis or embolic event in a patient who was previously asymptomatic[12].

PAD describes a loss of vascular conductance in the peripheral arteries primarily through atherosclerosis and associated thrombosis. Atherosclerosis is a process where the buildup of cholesterol and other substances causes arteries to become hardened and narrow. These plaques continue to grow as infiltrating monocytes differentiate into macrophages, engulf the lipids within the plaque, and become foam cells[30,31]. Vascular smooth muscle cells (SMC) will also migrate to the plaque and synthesize extracellular matrix (ECM) forming a fibrous cap[31]. As the plaque continues to grow, the internal stresses exceed the material strength of the fibrous cap leading to plaque rupture[31,32]. A ruptured plaque exposes the underlying thrombogenic matrix to the blood, allowing circulating platelets to adhere and aggregate activating the coagulation cascade leading to atherothrombosis[30,33]. As the thrombus continues to grow, cross sectional area of the artery is reduced and blood flow is progressively restricted[30].

Under healthy conditions, the body will attempt to increase blood supply to the affected limb through the activation of angiogenesis and arteriogenesis[34]. Once tissue damage has occurred, vascular remodeling, inflammation and various apoptotic pathways will be activated to facilitate repair[34]. Unfortunately, for patients with CLI, these healing processes are ineffective due to various compounding issues such as loss of angiogenic potential or the presence of other comorbidities[34-36]. CLI patients will often develop ulcers and gangrene due to ischemia and necrosis.

Table 1. Fontaine classification for peripheral artery disease[37].

Stage I	Asymptomatic arteriopathy
Stage II	Exercise-induced ischemia
Iia	Intermittent claudication, pain during walking Relief of symptoms when standing Compensated disease: walking distance > 100 m
Ibis	Decompensated disease: walking distance < 100 m
Stage III	Ischemia-driven symptoms at rest
IIIa	Ankle Pressure Index > 50 mmHg
IIIb	Ankle Pressure Index < 50 mmHg
Stage IV	Trophic ulcers and gangrene
Iva	Limited gangrene
Ivb	Extensive gangrene

Role of Inflammation in Vascular Repair

Inflammation is the initial response to injury within the body carrying out multiple functions including fighting infection, clearance of cellular debris, ECM remodeling and induction of neovascularization. The immune response begins with the recruitment of leukocytes to the affected region. After an injury, platelets and polymorphonuclear leukocytes (PMNs) form a blood clot. Proinflammatory cytokines such as tumor necrosis factor-α (TNFA) and interferon-γ (IFNG) at the site of injury upregulate various adhesion

molecules that facilitate leukocyte adhesion[38]. Neutrophils infiltrate infected tissue where they begin to debride devitalized tissue and clear any infectious agents[38,39]. In the absence of continued stimuli for recruitment, the infiltration of neutrophils subsides within 2 – 3 days. Residual neutrophils are then phagocytosed by monocyte-derived macrophages[39]. Adhesion and diapedesis of monocytes is facilitated primarily through the upregulation and activation of adhesion molecules such as the platelet-endothelial adhesion molecule-1 (PECAM-1 or CD31) and endothelial-leukocyte adhesion molecule-1 (ELAM-1 or E-selectin) through mechanical and chemical mechanisms[40,41]. As monocytes extravasate from the blood into the tissue they differentiate into activated macrophages. While the complete function of macrophages in wound repair is incompletely understood, it is known that activated macrophages function as antigen presenting cells, phagocytes and as important synthesizers of potent growth factors, cytokines and matrix modulation proteins. The fundamental role of macrophages in tissue repair and recovery has been exemplified in numerous studies where macrophage depletion/impairment led to lack of debridement and delayed fibrosis and wound closure[42-44].

Current Therapies and Limitations

The first line of defense for PAD is management and prevention which focuses on the reduction of risk factors[45,46]. For example, physical exercise programs have been recommended to increase waking distance and for prognostic measures[34,47]. Exercise; however, may prove problematic as PAD patients have an exercise capacity of < 5 metabolic equivalents[48,49]. Weight loss and abstinence from drinking have also been significantly associated with lower risk of PAD[50,51]. Smoking in particular is one of the major risk factors for PAD along with diabetes and other CVDs[1,33]. As a result, smoking

cessation has one of the strongest correlations with a decrease in the incidence and progression of PAD[15,50,51]. Unfortunately, reduction of risk factors largely depends on the patient's own resolution. For instance, in a survey of 112 individuals, 71% reported a lack of regular exercise and < 14% had a measured their blood pressure within a year[52]. As a result, early PAD is often managed through pharmacological means.

Pharmacological treatment for PAD is often a complicated task due to the likelihood of PAD patients to also exhibit comorbid CVDs that would negatively respond to treatment[53]. Furthermore, endovascular interventions, including angioplasty and stenting, are often subject to fracture, restenosis and thrombosis[54]. As a result, alternative therapies for treating ischemic diseases have recently centered around growth and regeneration of blood vessels in ischemic tissues through neovascularization.

Neovascularization

Neovascularization describes the general process of cardiovascular repair and regeneration. Neovascularization is presumed to occur through three distinct processes: angiogenesis, arteriogenesis and vasculogenesis[55,56]. The ultimate goal of any ischemia therapy is to induce or magnify the manifestation of any combination of these three processes.

Angiogenesis

Angiogenesis describes the formation of new blood vessels from preexisting vasculature. This process is initiated through the hypoxia inducible factor-1 alpha (HIF-1α) which, in an oxygen deprived environment, binds to enhancer regions that upregulate

angiogenic genes such as the vascular endothelial growth factor (VEGF) signaling pathways[57,58]. VEGF increases vascular permeability through redistribution of platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and vascular endothelial-cadherin (VE-cadherin or CDH5)[59]. The degree of vascular permeability is regulated by Angiopoietin-1 (ANGPT1) which reduces plasma leakage without affecting endothelial proliferation[60]. As endothelial cells migrate toward ischemic tissue, they begin to form cords. These cords will form lumens as endothelial cells thin and anastomose. The growth of this nascent vasculature is encouraged and supported by recruited macrophages[61,62]. Hypoxia recruits inflammatory cells that release cytokines and chemokines that stimulate endothelial migration and sprouting (Figure 1A)[63].

Vasculogenesis

Vasculogenesis is the process of *de novo* blood vessel growth or the differentiation of a progenitor population into new endothelial cells to form or contribute to a vascular network[64]. This process happens within the embryo when mesodermal cells differentiate into hemangioblast cells that, in turn, differentiate into angioblasts and eventually endothelial cells[65]. The vasculogenic potential of adults is severely limited; however, there remains a small population of endothelial progenitor cells (EPCs) capable of recruiting from the bone marrow to a site of injury, differentiating into endothelial cells and incorporating into the vasculature (Figure 1B)[66,67].

Arteriogenesis

Arteriogenesis refers to the maturation of pre-existing collateral arteries increasing vascular conductance. Unfortunately, the details of this process are not yet fully

understood. The accepted premise is that altered flow conditions and circumferential wall stresses trigger proliferation of vascular SMCs and induce "muscularizaton" of the nascent vasculature where they begin to expand and contract, thereby increasing the diameter of the lumen as shown in Figure 1C[58,68]. After a significant stenosis has occurred in a major artery, the blood is redirected to the path of least resistance through preexisting arterioles. This increased shear stress activates the endothelial cells to release nitric oxide and cytokines such as monocyte chemoattractant protein-1 (CCL2), granulocyte-macrophage colony stimulating factor (GM-CSF) and adhesion molecules such as selectins, intercellular adhesion molecules (ICAM1 and ICAM2), and vascular cell adhesion molecules (VCAMs)[59,69,70]. These secreted cytokines stimulate integrin expression on circulating monocytes which then bind to the activated adhesion molecules present on endothelial cells, allowing migration through the vascular wall[69]. Recruited monocytes will then differentiate into macrophages and secrete various extracellular matrix (ECM) proteins, proteoglycans and proteases to remodel the microenvironment[59,71]. Inflammatory cells secrete multiple growth factors, in particular those from the fibroblast growth factor (FGF) family, to induce endothelial and SMC migration and proliferation. This proliferation is supervened by the migration of SMC into the vessel wall to form a neointima. These SMCs, along with local and recruited fibroblasts and macrophages, remodel the vessel through the secretion of ECM enzymes such as matrix metalloproteinases (MMPs) and plasmin in order to provide more space for additional cell layers[59,69]. The increased diameter of the remodeled vessel reduces fluid shear stress which then signals vascular maturation. Furthermore, the initial thinning of the vascular wall increases circumferential wall stress which is a proliferative stimulus for SMCs. The

proliferation of SMCs acts as a negative feedback to normalize the circumferential wall stress and end vascular remodeling.

Molecular Therapies for CLI

There have been several angiogenic growth factors investigated as therapies for ischemic cardiovascular disease including VEGF, FGF, hepatocyte growth factor (HGF) and HIF-1[72-75]. A variety of clinical trials have been conducted using VEGF to treat limb ischemia[74,76-78]. Overall, treatment with VEGF has largely proven ineffectual with treated patients having very modest gains. A potential cause for this lack of recovery is that VEGF, while a potent and effective stimulator of endothelial proliferation and vascular growth, is only capable of inducing immature, leaky, unstable vessels[79]. There were a few attempts to use HIF-1, the transcription factor that precedes the angiogenic cascade; however, the results of the trials were underwhelming in that there was no significand difference between treatment and placebo groups[73,80]. Other trials using FGFs have reported similarly modest results[81-83]. One such study was stopped prematurely due to clinically significant proteinurea[81]. As a result, future clinical trials developed delivery strategies to mitigate the side-effects of FGF administration[72]. HGF has been shown to increase the ABI in PAD patient with little to no side effects of gene therapy[75,84,85]. These benefits have shown some promise in translating to CLI[86]. Overall, the use of growth factors to treat limb ischemia have been largely ineffective leading to thoughts that a combination of factors may be necessary. One solution is to use cells as a growth factor releasing vessel that responds to the microenvironment.

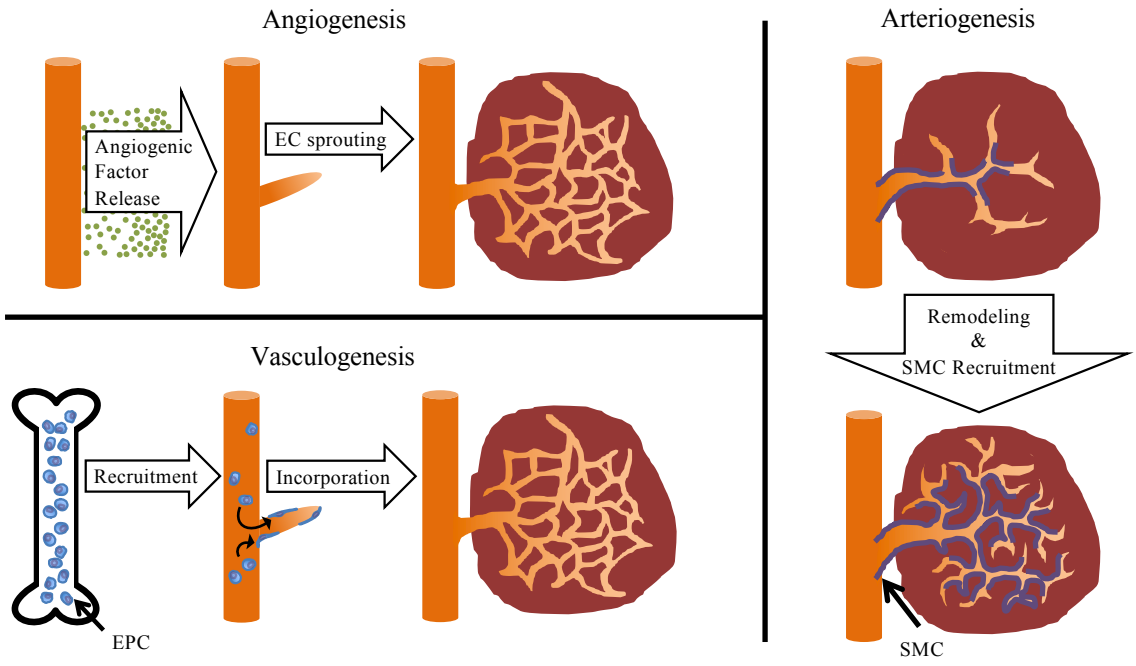

Figure 1. Schematic depicting the three major processes of neovascularization. (A) angiogenesis, (B) vasculogenesis and (C) arteriogenesis.

Cell Therapies for CLI

Endothelial Progenitor Cells

The discovery of a population of peripheral blood $CD34^+$ cells by Asahara et al. (1997) established the existence of EPCs that can differentiate *ex vivo* into ECs[66]. Classically, EPCs are identified through expression of both stem (such as CD133) and progenitor markers (such as KDR; kinase insert domain receptor)[87]. In culture, EPCs have been shown to form early-outgrowth and late-outgrowth colonies[66,67]. Early outgrowth cells express hematopoietic lineage markers and are clearly angiogenic but do not give rise to endothelial cells; however, late outgrowth cells show a similar cell morphology, proliferation rate and surface marker expression to ECs and lack hematopoietic lineage markers[88,89]. The application of EPCs to therapeutic approaches however, has been limited

by their broad definition, and the lack of standardized methods for isolation and culture[90,91]. As a result, clinical trials using EPCs for cardiac and vascular repair have been performed using the parent population of bone marrow (BM) and peripheral blood derived mononuclear cells (MNC) or subsequently sorted $CD34^+$ cells under the premise that EPCs are a subpopulation of transplanted cells[92,93]. Other proposed parent populations of EPCs include $CD31^+$ cells and $CD14^+$ cells[10,94]. $CD14^+$ cells have been tested in clinical trials with limited success[95]. Clinical trials using EPCs have consistently shown statistically significant improvements in vascular perfusion, infarct reduction, and transcutaneous oxygen pressure ($TcPO_2$) levels in patients; however, vascular improvements remain modest[96-99]. The underwhelming performance of EPCs in clinical trials can likely be attributed to a reduction in the proliferative, migrational and functional capacity of EPCs associated with cardiovascular disease, aging and smoking[100-102]. EPCs are also a rare population of cells accounting less than one for every 10,000 MNCs[103,104].

Mesenchymal Stem Cells

MSCs have been investigated as potential candidates for stem cell therapy due to their angiogenic gene expression, immunosuppressive effects, and transdifferentiation potential[105,106]. Studies have shown that MSCs can differentiate into adipocytes[107,108], chondrocytes[108-110], osteoblasts[108,111], myocytes[112], neural cells[113] and endothelial cells[114]. However, there remains questions about the differentiation capacity of MSCs outside of the adipogenic, chondrogenic and osteogenic lineages[115,116]. Furthermore, MSCs have been shown to form tumors and promote tumor growth after transplantation into animal models[117-119]. In clinical trials, MSCs have demonstrated significant trends toward

increased cardiac output, muscle mass, and wound healing; however, their therapeutic effects remain marginal[120-122].

Overall, while the use of adult stem cells, including EPCs and MSCs, in clinical trials has been shown to collectively decrease limb amputation and increase amputation free survival of critical limb ischemia patients, the effects have been marginal[123].

Effector cells ($CD31^+$ cells)

$CD31^+$ ($PECAM\text{-}1^+$) cells are highly angiogenic and vasculogenic cells found in the bone marrow and circulating in peripheral blood[9]. As the major mechanisms underlying therapeutic effects for BM-derived stem and progenitor cells were determined to be largely paracrine effects, Kim et al. identified a cell population enriched for the angiogenic fraction[9,10]. Furthermore, direct isolation using a surface marker circumvented negative phenotypic changes associated with *in vitro* culture and the use of animal serum. Isolation of $CD31^+$ cells directly from BM were shown to exclusively exhibit hematopoietic stem or progenitor cell activities without the need for expansion *in vitro*[10,11]. Furthermore, $CD31^+$ cells were enriched for both angiogenic and vasculogenic cells, displaying spontaneous tube formation *in vitro* and incorporation into host vasculature *in vivo*[10,11,124]. It was recently shown that spherical culture of myeloid cells produces highly angiogenic $CXCR4^+CD31^+$ clusters[125]. These angiogenic cell clusters contained many cells double positive for CD14 and CD31[125].

CHAPTER 3 IDENTIFICATION AND CHARACTERIZATION OF PERIPHERAL BLOOD MONONUCLEAR CELL FRACTIONS

Introduction

Ischemic cardiovascular disease (CVD) is the leading cause of death in the United States[1,126]. Unfortunately, the current medical therapies used to treat CVD are often only effective at slowing disease progression and relieving symptoms of underlying disease[53,54,127]. Peripheral artery disease (PAD) treatment often will require the administration of multiple therapies in combination; however, this may become problematic with the limited tools available for treating CVD[128-130]. The major cause of ischemic CVD is atherosclerosis, which leads to atherothrombus formation and the occlusion of arteries[12,30]. The body will attempt to increase blood supply to the affected limb through the activation of angiogenesis and arteriogenesis; however, for patients with CLI, these healing processes are ineffective due to various compounding issues such as loss of angiogenic potential or the presence of other comorbidities[34-36].

Angiogenic Cells for Cardiovascular Disease

For the past few decades, cell therapy has been investigated as a potential solution to inducing angiogenesis and vascular repair. For example, endothelial progenitor cells (EPCs) have been extensively investigated for their angiogenic potential[90,131,132]. EPCs have consistently displayed strong secretion of angiogenic growth factors such as VEGFA, FGF, HGF and CXCL12[133]. Given that EPCs originate out of the mononuclear cell (MNC) population, MNCs have also been shown to express angiogenic factors to a lesser extent.

Similarly, mesenchymal stem cells (MSC) have been investigated as a promising proangiogenic cell due to their expression of angiogenic factors VEGF, FGF2, and ANGPT1[134,135] . Recently, bone marrow (BM) and peripheral blood (PB) $CD31^+$ cells have been investigated for their highly angiogenic expression[10,11]. $CD31^+$ cells have been demonstrated to be enriched with multiple proangiogenic factors such as VEGFA, HGF, FGF2, CCL2, ANGPT1, insulin-like growth factor 1 (IGF1) and platelet derived growth factor beta (PDGFB)[11,124]. The angiogenic effects of these various cells has been extensively investigated and characterized; however, each cell population, while angiogenic, also has shortcomings toward its application to treat CVD in humans.

The application of EPCs is limited by their broad definition, and the lack of standardized methods for isolation and culture[90,91]. As a result, clinical trials using EPCs for cardiac and vascular repair have been performed using the parent population of BM and PB derived MNCs or subsequently sorted CD34+ cells under the premise that EPCs are a subpopulation of transplanted cells[92,93]. Furthermore, EPCs are an extremely rare population of cells accounting less than 100 parts per million MNCs[103,104]. In the case of MSCs, a number of claims have been made about their angiogenic potential, vasculogenic capability, immune privileged nature and other bold claims; however, it is well documented that MSCs are indistinguishable from fibroblasts[136]. MSCs also suffer from a lack of consensus in the literature defining their expression and characterization[137]. Furthermore, one of the criteria critical to the identification of MSCs requires their maintenance in culture, which is known to have significant transcriptional consequences as early as passage 0[136,138]. $CD31^+$ cells show great promise as an angiogenic cell therapy with

vasculogenic potential; however, given their heterogeneous nature, it is unclear if this population of cells may be further enriched for proangiogenic cell types.

CD14 for the Enrichment of PB-CD31$^+$ Cells

It was reported that approximately 40% of CD31$^+$ cells also express CD14[11]. CD14 is a differentiation antigen expressed by monocytes, macrophages and activated granulocytes; however, in the case of PB-CD31$^+$ cells, CD14 is a monocyte marker[139]. This may be concluded since macrophages are, in part, defined by their location within tissue and derivation from circulating monocytes[140]. Granulocytes are removed in the process of MNC isolation from whole blood[141]. Monocytes are known to be critical to the angiogenic process with important functions in growth factor secretion, matrix remodeling and clearance of debris[71,142]. Monocytes have also been proposed as the true phenotype of EPCs in circulation[94,132]. However, there exists some controversy over the contribution of circulating monocytes to the proinflammatory, anti-healing M1 phenotype of macrophages[143-145]. Furthermore, in a CD14 knockout mouse, it was shown that diabetic mice had reduced adiposity, lower blood pressure and improved glucose homeostasis[146].

For this thesis, we investigated the angiogenic potential of PB- CD31$^+$CD14$^+$ cells. We hypothesized that the CD14$^+$ population of PB-CD31$^+$ cells would contain the more angiogenic subset of cells. Using rigorous characterization methods, we demonstrate that PB- CD31$^+$CD14$^+$ cells are indeed the highly angiogenic subfraction of PB-CD31$^+$ cells. We also uncovered a highly arteriogenic population of cells in the PB- CD31$^+$CD14$^-$ fraction. Our results uncover two independent populations of cells with high therapeutic potential for cardiovascular regeneration.

Methods

Isolation and culture of PB cells.

Human PB samples were collected from healthy male and female volunteers. Isolation of MNCs was performed using a modification of a previously reported method[147]. Briefly, each PB sample was diluted with one volume of calcium- and magnesium-free phosphate-buffered saline (PBS) containing 2% bovine serum albumin (Miltenyi Biotec, Auburn, California), layered on one volume of Histopaque-1077 (Sigma, St. Louis, Missouri), and centrifuged at 400 g for 35 min. The MNCs were harvested from the interface and washed with magnetic-activated cell sorting (MACS) buffer (Miltenyi Biotec). MNCs were centrifuged again at 400 g for 10 min to remove platelets. The cells were counted and resuspended in MACS buffer blocking reagent.

For FACS isolation, cells were incubated for 20 min at 4°C with phycoerythrin (PE)-conjugated mouse anti-human CD14 and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD31 antibodies (BD Bioscience, San Jose, California). Proper isotype-identical immunoglobulin G (IgG) served as controls. For MACS isolation, human CD14 microbeads (Miltenyi Biotec) were sequentially added and incubated at 4°C for 20 min. The cells were washed and resuspended in MACS buffer. $CD14^+$ and $CD14^-$ cells were isolated using magnetic LS columns (Miltenyi Biotec) according to the manufacturer's instructions. $CD14^-$ cells were then washed with MACS buffer and incubated with Human CD31 microbeads (Miltenyi Biotec) at 4°C for 20 min. $CD31^+CD14^-$ and $CD31^-$ cells were isolated using magnetic LS columns (Miltenyi Biotec) according to the manufacturer's instructions.

For conditioned medium experiments, PB cells were plated at a density of 1×10^6 cells/cm^2 on 0.1% fibronectin in endothelial basal media 2 supplemented with 2% or 0.5% fetal bovine serum (Lonza Cologne AG, Cologne, Germany), and incubated at 37°C in 5% CO^2. Conditioned medium was collected after 24 h, centrifuged and sterile filtered through a 0.22 um filter. Human umbilical vein endothelial cells (HUVEC) were plated at a density of 1×10^6 cells/cm^2 on 0.1% gelatin coated plates. HUVECS were cultured in endothelial growth media 2 supplemented with 15% fetal bovine serum and the cytokine cocktail, SingleQuots (Lonza Cologne AG, Cologne, Germany) and incubated at 37°C in 5% CO^2. Media was changed after 24 h and every 2 to 3 days. Cells were detached by incubation with 0.05% trypsin–ethylenediamine tetraacetic acid (Gibco, Gaithersburg, Maryland) for 5 min at 37°C. When the cells reached 70% confluence, they were either passaged, used in other assays, or frozen in freezing media (90% fetal bovine serum and 10% dimethyl sulfoxide) for future use.

Flow cytometry.

PB-MNCs were resuspended in PBS and incubated for 20 min at 4°C with direct phycoerythrin-, R-phycoerythrin-cyanine 5 (PE-Cy5)- or fluorescein isothiocyanate (FITC)–conjugated antibodies or nonconjugated antibodies followed by a FITC-conjugated rabbit anti-mouse IgG. Proper isotype-identical IgG served as controls. After staining, the cells were fixed in 2% paraformaldehyde and analyzed on a flow cytometer (Becton Dickinson, San Jose, California). Antibodies used included the following: CD3, CD4, CD8, CD11b, CD11c, CD14, CD16, CD19, CD31, CD34 (all from BD Bioscience, San Jose, California), CD56 (R&D Systems, Minneapolis, Minnesota) and CD133

(AC133; Miltenyi Biotec). Flow cytometric data were analyzed with FlowJo (Tree Star, Inc., Ashland, Oregon) using appropriate isotype-matched controls.

qRT-PCR.

Total RNA from PB-MNCs, -$CD14^{+}CD31^{+}$ cells, -$CD31^{+}CD14^{-}$ cells, -$CD31^{+}$ cells and -$CD31^{-}$ cells was isolated using RNeasy Plus Mini Kit (Quiagen, Hilden, Germany) according to manufacturer's instructions. Extracted RNA was reverse-transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, California) according to manufacturer's instructions. The complimentary DNA was subjected to qRT-PCR using human-specific primers and probes (Table 3). Quantitative assessment of RNA levels was performed using an ABI PRISM 700 Sequence Detection System (Applied Biosystems). Housekeeping gene human large ribosomal protein P0 (RPLP0) was used as an internal expression control. Relative gene expression was calculated using the ΔΔCt method with the MNC parent population as the standard. It is recommended that our qRT-PCR data be analyzed using a non-parametric statistical method due to the low sample size and generally high variability within our samples; however, we opted to use ANOVA and Tukey's post hoc analysis due to their general robustness to normality. The intention of our qRT-PCR analysis is to determine if the mean of one population of cells is generally higher or lower than that of the others. In this regard, ANOVA and Tukey's post hock perform adequately.

RNA-Seq

Total RNA from PB-MNCs, -$CD31^{+}CD14^{+}$ cells, -$CD31^{+}CD14^{-}$ cells, -$CD31^{+}$ cells and -$CD31^{-}$ cells was isolated using RNeasy Plus Mini Kit (Quiagen, Hilden, Germany)

according to manufacturer's instructions. 50 µl at 20 ng/µl of RNA per sample was sequenced using Illumina sequencing technology (Macrogen, Seoul, South Korea). Raw fastq files were analyzed using the open-source, web-based platform Galaxy (usegalaxy.org). Raw data files were uploaded to the Galaxy server and quality controls were generated using FastQC All samples were found to be of high quality so no truncation of reads was necessary (Figure 2). Sequences were aligned using the hierarchical indexing for spliced alignment of transcripts 2 (HISAT2) system. Count files were generated using gene transfer files for Genome Reference Consortium human genome build 38 (GRCh38) available from gencodegenes.org and the high throughput sequencing (HTSeq) Python package. Further analysis of count files was done in R programming (Figure 25). Differential gene expression was determined using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system[148,149]. A statistical overrepresentation test was used to determine ontological significance of differentially expressed gene list using the PANTHER method of comparing the percent representation of a particular PANTHER category in a reference/test list to the experimental list. PANTHER uses a binomial comparison test to determine significant representation of a given gene ontology category.

GSEA Analysis.

We used the gene set enrichment analysis (GSEA) software from the Broad Institute to analyze significant enrichment of gene sets between groups. A priori gene sets were taken from the Broad Institute gene ontology database and used to perform GSEA between peripheral blood cell fractions.

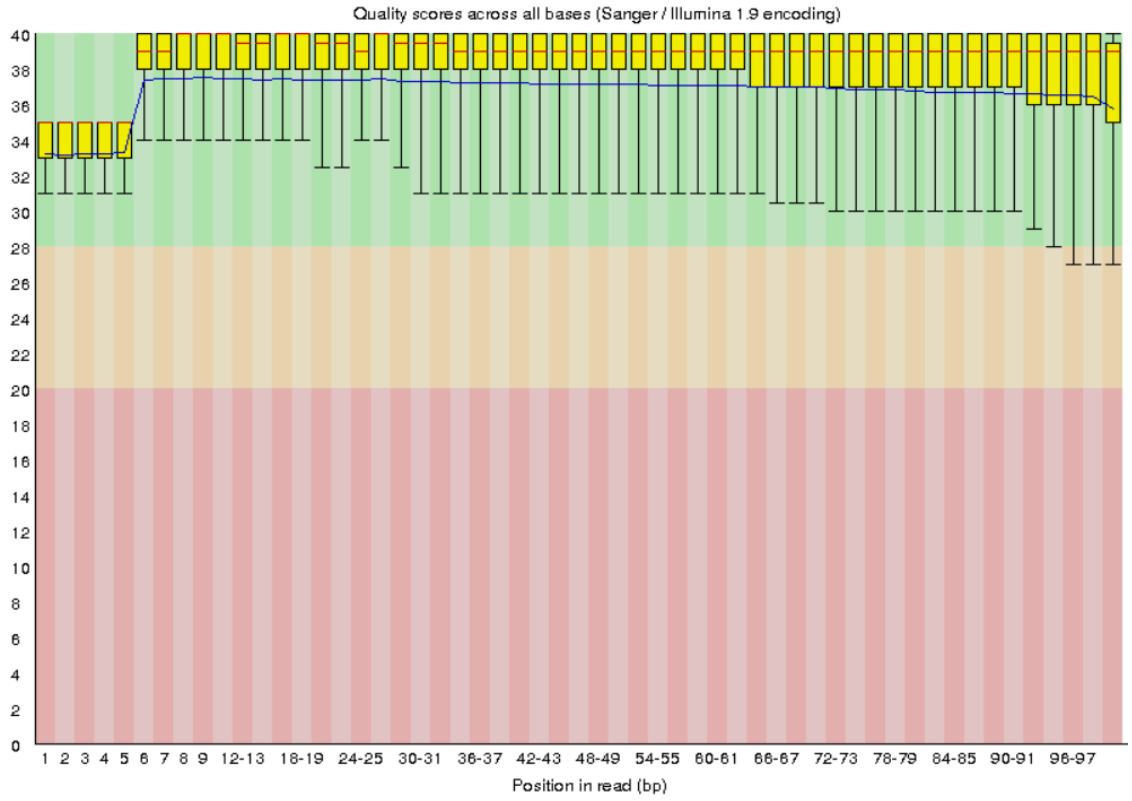

Figure 2. Representative quality control report for RNA sequencing data.

Wound healing assay

The endothelial wound healing assay was done by plating HUVECs between passage 5 and 7 on gelatin-coated 12-well plates at a density of 1 x 10^5 cells / cm^2 in endothelial growth media 2 supplemented with 15% fetal bovine serum and the cytokine cocktail (EGM-2), SingleQuots (Lonza Cologne AG, Cologne, Germany) and incubated at 37°C in 5% CO^2. After 24 hrs, media was changed to endothelial basal media 2 supplemented with 0.5% fetal bovine serum (0.5% EBM-2) and incubated overnight at 37°C in 5% CO^2. Plates were imaged to confirm confluency and health of the cells. A 200 µl sterile pipette tip was scraped vertically from bottom to top across the 12-well plate through the confluent monolayer of cells to create a uniform cell-free zone. Immediately,

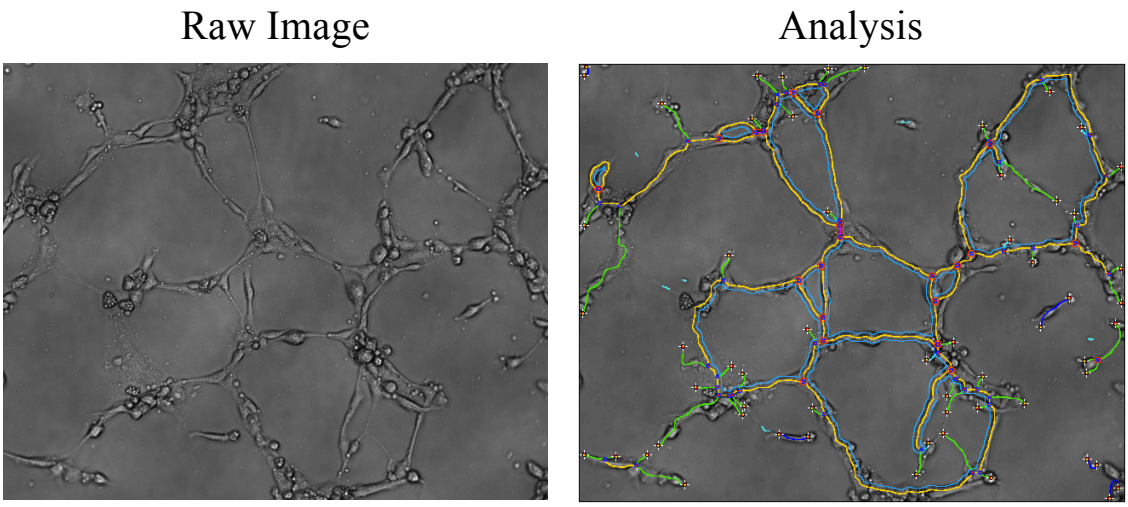

Figure 3. Representative image of Angiogenesis Analyzer overlay in ImageJ.

the wells were gently rinsed with PBS to remove cell debris and old media. Conditioned medium was added to each treatment group and EGM-2 and 0.5% EBM-2 were used as positive and negative controls, respectively.

Tube forming assay

Growth factor reduced (GFR) Matrigel was thawed overnight at 4°C. Chilled 96-well plates were placed on ice and chilled pipette tips were used to pipette 50 µl of Matrigel in each well. Matrigel was allowed to polymerize for 30 min in an incubator at 37°C in 5% CO^2. HUVECs between passage 5 and 7 were suspended in 2% EBM-2, EGM-2, or conditioned medium and layered on top of the Matrigel at 1 x 10^4 cells per well. Each well was imaged following 6 hrs incubation at 37°C in 5% CO^2. Tube formation was analyzed in ImageJ using the Angiogenesis Analyzer tool (Figure 3).

Statistical Analysis

All data are represented as means ± S.E.M and statistical analysis was conducted in R (Figure 27). Student's t test was used for the statistical analysis for continuous variables between two groups and ANOVA followed by Tukey-Kramer or Fisher least significant difference (LSD) method for variables among more than 2 groups. A p value < 0.05 was considered statistically significant.

Results

$CD14^+$ cells are contained exclusively within the $CD31^+$ fraction of cells.

We used FACS analysis to isolate each cell fraction. The gating system we used was as follows: 1) use the side scatter (SSC) and forward scatter (FSC) area channels to remove cellular debris from our target population; 2) use that slection and used the FSC height and FSC area channels to remove doublets and triplets from the selection; gate for cells based on their fluorescnce intensity of CD14- and CD31-conjugated fluorophores. The scatterplot of single MNCs shows that all PB-$CD14^+$ cells are also positive for CD31 (Figure 4A). This result is consistent with the literature where CD14 was not detected in the PB-$CD31^-$ population of cells[11]. We used this data to develop a MACS sorting protocol for isolation of cells positive for both CD14 and CD31 using the scheme depicted in Figure 4B. The MACS sorting procedure is more crude and prone to lower sorting purity that FACS so we used flow cytometry to determine the sorting efficiency and purtiy of MACS sorted cells. Flow cytometric analysis on MACS sorted cells shows that > 95% of MACS isolated $CD14^+$ cells and < 2% of $CD14^-$ cells express CD14. Similarly, > 95% of MACS sorted $CD31^+$ cells express CD31 (Figure 4C). Notably, just under 12% of MACS sorted $CD31^-$ cells express CD31 indicating a loss of sorting efficiency when using MACS. While

positive selection using MACS results in high purity of isolated populations, there is still potential contamination of $CD31^+$ cells in the flowthrough after MACS sorting using CD31 microbeads. We concluded that these contaminating $CD31^+$ cells lowly express CD31 and are such a minor portion of the $CD31^-$ population that they would not significantly alter the function of the population as a whole; however, in order to avoid the introduction of any artifacts in our evaluation, we used FACS sorted cells for all in vitro assays.

$CD31^+CD14^+$ cells are enriched with stem and vascular repair blood lineages

Given the heterogeneity of PB cells, it is important to determine which cell lineages remain present in each isolated cell fraction. This information is important for deducing how each cell fraction is likely to function during injury repair. We decided to use lineage markers for T-cells (CD3), T-helper cells (CD4), cytotoxic T-cells (CD8), monocytes (CD11b and CD16), dendritic cells (CD11c), B-cells (CD19), natural killer cells (CD56) and hematopoietic stem and progenitor cells (HPSC) (CD34 and CD133). These lineages together constitute the mononuclear cell portion of leukocytes and all have a unique role in injury and cardiovascular repair[150]. In particular, T-cell recruitment and polarization plays an important role in macrophage polarization and cell clearance while B-cells initiate and facilitate the T-cell immune response and humoral immunity[151-153]. Dendritic cells modulate the immune response while monocytes differentiate into macrophages and aid in tissue clearance, remodeling and wound healing[39,150,154]. Using flow cytometry to determine lineage expression we determined PB-$CD31^+CD14^+$ cells were significantly enriched with expression of monocyte markers CD11b, CD16, and HPSC marker CD34 when compared to all other cell fractions cells. PB-$CD31^+CD14^+$ cells were also highly enriched for dendritic cell marker CD11c and HSPCS marker CD133 compared to all other

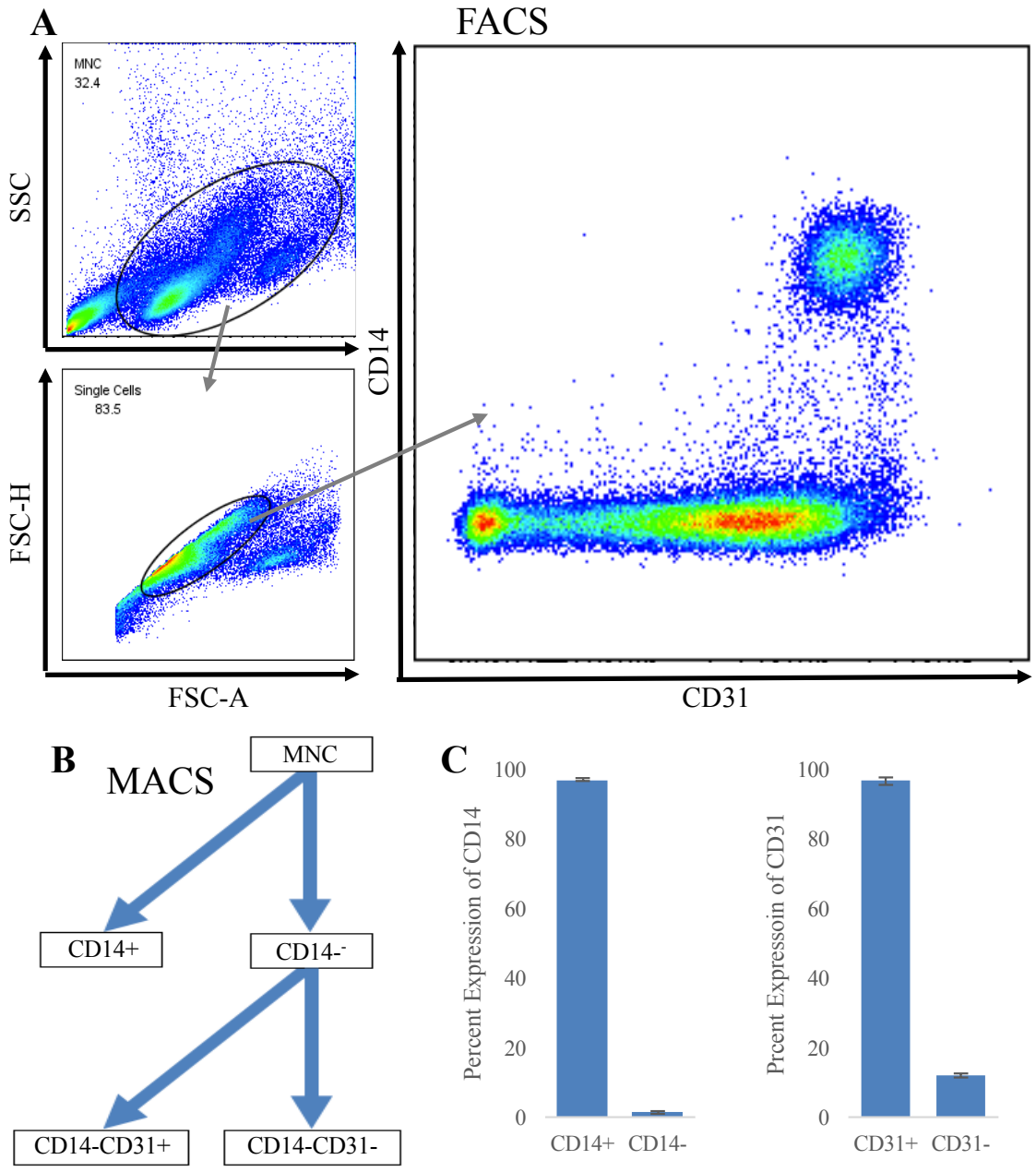

Figure 4. Isolation of PB-MNC fractions. (A) FACS staining of PB-MNCs with CD14 and CD31. (B) MACS isolation procedure of PB-MNCs. (C) MACS sorting efficiency. n = 3.

cell populations except PB-CD31^{+} cells (Figure 5). Markers indicative of T-cell and B-cell lineage were not significantly different between cell fractions. However, CD56, a marker for natural killer cell lineage, was significantly lower in the PB-CD31^{+}CD14^{+} cell population than in the PB-CD31^{-} fraction. While the enrichment of monocyte markers

within the PB-$CD31^+CD14^+$ fraction is expected, enrichment of dendritic cell marker CD11c implies a potential immunomodulatory function. Furthermore, the overrepresentation of the HSPC markers CD34 and CD133 imply that if a progenitor cell population is to be isolated, it would be found in the PB-$CD31^+CD14^+$ population of cells.

We next used flow cytometry to determine the representation of $CD31^+CD14^+$ and $CD31^+CD14^-$ cells within various cell lineage groups. $CD41^-CD31^+$ cells were more highly represented in the T-cell (CD3) fraction compared to $CD31^+CD14^+$ cells. The B-cell (CD19) and HSPC (CD34 and CD133) populations were shown to have a significant portion of cells with $CD31^+CD14^+$ expression (Figure 6). The observation that roughly 75% of PB-$CD34^+$ and $CD133^+$ cells are $CD31^+CD14^+$ further supports the idea that circulating progenitor cells are contained within the PB-$CD31^+CD14^+$ fraction.

PB-$CD31^+CD14^+$ cells and PB-$CD31^+CD14^-$ cells express angiogenic and arteriogenic factors, respectively.

We then used RT-PCR to determine differences in mRNA expression between groups. In order to accurately compare gene expression, it is necessary to use a reference gene that is constitutively expressed at a similar level for all sample groups. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is by far the most commonly used gene for these studies; however, there have been conflicting studies about the suitability of GAPDH as an internal reference control[155-157]. In order for a gene to be considered suitable as a standard, it must have a standard deviation of less than 1[158]. We chose to compare two genes, GAPDH and human large ribosomal protein P0 (RPLP0) as they have both been indicated as suitable housekeeping genes for hematopoietic cells[155,157]. We first compared the Ct

value of RPLP0 and GAPDH for every cell fraction using peripheral blood from three donors/samples (Figure 7A). Notably, we see variations in both genes across all samples except for RPLP0 in Sample B. Interestingly, there is a consistent trend in the GAPDH expression implying a relative difference in expression specific to each cell fraction. When we determined the standard deviation of each gene across all PB cell fractions, we found that RPLP0 and not GAPDH was consistently below a standard deviation of 1 (Figure 7B). Figure 7C shows that GAPDH expression is highly variable from donor to donor when RPLP0 is used as the internal standard. As a result, we chose RPLP0 as our reference gene for our qRT-PCR studies. These studies also demonstrate the importance of first normalizing the expression of each cell population to a control population (i.e. the MNC population) before pooling data from different donors/samples due to the differing baseline expression of the reference gene from donor to donor.

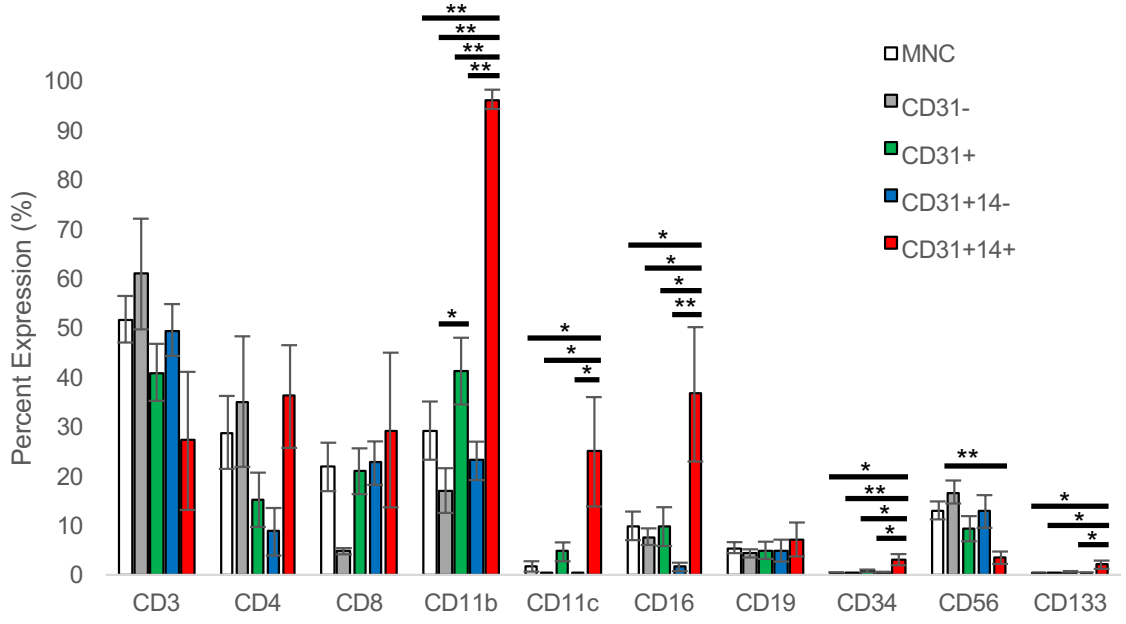

Figure 5. Lineage expression within PB derived cell populations. * p < 0.05, ** p < 0.01. n = 5.

In order to determine the expression profiles of PB- $CD31^{+}CD14^{+/-}$ cells we used qRT-PCR analysis for angiogenic, arteriogenic and immunogenic growth factors, cytokines and enzymes. Hallmark angiogenic factors VEGFA and HGF were significantly upregulated in PB-$CD31^{+}CD14^{+}$ cells compared to PB-$CD31^{+}CD14^{-}$ cells and $CD31^{-}$ cells. Similarly, PB-$CD31^{+}$ cells had increased expression of VEGFA and HGF compared to PB-$CD31^{-}$ and $CD31^{+}CD14^{-}$ cells, respectively. Expression of IGF was markedly low in PB-$CD31^{+}CD14^{+}$ cells compared to other groups; however, this difference was not significant. PB-$CD31^{+}CD14^{+}$ cells and PB-$CD31^{+}$ cells displayed higher expression of angiogenic factors ANGPT1 and CXCL8 compared to PB-$CD31^{+}CD14^{-}$ cells and PB-$CD31^{-}$ cells although these differences in expression were not significant (Figure 8).

Arteriogenic factor PDGFB was significantly highly expressed in both PB-$CD31^{+}CD14^{-}$ cells and PB-$CD31^{-}$ cells compared to PB-$CD31^{+}CD14^{+}$ cells and PB-$CD31^{+}$ cells. Furthermore, ECM remodeling enzyme MMP2 was significantly expressed in PB-

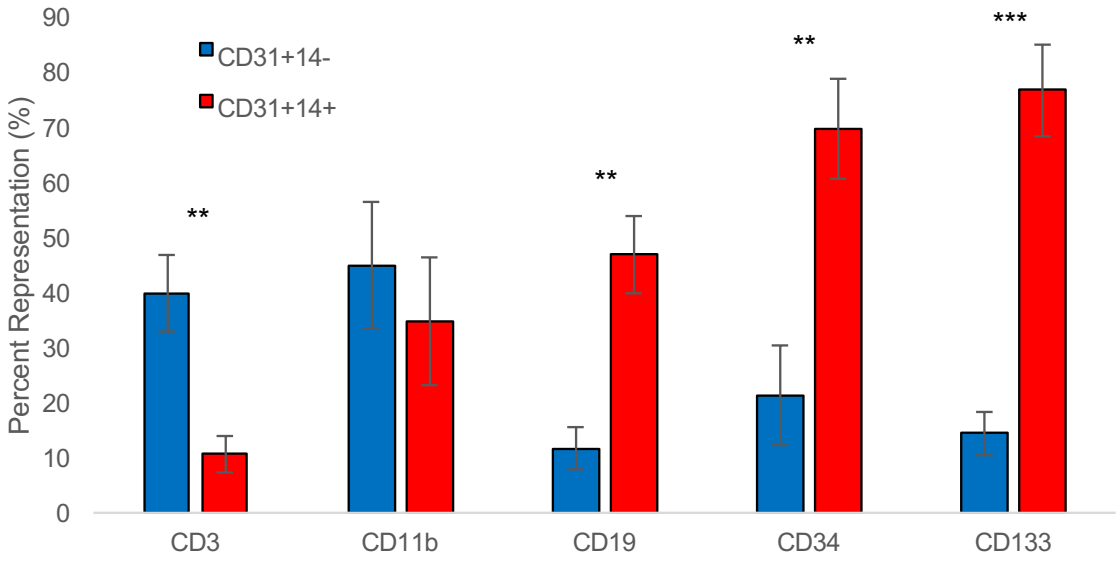

Figure 6. Percent representation of PB-$CD31^{+}CD14^{-}$ and PB-$CD31^{+}CD14^{+}$ cells within notable blood cell lineages. ** $p < 0.01$, *** $p < 0.001$. $n = 5$.

$CD31^{+}CD14^{-}$ cells compared to all other cell fractions. Interestingly, Arteriogenic factor FGF2 appeared to have reduced expression in the $CD31^{+}CD14^{+}$ cell fraction while CCL2 showed higher expression although these differences were not significant (Figure 9).

Among immune response regulatory cytokines, interleukin 10 (IL10) appeared to show higher expression in the PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}$ populations and cytokine

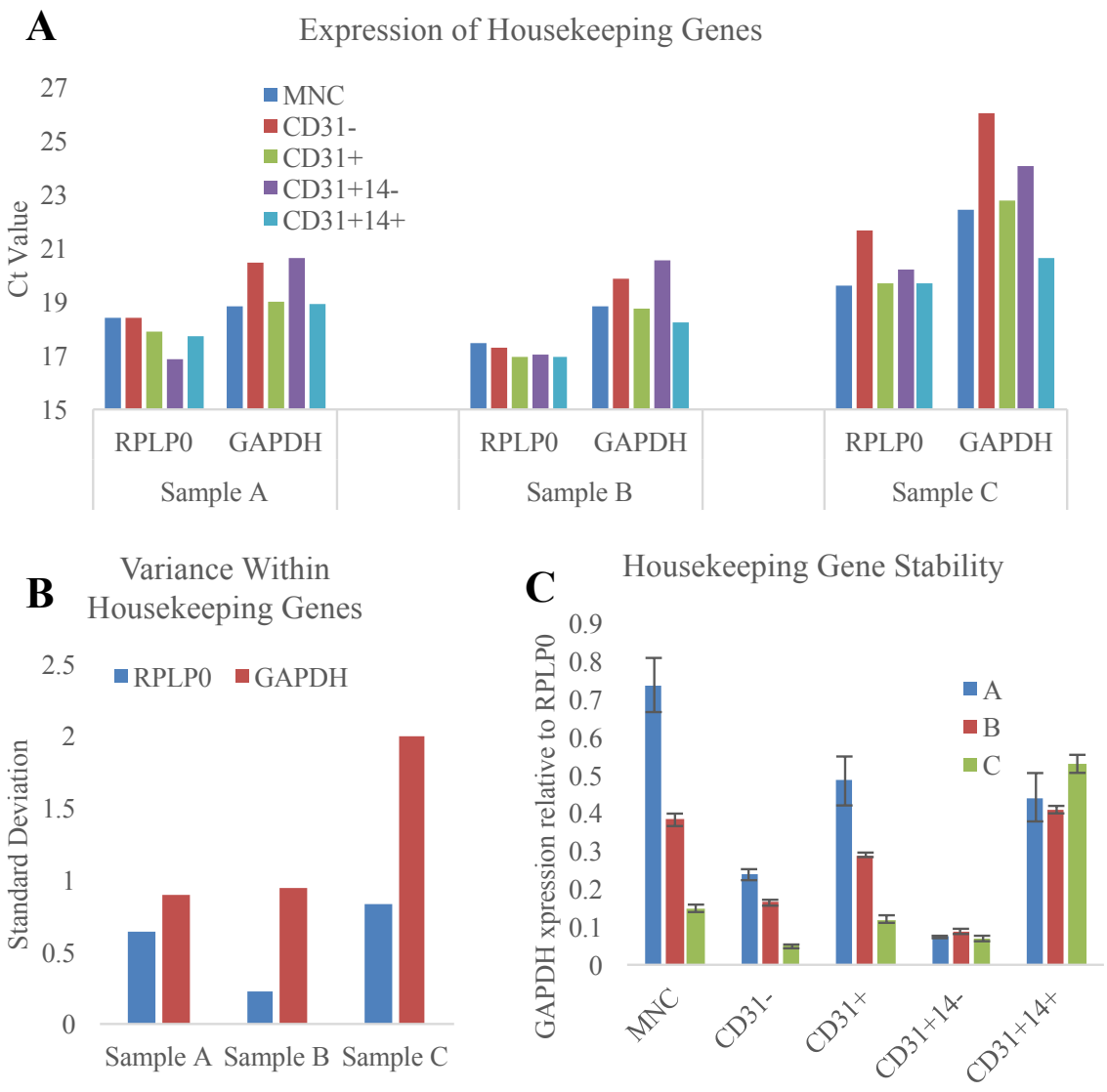

Figure 7. Comparison of RPLP0 and GAPDH as suitable reference genes for RT-PCR. (A) Comparison of doubling cycles necessary to reach threshold gene quantification. (B) Comparison of variations of each gene across all cell fractions within each sample. (C) Expression of GAPDH using RPLP0 as a reference gene.

IFNG appeared to show higher expression in the PB-CD31$^+$CD14$^-$ and PB-CD31$^-$ populations however these differences in expression were not significant (Figure 10). The observed expression profiles imply a pro-angiogenic secretion profile for both PB-CD31$^+$ and PB-CD31$^+$CD14$^+$ cells and a pro-arteriogenic secretion profile for PB-CD31$^+$CD14$^-$ cells.

PB-CD31$^+$CD14$^+$ cells have highly correlated gene expression with PB-CD31$^+$ cells.

In order to investigate the full expression profile of each cell fraction, we used RNA-Seq. Total RNA from each cell group was isolated, sequenced and analyzed for expression of angiogenesis and immune related genes. The gene sets used for analysis of expression correlation were the gene ontology (GO) angiogenesis (279 genes) and GO Immune response (1075 genes) gene sets. A scree plot was generated to show the variances among calculated components of the sequencing data. As shown in Figure 11A, the contribution of variance from each eigenvector clearly begins to level between components 2 and 3. We decided to determine population groupings along the primary (PC1) and secondary (PC2) principal components in order to account for 70% of the variance in the data. A principal component analysis plot of population groupings among angiogenic and inflammatory genes reveals that PB-CD31$^+$CD14$^+$ cells are highly correlated with PB-CD31$^+$ cells along PC1 while PB-CD31$^+$CD14$^-$ cells are more similar to PB-CD31$^+$ cells along PC2 (Figure 11B). Furthermore, PB-CD31$^+$CD14$^+$ and -CD31$^+$CD14$^-$ cells are dissimilar from each other along both PCs and the same is true for the PB-CD31$^+$ and -CD31$^-$ populations. Most of the variance within the data is found along PC1, showing a stronger correlation between PB-CD31$^+$CD14$^+$ and -CD31$^+$ cells than between PB-CD31$^+$CD14$^-$ cells and -CD31$^+$ cells. The PB-CD31$^+$ population has strong correlation

along both PCs showing that angiogenic and immune genes are highly expressed in this population. The distribution of cells within the plot show that the $CD31^{+}CD14^{+}$ and $CD31^{+}CD14^{-}$ subpopulations completely split relative expression of these correlated genes

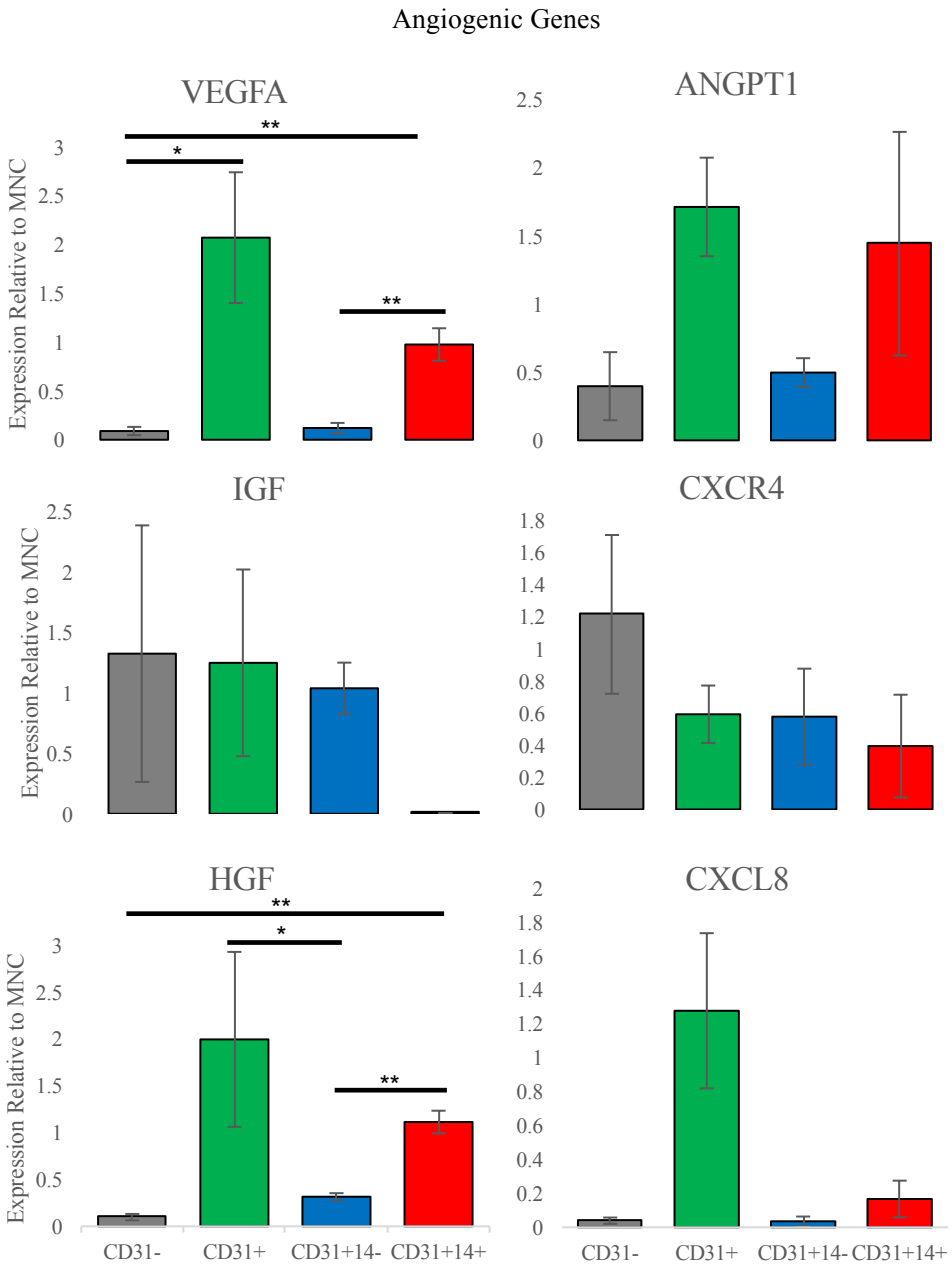

Figure 8. qRT-PCR analysis for angiogenic growth factors and cytokines. * $p < 0.05$, ** $p < 0.01$. n = 3.

with many angiogenic and immune response genes most highly expressed within the $CD31^{+}CD14^{+}$ fraction.

Using R, we generated a heatmap where the angiogenic and immune response genes were ordered from high expression in PB-$CD31^{+}$ cells to high expression in PB-$CD31^{-}$ cells (Figure 12). Interestingly, the dendogram confirms the strong correlations between the PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}$ populations through their similar expression patters. Similarly, the PB-$CD31^{+}CD14^{-}$ and PB-MNC populations are more closely related while the CD31- population is most different from all other populations. This data implies that the highly angiogenic features previously published about PB-$CD31^{+}$ cells would still be retained within the PB-$CD31^{+}CD14^{+}$ fraction.

To determine differentially expressed (DE) genes between PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}CD14^{-}$ cells we used the following pre-established gene ontology datasets available from the Broad Institute website: angiogenesis (279 genes), endothelial differentiation (67 genes), protein secretion (95 genes), cell-cell adhesion (604 genes), cell-matrix adhesion (105 genes), and immune response (1075 genes). We combined the protein secretion and cell-cell adhesion gene sets into the broader cell signaling expression category. Likewise, the angiogenesis, endothelial differentiation, cell-matrix adhesion and immune response gene sets were relabeled as angiogenic, vasculogenic, cell migration and immunogenic gene categories, respectively. Out of the total list of 2225 genes, we found 630 genes to be differentially expressed between the PB-$CD31^{+}CD14^{+}$ group and the PB-$CD31^{+}CD14^{-}$ group (Figure 13A). The PB-$CD31^{+}CD14^{+}$ fraction had increased expression in 42 angiogenic genes and lower expression in 36 genes when compared to PB-$CD31^{+}CD14^{-}$ cells. Among inflammation related genes, 172 were significantly upregulated

in the $CD31^{+}CD14^{-}$ population while 116 genes were upregulated in PB-$CD31^{+}CD14^{+}$ cells. Genes related to endothelial differentiation and cell communication were nearly equally upregulated in both cell populations with 9 angiogenic genes upregulated in both populations and 112 and 109 cell communication genes upregulated in PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}CD14^{-}$ cells, respectively (Figure 13B). The expression distribution from

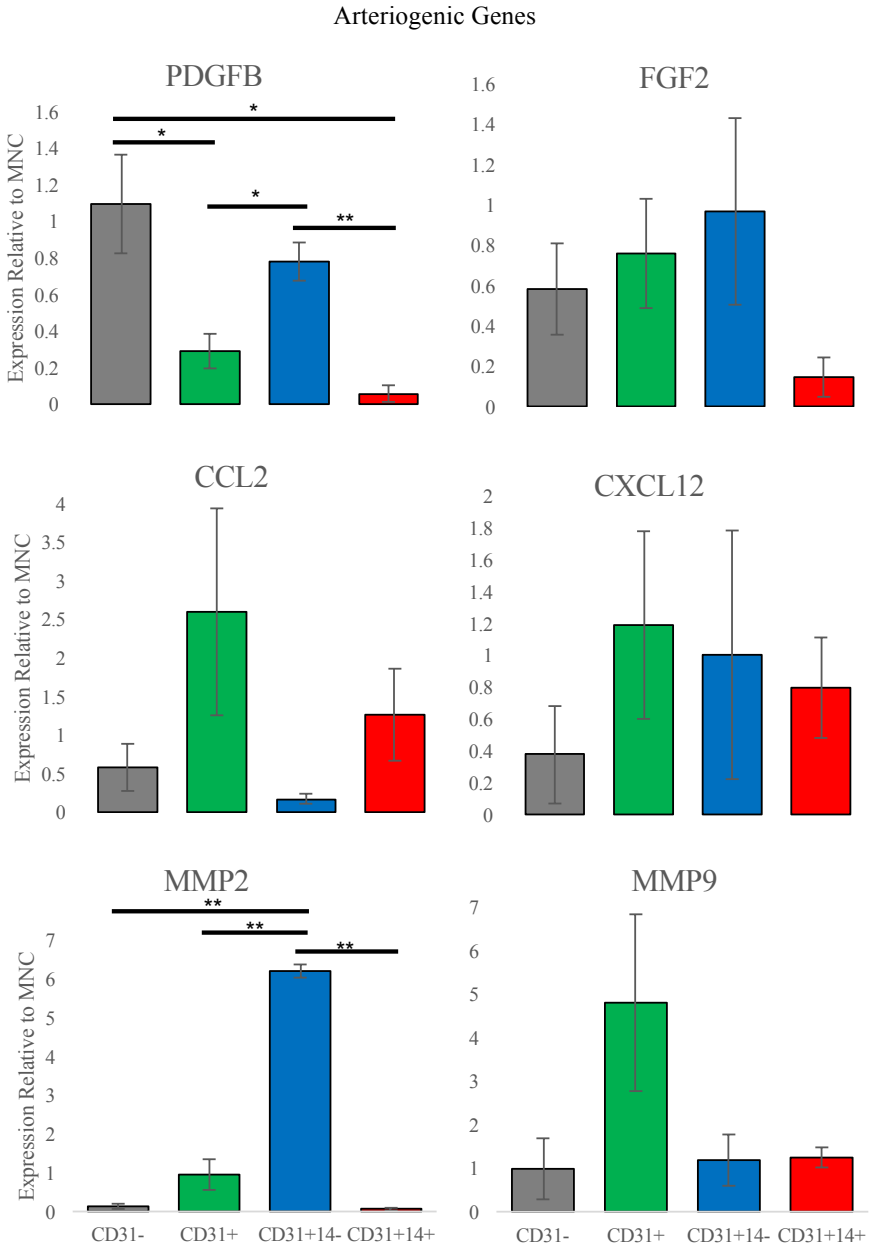

Figure 9. qRT-PCR analysis for arteriogenic growth factors, cytokines and enzymes. * $p < 0.05$, ** $p < 0.01$. n = 3.

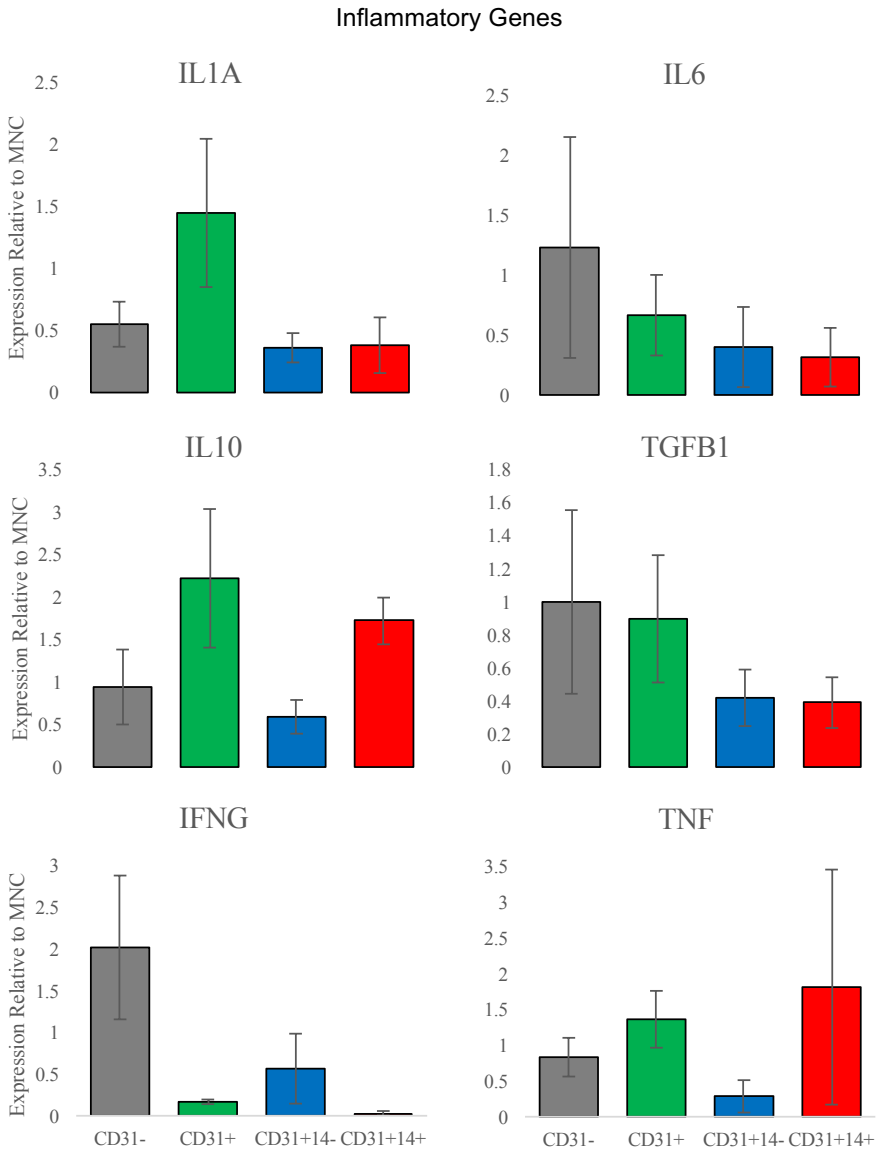

Figure 10. qRT-PCR analysis for inflammatory growth factors and cytokines. n = 3.

these gene ontology groupings display how both cell fractions are involved each of the various aspects of vascular regeneration and tissue repair; however, the specific expression signatures of these populations will provide greater insight into the mechanisms through which each of these populations primarily acts.

PB-CD31^{+}CD14^{+} cells significantly differentially express pro-angiogenic genes

In order to investigate potential mechanisms through which the PB-CD31^{+}CD14^{-} and PB-CD31^{+}CD14^{+} cell fractions function, we created a list of the top 50 most differentially expressed genes for each group (Table 1). Notable genes upregulated in the PB-CD31^{+}CD14^{+} population include FLT4 (vascular endothelial growth factor receptor 4), UNC5B (unc-5 netrin receptor B), VCL (vinculin), TGFB1 (or TGFB), CMKLR1 (chemerin chemokine-like receptor 1) and ICAM1. FLT4 is well established as an important mediator of lymphangiogenesis; however, recent research has demonstrated its NOTCH-regulated involvement in endothelial sprouting during angiogenesis[159,160]. UNC5B is a receptor expressed in endothelial tip cells during vessel formation where it plays a role in neural guidance during development[161]. Endothelial-specific knockouts of *Unc5b* in mice and zebrafish showed a reduction in fetal arterioles[162]. Another important process in initiation of angiogenesis is the recruitment of vinculin to cell-cell focal adherens junctions (FAJ) within the vasculature[163]. Vinculin, while not necessary for FAJ formation, has been shown to be required for FAJ remodeling by cytoskeletal forces[163,164]. TGFB is primarily known for its role in angiogenesis in the promotion of tumor growth. TGFB has also been shown to induce angiogenesis when administered subcutaneously in both mice and rats[165,166]. Chemerin induces endothelial proliferation through activation of the MAPKinase and Akt signaling pathways[167]. Lastly, ICAM1 is an important molecule involved in polymorphonuclear cell induced angiogenesis[168]. Each of these factors is closely related to increasing angiogenesis through influencing either endothelial migration, proliferation or tip-cell formation.

PB-CD31^{+}CD14^{-} cells significantly differentially express genes promoting vascular stability and maturation

In the PB-CD31^{+}CD14^{-} cell population, notable expressed genes include PDE3B (phosphodiesterase 3B), ROBO4 (roundabout guidance receptor 4), CLDN11 (claudin 11), PML (promyelocytic leukemia), ITGB2 (integrin subunit beta 2), JUP (junction plakoglobin), SHC1 (src homology 2 domain containing transforming protein 1), ANGPT4 (angiopoietin 4), CDH5 (vascular endothelial cadherin), RAP1A (ras-related protein krev-

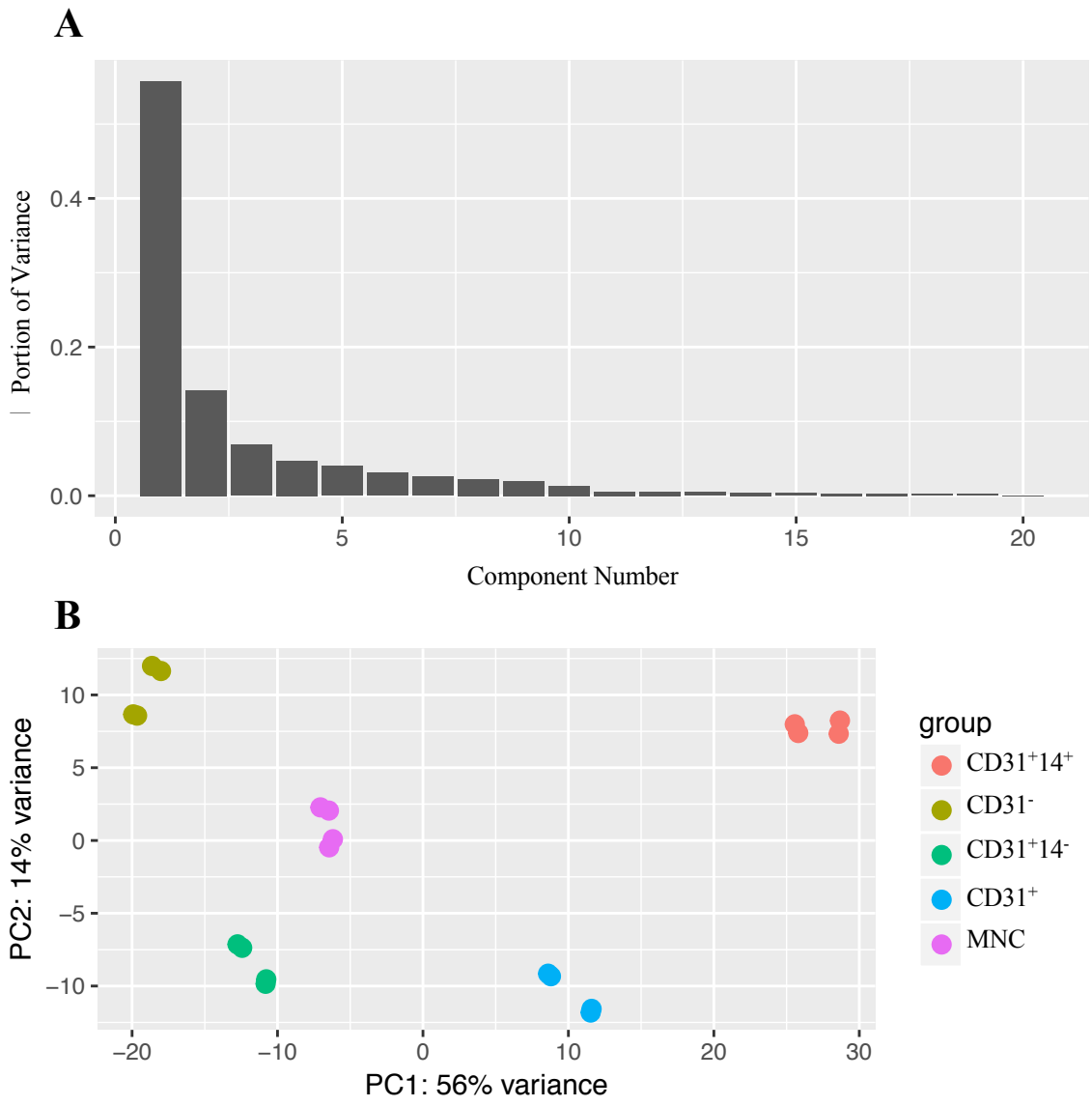

Figure 11. Principal component analysis of PB cell fractions along angiogenesis and immune response genes. (A) Scree plot displaying variance within first five components. (B) PCA scatter plot showing separation of groups along primary and secondary principal components.

1), FGFR1 (fibroblast growth factor receptor 1), EGR3 (early growth response 3), ZNF304 (zing finger protein 304). Expression of PDE3B is regulated by the cAMP signaling

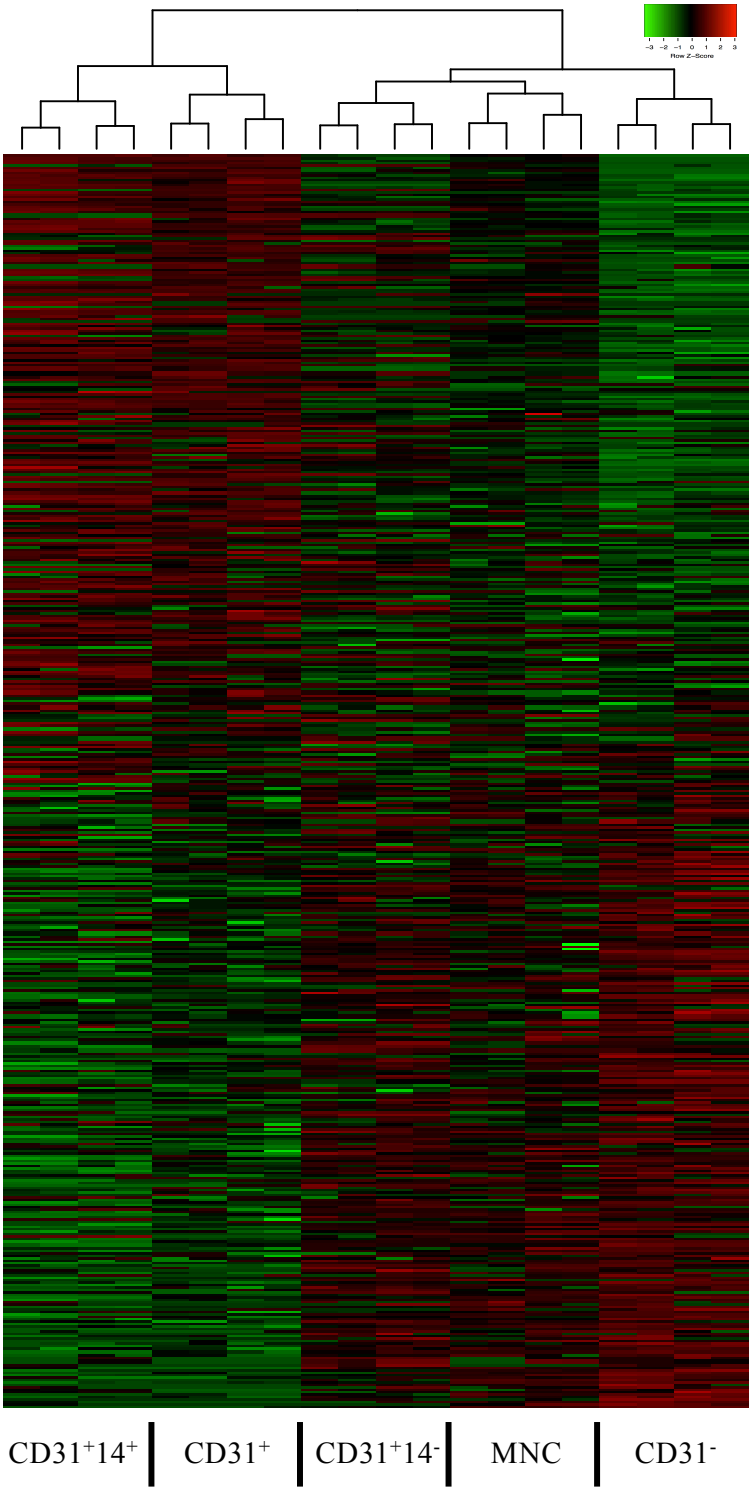

Figure 12. Heatmap of angiogenic and immunogenic genes.

pathway where it then binds with activated RAPGEF3 where it decreases vascular permeability[169,170]. Similarly, ROBO4 has been shown to interact with UNC5B to reduce VEGF signaling, and reduce vascular permeability[171]. CLDN11 is a cell-cell junction protein that is important in maintaining epithelial and endothelial barrier function[172]. It was recently shown that siRNA mediated knockdown of CLDN11 in cultured Schlemm's canal endothelial cells resulted in compromised barrier integrity and increased permeability[173]. PML is most known for its role in tumor suppression and its role in regulating the TNF, TGFB, and IFN-alpha signaling pathways[174,175]. PML represses TGFB which strongly induces the secretion of growth factors such as IL1 and the production of collagen and fibronectin from macrophages to induce angiogenesis[176-180]. Knockdown of PML has been shown impair angiogenesis by upregulating ITGB1 and reducing endothelial migration[181]. ZNF304, another regulator of ITGB1, has been shown to associate with the ITGB1 promoter to prevent anoikis in ovarian cancer[182]. Likewise, ITGB2 is also known to play a role in modifying endothelial invasion and matrix remodeling during angiogenesis[183,184]. JUP binds with CDH5 to form adherens junctions and promote adhesion between endothelial cells[185]. FGFR1 signaling is highly expressed during collateral artery growth and leads to increased phosphorylation and activation of SHC1[186,187]. SHC1 is a downstream signaling molecule of the angiopoietin receptor TEK/TIE2 where it plays a role in collateral remodeling and arteriogenesis[188,189]. Antithetically, ANGPT4 functions as a disruptor of CDH5 and claudin-5 vascular junctions, thereby increasing vascular permeability[190]. Interestingly, RAP1A plays a critical role in attaining mature endothelial junctional barrier function[191,192]. EGR3 has primarily been studied in lymphocyte and neuromuscular development; however, EGR3 is also a critical mediator of the endothelial

response to VEGF[193]. The combination of these factors are associated with the downregulation of vascular permeability and endothelial migration except for in the case of ANGPT4. Overall, $CD31^{+}CD14^{-}$ cells are likely modulators of vascular maturation and ECM remodeling.

$CD31^{+}CD14^{-}$ cells promote cell survival while $CD31^{+}CD14^{+}$ cells promote immune function

We sought to determine the ontological expression signature of DE genes between PB-$CD31^{+}CD14^{-}$ and PB-$CD31^{+}CD14^{+}$ cells using the (PANTHER) classification system. The PANTHER system tests statistical overrepresentation by comparing the representation of a functional set of genes within the list of DE genes and comparing that representation to a reference list. Using PANTHER, we analyzed DE genes for significant gene ontological and pathway function (Figure 14). DE genes from the PB-$CD31^{+}CD14^{-}$

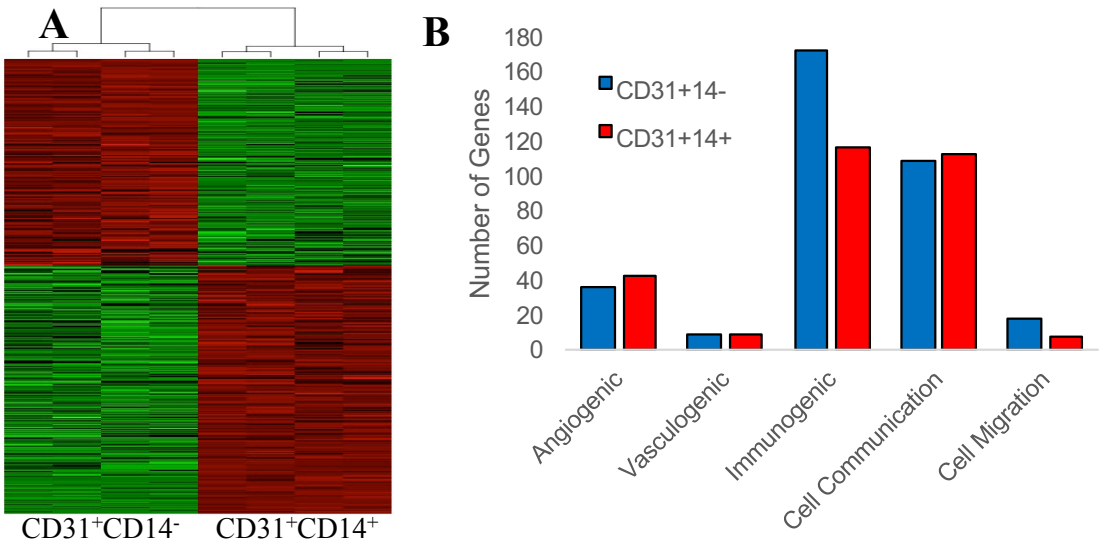

Figure 13. Differential gene expression. (A) Heatmap of differentially expressed genes. (B) Quantification of genes significantly upregulated by ontological function.

Table 2. Top 50 differentially expressed genes between PB-CD31^{+}CD14^{-} and PB-CD31^{+}CD14^{+} cells and their corresponding ontological groupings.

CD31+14+					CD31+14-			
Gene Name	p-value	Gene Name	p-value		Gene Name	p-value	Gene Name	p-value
NCF2	1.4531E-09	TFEB	4.7362E-06		BTN3A2	4.1521E-10	ADARB1	1.7097E-06
CTSS	1.2003E-08	ARFIP1	6.5687E-06		BMPR2	6.6684E-09	SLC11A1	2.0274E-06
NOD2	6.5708E-08	PIP5K1C	7.251E-06		PDE3B	1.12E-07	HIST1H2BC	2.0841E-06
DEFB134	1.1462E-07	STX7	7.3966E-06		GBP2	1.136E-07	SLC12A6	2.3468E-06
ARVCF	1.2944E-07	COL4A3BP	8.3449E-06		TNFRSF1A	1.7083E-07	SATB1	2.6786E-06
STX12	2.2883E-07	CSK	8.7199E-06		ARHGAP6	1.7344E-07	RAP1A	3.2137E-06
PCDHB10	2.4337E-07	TNFRSF10D	9.3055E-06		ROBO4	1.7705E-07	FGFR1	4.5253E-06
CD3D	6.3146E-07	SIRPB1	1.0438E-05		PCDHA3	2.3116E-07	IFITM1	5.8766E-06
FLT4	7.0613E-07	CD83	1.1657E-05		ACTN2	2.5376E-07	MYLPF	7.0688E-06
LY86	7.0778E-07	LYST	1.1692E-05		CLDN11	2.7649E-07	IL27RA	7.2967E-06
C5	7.3917E-07	PCDHB13	1.2063E-05		PML	3.2685E-07	PPP3CA	8.2207E-06
UNC5B	1.0863E-06	JAK3	1.6026E-05		ITGB2	3.3936E-07	EGR3	9.1148E-06
STAT5B	1.0983E-06	CCR2	1.623E-05		CARD11	4.1261E-07	PAX5	1.0287E-05
DOCK8	1.1666E-06	APOD	1.6251E-05		NLRP1	4.2619E-07	IL5RA	1.0425E-05
RC3H1	1.2756E-06	LILRA1	1.904E-05		JUP	5.0003E-07	SUSD2	1.0859E-05
VCL	2.3997E-06	PTGER4	2.3862E-05		NLRP6	5.4415E-07	OTUD7B	1.295E-05
TGFBI	2.4601E-06	CDH16	2.59E-05		SHC1	5.9059E-07	ATP6V1B1	1.3115E-05
CCR4	3.5392E-06	TNFSF9	2.6171E-05		IFNW1	6.1835E-07	HIST1H2BI	1.4254E-05
CLTA	3.6435E-06	PDE2A	2.6443E-05		ARF1	6.8735E-07	ELMO2	1.6378E-05
TICAM1	3.9504E-06	PDCD6	3.2558E-05		LILRB4	7.8271E-07	IRF4	1.7687E-05
OAS3	4.1231E-06	ICAM1	3.2683E-05		ITGA3	7.9931E-07	ZNF304	1.9375E-05
CMKLR1	4.441E-06	NCF1	3.3434E-05		ANGPT4	8.0327E-07	RPS6	2.0027E-05
BST1	4.4674E-06	PCDHB14	3.6369E-05		CDH5	1.0205E-06	CNTN4	2.05E-05
TNFSF10	4.6158E-06	TNFRSF10A	3.6381E-05		HIST1H2BG	1.2566E-06	CDHR2	2.18E-05
TRIL	4.7046E-06	CAMK2G	3.78E-05		RHOH	1.6625E-06	HIST1H2BE	2.68E-05

Immune Response
Cell-Cell Adhesion
Protein Secretion
Angiogenesis
Endothelial Differentiation
Cell-Matrix Adhesion

fraction were uniquely significantly associated with the following biological functions: cell motility (chemotaxis, cell migration, motility, and projection assembly, regulation of locomotion, taxis, negative regulation of cell-cell adhesion), cell survival (apoptotic signaling, negative regulation of both apoptotic process and programmed cell death), and humoral responses (cytokine-mediated signaling pathway, regulation of signal transduction) and an number of major signaling pathways (I-Kappa B/NF- Kappa B, JAK-STAT, and MAPK signaling cascades) (Figure 14A).

The strong representation of genes involved in various aspects of cell migration supports the notion that PB-CD31^{+}CD14^{-} cells induce chemotaxis and recruitment of various cell types. Furthermore, the strong representation of a number of cell survival genes indicate that PB-CD31^{+}CD14^{-} cells play an important role in the prevention of necrosis

and cell viability. The most insightful information is found in the signaling pathways associated with DE genes. The I-Kappa B/NF-Kappa B signaling pathway is a cyclical feedback signaling cascade where I-Kappa B is an inhibitor of NF-Kappa B DNA binding and NF-Kappa B aids in regulation of I-Kappa B expression[194]. This signaling pathway is known to be activated in smooth muscle cells after arterial injury where it induces expression of VCAM-1, MCP-1 and nitric oxide synthase[195]. VCAM-1 expression in endothelial cells is also highly regulated by NF-Kappa B expression[196]. JAK-STAT signaling is a critical signaling medium for a plethora of growth factors and cytokines[197]. The JAK-STAT pathway is responsible for multiple vascular repair processes including MMP secretion in SMCs[198] and VEGF and VCAM upregulation in endothelial cells[199]. Furthermore, IL-6 and IFNG are well-known STAT activators[200]. The MAPK signaling cascade is critically involved in various complex cellular processes including proliferation, differentiation, transformation and apoptosis[201]. MAPKs are also regulators of the JAK-STAT pathway due to their role in the phosphorylation and regulation of various STAT proteins.

Notable ontological functions associated with DE genes upregulated in PB-$CD31^{+}CD14^{+}$ cells include programmed cell death (T-cell differentiation, extrinsic apoptotic signaling and positive regulation of apoptotic process) and protein production (positive regulation of macromolecule biosynthetic process and protein metabolic process and regulation of cytokine biosynthetic process) (Figure 14B). Interestingly, the significant expression of pro-apoptotic genes in PB-$CD31^{+}CD14^{+}$ cells directly counteracts the anti-

apoptotic expression signature found in significantly expressed genes from PB-CD31^{+}CD14^{-} cells.

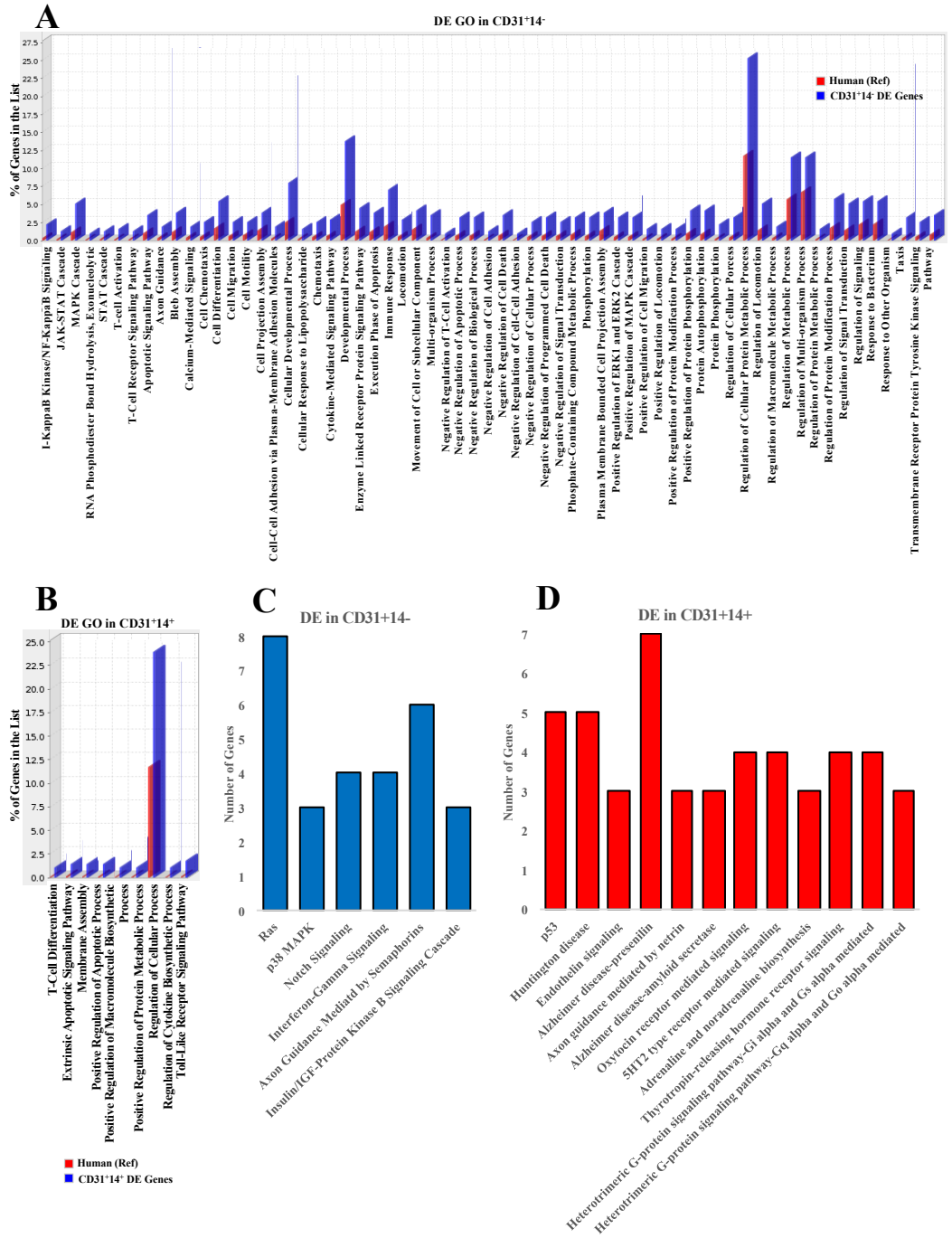

Figure 14. Gene ontology of differentially expressed genes between CD31^{+}CD14^{-} and CD31^{+}CD14^{+} cell fractions. Gene ontology set representation for DE genes in CD31^{+}CD14^{-} (A) and CD31^{+}CD14^{+} (B) fractions. Biological pathways associated with DE Genes in CD31^{+}CD14^{-} (C) and CD31^{+}CD14^{+} (D) fractions. DE = differentially expressed. GO = gene ontology.

$CD31^{+}CD14^{-}$ cells promote cell proliferation while $CD31^{+}CD14^{+}$ cells initiate stress response signalling pathways

In order to further determine likely mechanism that differentiate PB-$CD31^{+}CD14^{-}$ cells from PB-$CD31^{+}$ $CD14^{+}$ cells, we examined DE genes for significant involvement in major pathways and signaling cascades. Our results show that PB-$CD31^{+}CD14^{-}$ cells are strongly associated with activity in the Ras, p38 MAPK, IGF signaling pathways (Figure 14C). These interconnected pathways initiate and regulate various cell processes involved in cell proliferation, migration and metabolism. Ras is a key regulator of cell growth and proliferation. Ras activates a series of Raf, MAPK and ERK kinases which, upon activation, phosphorylate cytoplasmic targets that stimulate various transcription factors[202]. Interestingly, Ras signaling can promote both cell death and cell survival through c-Myc and PI3K, respectively[203]. Upregulation of p38-MAPK signaling is required for the initiation of apoptosis[204]. The genes associated with p38-MAPK signaling that are upregulated in PB-$CD31^{+}CD14^{-}$ cells are MAP3K5, SRF and MEF2C. MAP3K is an activator of p38 signaling and therefore apoptosis; however, SRF and MEF2C are suppressors of apoptosis signaling[205-208]. IGF signaling plays a role in both the upregulation and inhibition of apoptosis[209,210]; however, IGF-kinase B signaling also has the effect of stimulating cell proliferation[211,212]. These data are support the notion that PB-$CD31^{+}CD14^{-}$ cells induce cell proliferation and regulate apoptosis.

Noteworthy pathways associated with genes differentially expressed in the PB-$CD31^{+}CD14^{+}$ cells are the netrin mediate axon guidance, oxytocin receptor signaling, 5HT2 type receptor signaling and association with Huntington and Alzheimer disease pathways (Figure 14D). These pathways are all associated with neuronal development,

function or disease. This is an interesting result as it implies that PB-CD31^{+}CD14^{+} cells may hold some potential in the improvement of some neurodegenerative disorders. Other pathways implicated by DE genes in PB-CD31^{+}CD14^{+} cells are p53, endothelin signaling, adrenalin and noradrenalin biosynthesis and heterotrimeric G-protein signaling pathways. The p53 pathway is most researched for its role in tumor suppression; however, p53 also has functions in the initiation of apoptosis and stress mediated cell cycle arrest[213,214]. Endothelin signaling plays various roles in the vascular system. Among the most important of these functions is endothelin mediated vasoconstriction and vasodilation[215]. Adrenaline and noradrenaline are stress response hormones capable of inducing vasoconstriction and counteracting hypotension at low doses[216]. Heterotrimeric G-protein signaling is upstream of VEGF receptor-2 phosphorylation in VEGF signaling[217]. Heterotrimeric G-protein signaling is also a modulator of vascular SMC contraction[218]. The given representation of signaling pathways implies that PB-CD31^{+}CD14^{+} cells function through the regulation of apoptosis, vasoconstriction and endothelial proliferation in response to stress.

It is important to note that there are a number of gene ontology groupings and signaling pathways represented by DE genes from both PB-CD31^{+}CD14^{-} and PB-CD31^{+}CD14^{+} cells. Among shared significant GO functions, PB-CD31^{+}CD14^{+} cells displayed notably higher representation of cell adhesion, cell activation and biological adhesion (Figure 15A). The higher representation of these GO groups are likely tied to the ability of PB-CD31^{+}CD14^{+} cells to recruit inflammatory cells. Interestingly, while both cell fractions show involvement in neovasculogenesis pathways, there are a greater number of DE gene for PB-CD31^{+}CD14^{-} cells involved in angiogenesis, FGF and PDGF signaling

pathways (Figure 15B). tConversely, a greater number of Wnt and cadherin signaling genes were found from the PB-CD31^{+}CD14^{+} cell fraction (Figure 15B).

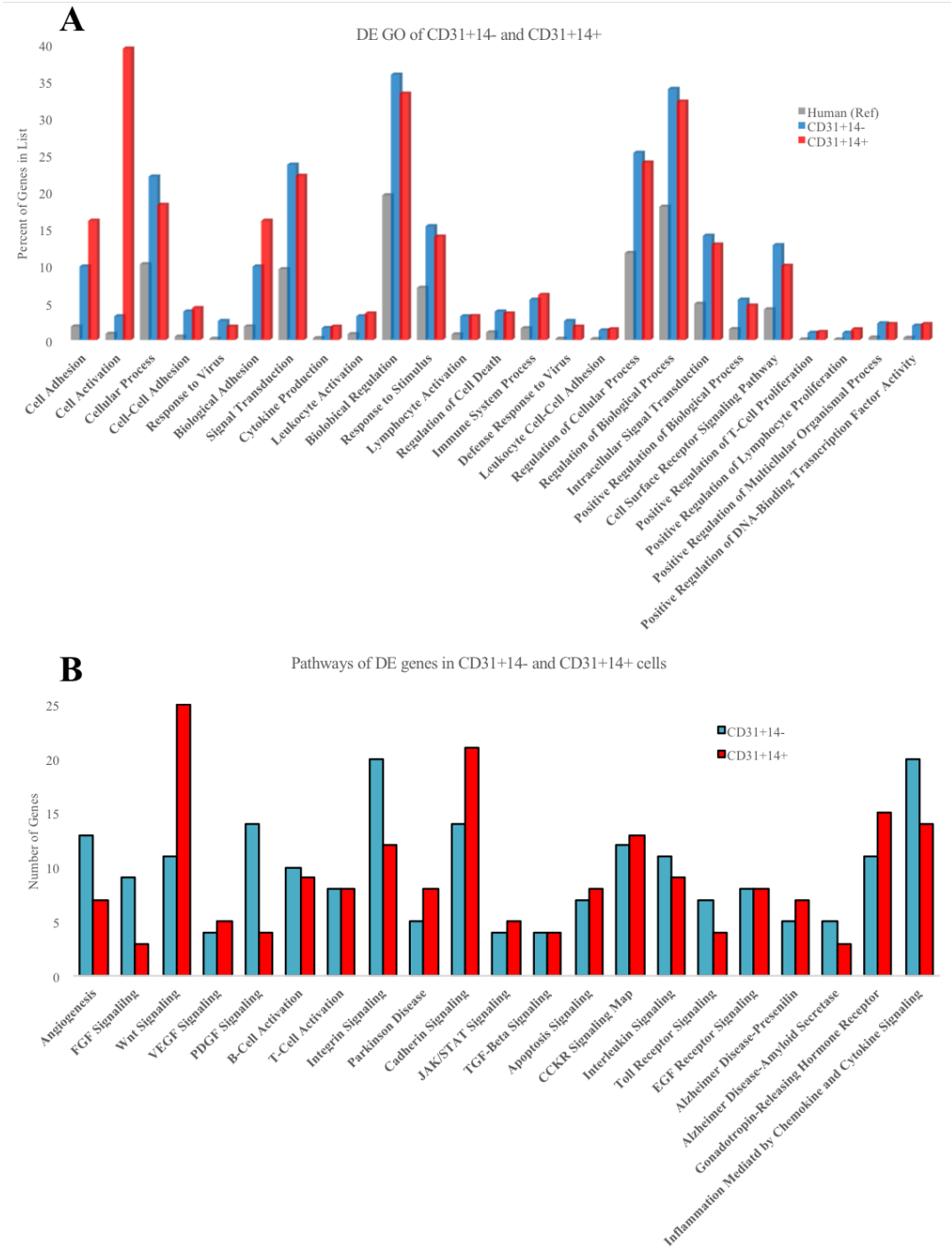

Figure 15. Gene Ontology and Pathway association of differentially expressed genes in PB-CD31^{+}14^{-} and PB-CD31^{+}14^{-} cells. DE = differentially expressed. GO = gene ontology.

Predetermined gene sets for angiogenesis, artery development, vessel remodeling, inflammatory response and ECM disassembly were used to determine enrichment. Angiogenic genes show favored expression in the $CD31^{+}CD14^{+}$ fraction while artery development, inflammatory and ECM and vascular remodeling genes are more enriched in $CD31^{+}CD14^{-}$ cells. None of the gene sets were enriched enough to show significance (Figure 16).

Paracrine factors from PB-$CD31^{+}CD14^{+}$ cells increase endothelial function.

In order to evaluate the paracrine effects of the various PB-MNC subpopulations, we harvested conditioned medium. An important component of angiogenesis is the migration of endothelial cells toward the ischemic region and the formation of collateral vessels. We used an in vitro wound healing assay to determine the impact of conditioned medium on endothelial migration. Wounds through a monolayer of confluent HUVECs were observed over the course of 12 hrs. Wound closure was calculated as the ratio of the

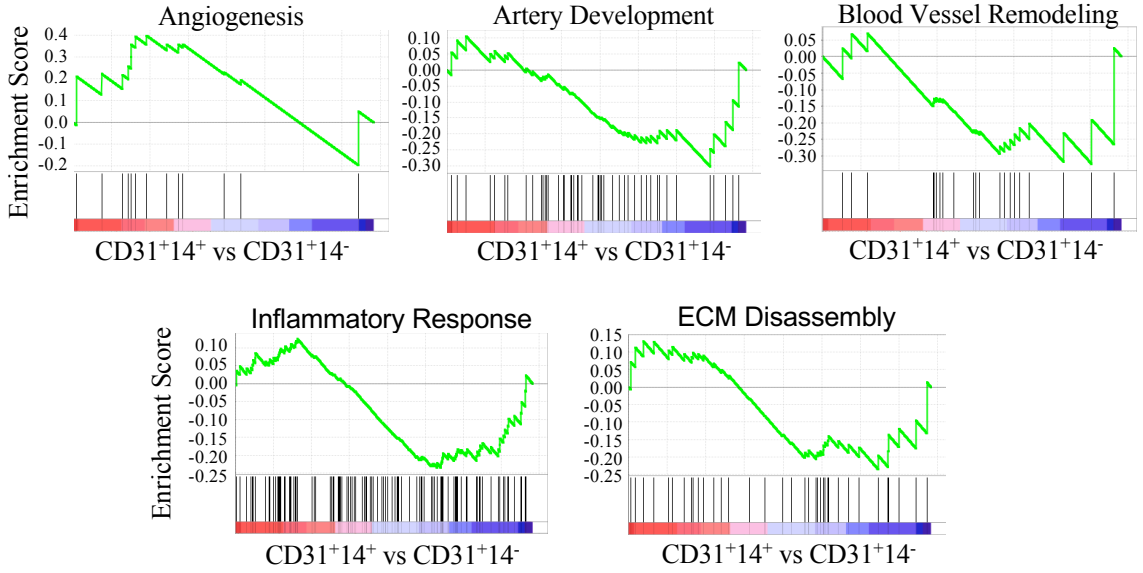

Figure 16. GSEA analysis comparing PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}CD14^{-}$ cell populations.

remaining gap width at 12 hrs and the original gap at 0 hrs (Figure 17A). After statistical analysis, the Fischer LSD groupings show that ECs treated with conditioned medium from $CD31^+CD14^+$ cells do not share a group with either the 0.5% EBM or the $CD31^-$ conditioned medium treated ECs. These results demonstrate that conditioned medium from PB-$CD31^+CD14^+$ cells significantly increased wound closure and endothelial migration when compared to $CD31^-$ CM and negative controls (Figure 17B).

An endothelial tube formation assay was then used to assess the effects of conditioned medium on endothelial function. Fischer LSD groupings show that PB-$CD31^+CD14^+$ CM-treated ECs do not share a grouping with 0.5% EBM CM treated and $CD31^-$ CM treated ECs. Therefore, CM from PB-$CD31^+CD14^+$ cells significantly increased endothelial tube formation compared to the $CD31^-$ CM and negative controls. (Figure 18A and Figure 18B). These data confirm the angiogenic function of PB-$CD31^+CD14^+$ cells.

Discussion

In this chapter CD14 was investigated as a selection marker for the isolation and enrichment of highly angiogenic cells from PB-$CD31^+$ cells. The purpose for this enrichment is to increase the potency and efficacy of cell therapy for critical limb ischemia which has historically underperformed either due to a minuscule source population, selection through in vitro culture methods corresponding to a lack of defined phenotype or the presence of ineffectual cell types[90,91]. To address these concerns, we hypothesized that the $CD14^+$ fraction of $CD31^+$ cells will retain the angiogenic phenotype. Our flow cytometry results indicate that the PB-$CD31^+CD14^+$ cells contain a significantly higher

portion of monocytes, dendritic cells (DC) and hematopoietic stem and progenitor cells. Similarly, we determined through FACS analysis that 70% of $CD34^+$ cells and 77% of $CD133^+$ cells are also positive for CD14. DCs are the inducers of immunity and hold the major function of distinguishing self from foreign[219]. In particular, the dendritic cell population is known to activate CD4 and CD8 T-cells during response to injury[220]. The

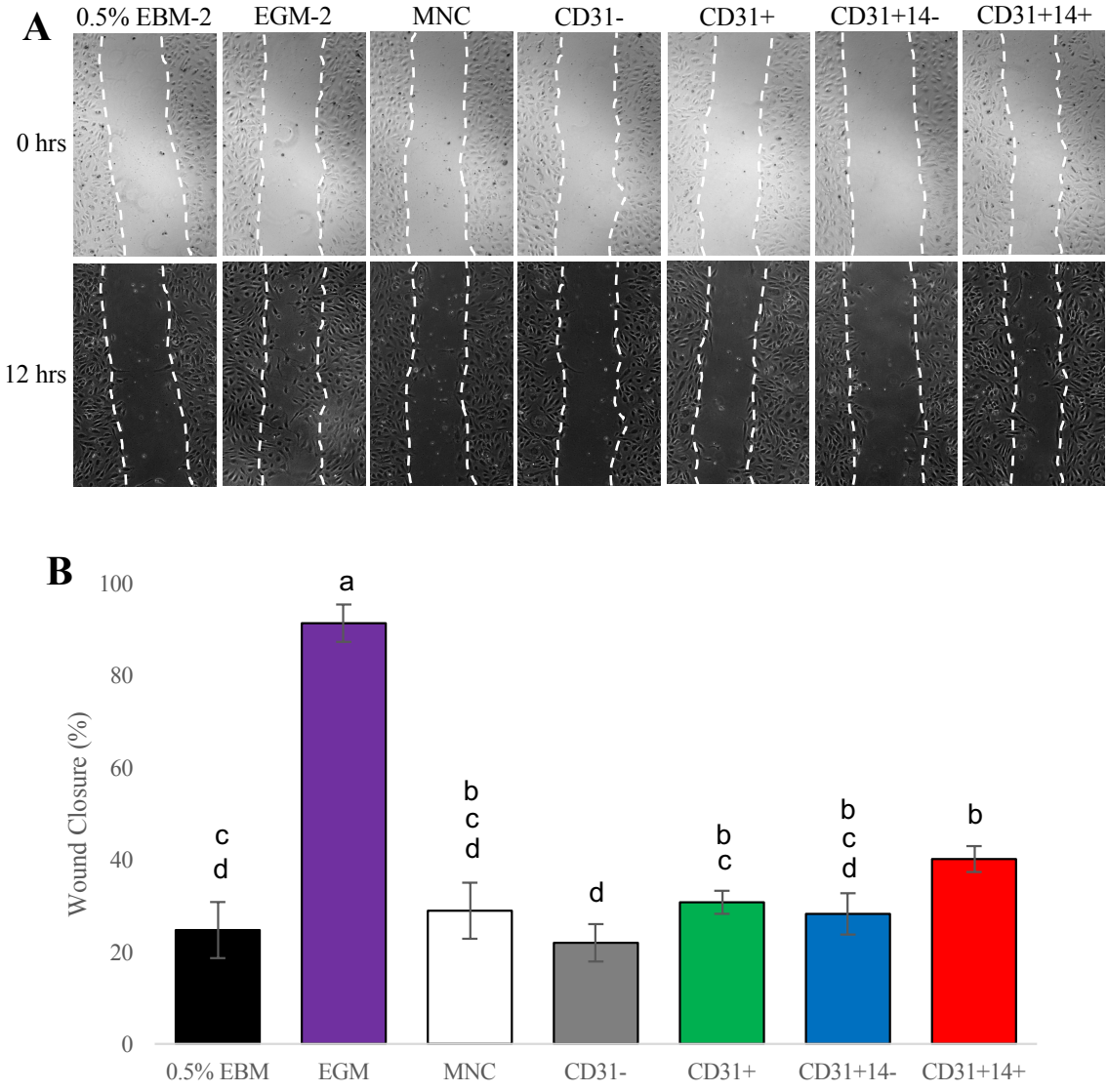

Figure 17. Progression of endothelial migration across a scratch wound in conditioned medium. (A) Representative images of endothelial migration into wound area over 12 hrs. White dotted lines indicate the wound borders at the beginning of the assay. (B) Quantification of wound closure after 12 hrs. a, b, c and d are the Fischer LSD groupings. n = 6.

enrichment of dendritic markers implies some immunomodulatory function of the $CD31^{+}CD14^{+}$ population. CD34 and CD133 are both proposed isolation markers for circulating EPCs[87]. The association between CD14 and EPCs has been previously proposed

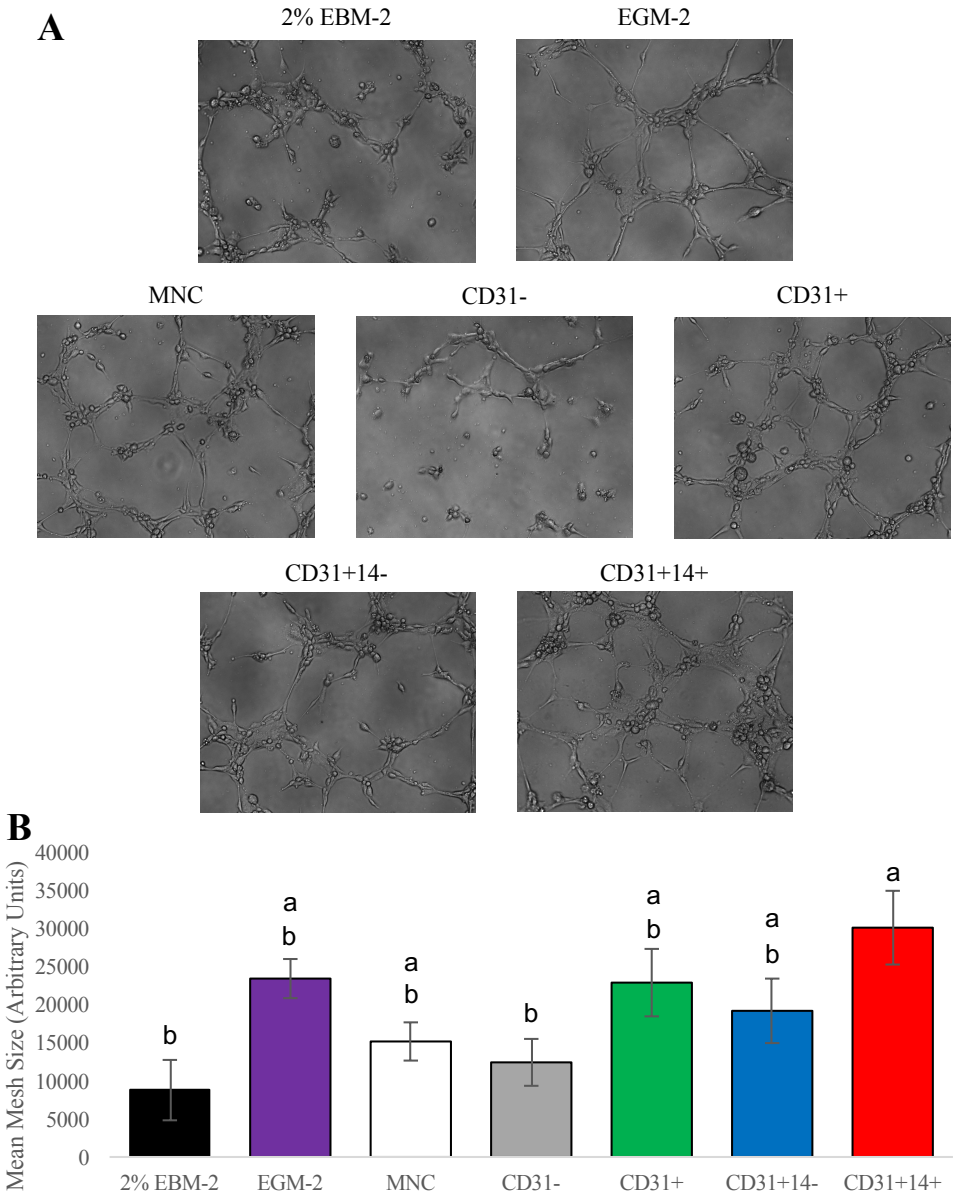

Figure 18. Effect of conditioned medium from PB cell fractions on endothelial tube formation. (A) Representative images of endothelial tube formation in conditioned medium from PB cells. (B) Analysis of endothelial tube formation in conditioned medium after 9 hrs of culture. a, b and c indicate Fischer LSD groupings. n = 6.

in the literature[132]. $CD14^+$ cells have been previously investigated for their expression of CD34 and used to treat hindlimb ischemia in mice[94,221]. Recently, $CD14^+CD34^{low}$ cells were used in a clinical trial with therapeutic benefits on par with MNC treatment[95].

Given the cellular composition observed in our isolated fractions, we sought to characterize their expression profiles using angiogenic growth factors and cytokines. While we did observe significantly higher expression of VEGFA and HGF in PB-$CD31^+CD14^+$ cells compared to PB-$CD31^+CD14^-$ cells, there was also notably high expression of angiogenic factors VEGFA, HGF, ANGPT1 and CXCL8 in the PB-$CD31^+$ population. VEGFA is the most potent and well characterized angiogenic factor. Due to is ubiquitous presence in the cancer and tumor field, VEGF has gained notoriety for inducing vascular growth to the point of pathology where vessels are immature and leaky[222]. VEGF promotes the sprouting of blood vessels and stimulates vasculogenesis through the recruitment of precursor cells[223,224]. CXCL8 both recruits monocytes to the vasculature and stimulates VEGF production[225,226]. $CD34^+$ progenitor cells have also been shown to be responsive to the chemotactic effects of CXCL8[227]. Comparing the expression of PB-$CD31^+$ cells to that of PB-$CD31^+CD14^+$ cells using the factors we have chosen, we must conclude that while the PB-$CD31^+CD14^+$ cells do indeed express an angiogenic profile similar to that of PB-$CD31^+$ cells, they are not more angiogenic than the parent $CD31^+$ population. This conclusion correlates with the data shown in the PCA plot and heatmap of angiogenic and inflammatory genes. The dendogram from the heatmap clearly demonstrates the resemblance between the PB-$CD31^+$ cell population and the PB-$CD31^+CD14^+$ cell populations. Furthermore, the combination of differentially expressed genes in the PB-

$CD31^+CD14^+$ cell imply an angiogenic phenotype. In particular, FLT4, TGFB, and ICAM1 are well documented pro-angiogenic proteins.

CCL2, a chemokine involved in leukocyte recruitment, matrix remodeling and arteriogenesis was more highly expressed in PB-$CD31^+$ cells and PB-$CD31^+CD14^+$ cells; however, this difference was not significant. Matrix remodeling protein MMP9 also showed high expression in PB-$CD31^+$ cells without significance. Interestingly, the growth factor PDGFB and matrix remodeling enzyme MMP2 were significantly upregulated in PB-$CD31^+CD14^-$ cells. FGF2 was also more highly expressed in PB-$CD31^+CD14^-$ cells compared to PB-$CD31^+CD14^+$ cells although this difference was not significant. In the literature, these factors are commonly categorized as angiogenic factors due to their known role in vascular growth and repair. Upon a more detailed review, the role of these factors is to increase vascular conductance through the recruitment of mural cells rather than increasing vascular growth. Secreted PDGFB creates a gradient that induces pericyte coverage of endothelial cells while FGF2 is a strong inducer of SMC migration[228,229]. FGF2 and PDGFB work together to maintain vascular maturation and stability and promote arteriogenesis[230-232]. Furthermore, MMP2 is a protein commonly used in the literature to determine arteriogenic activity due to its involvement in vascular remodeling[233,234].

Among inflammatory cytokines, PB- $CD31^+CD14^-$ cells appeared to have higher expression of inflammatory marker IFNG whereas PB-$CD31^+CD14^+$ cells, similar to PB-$CD31^+$ cells, appeared to have higher expression of inflammatory cytokines TNF and IL10 although these differences were not significant. IFNG plays a critical role in immunological resistance to infection and bacteria[235]. Likewise, TNF functions to promote inflammation through stimulation of proinflammatory responses in capillary endothelial cells[236].

However, IL10 functions as a repressor of excessive inflammatory responses[237]. Expression of inflammatory factors was fairly mixed between all cell fractions. This mix of inflammatory marker expression carried over into the list of differentially expressed genes. The majority of genes expressed in both the PB-CD31^{+}CD14^{-} and PB-CD31^{+}CD14^{+} cell populations were inflammatory and had functions involved in recruitment, migration and activation of various leukocyte lineages. However, this outcome is expected as each of the cell populations are composed of various combinations and representations of leukocytes.

A number of genes significantly differentially expressed in PB-CD31^{+}CD14^{-} cells are also heavily involved in regulating the strength of endothelial cell junctions. PDE3B, ROBO4, RAPGEF3, JUP, CDH5, and RAP1A all function to strengthen tight junctions or adherens junctions between endothelial cells. ANGPT4 was the only expressed gene with a known function in the loosening of vascular junctions. Interestingly, a couple of significantly differentially expressed genes in PB-CD31^{+}CD14^{-} cells, specifically PML, ITGB1, ZNF304 and EGR3, actively disrupt, inhibit or reduce angiogenesis and endothelial migration. The strengthening of endothelial bonds and reduction of vascular permeability is a major component of arteriogenic remodeling. The conclusion of RT-PCR and RNA-Seq data strongly imply a pro-arteriogenic and vascular maturing phenotype of PB-CD31^{+}CD14^{-} cells.

While there were fewer significant unique ontological functions associated with DE genes from PB-CD31^{+}CD14^{+} cells, there was a trend toward the initiation and regulation of programmed cell death. This feature was opposite of the apoptosis suppression ontological function associated with DE genes from PB-CD31^{+}CD14^{-} cells. Other

significant functions of PB-CD31^{+}CD14^{-} cells include the encouragement and promotion of cell migration, proliferation and cytokine signaling. These functions are supported by the pathway expression associated with DE genes. Interestingly, the representation of adrenaline, p53 and heterotrimeric G-protein signaling in PB-CD31^{+}CD14^{+} cells shows that these cells are perhaps an effective population for the local management of stress during an ischemic event. There is also a curiously high representation of axonal guidance signaling pathways associated with PB-CD31^{+}CD14^{+} cells; however, the extent of this association unclear and outside the scope of this thesis.

Our quantitative expression data converge on the theory that PB-CD31^{+}CD14^{+} cells are angiogenic, inducing endothelial migration and proliferation, while PB-CD31^{+}CD14^{-} cells are arteriogenic, functioning to increase matrix modulation and reduce vascular permeability. The results of the endothelial tube formation and migration assays further support this premise in that PB-CD31^{+}CD14^{+} cells secrete various growth factors that work to increase endothelial migration and angiogenic function. It was also shown that PB-CD31^{+}CD14^{-} and PB-CD31^{+}CD14^{+} play opposing roles in cell survival and programmed cell death, respectively. These opposing functions of both cell populations could contribute to a reduction of therapeutic efficacy when transplanted together.

CHAPTER 4 EVALUATION OF THE THERAPEUTIC POTENTIAL OF PERIPHERAL BLOOD MONONUCLEAR CELL FRACTIONS

Introduction

Critical limb ischemia (CLI), a state of unrelenting rest pain, is the most severe form of peripheral artery disease (PAD). With the current state of medical advancement, CLI has a 1-year amputation and mortality rate of 20% and 40%, respectively, for individuals without revascularization options[5-7]. Unfortunately, little is known about the optimal treatment of CLI[238,239]. Pharmacological treatment for PAD is often a complicated task due to the likelihood of PAD patients to also exhibit comorbid CVDs that would negatively respond to treatment[53]. Furthermore, endovascular interventions, including angioplasty and stenting, are often subject to fracture, restenosis and thrombosis[54]. An ideal treatment for CLI would involve restoration of ischemia through enhanced vessel formation and maturation. As a result, neovasculogenic cells have been investigated as alternative therapies for CLI.

Cell Therapy for CLI

Endothelial progenitor cells have been the most extensively investigated cell population for the treatment of CLI. Clinical trials using EPCs have been fairly successful, consistently showing statistically significant improvements in vascular perfusion, infarct reduction, and transcutaneous oxygen pressure ($TcPO_2$) levels in patients[96,97]. Unfortunately, vascular improvements attributable to EPC injection has been modest[98,99]. The underwhelming performance of EPCs in clinical trials can likely be attributed to a

reduction in the proliferative, migrational and functional capacity of EPCs associated with cardiovascular disease, aging and smoking[100-102]. Furthermore, the application of EPCs to therapeutic approaches is limited by their broad definition, and the lack of standardized methods for isolation and culture[90,91]. Mesenchymal Stem Cells (MSCs) have also been investigated as a cell therapy for CLI. MSCs, similar to EPCs, suffer from a lack of unique characterization markers[137]. Clinical trials of ischemic vascular disease using MNCs have demonstrated significant trends toward increased cardiac output, muscle mass, and wound healing; however, their therapeutic effects remain marginal[120-122]. Overall, while the use of adult stem cells, including EPCs and MSCs, in clinical trials has been shown to collectively decrease limb amputation and increase amputation free survival of critical limb ischemia patients, the effects are still marginal[120-123]. Many clinical trials using EPCs for cardiac and vascular repair have been performed using the parent population of bone marrow (BM) and peripheral blood (PB) derived mononuclear cells (MNC) under the premise that $CD34^+$ EPCs are a subpopulation of transplanted cells[92,93]. $CD34^+$ cells have also been demonstrated to be a subset of PB-$CD14^+$ cells. As a result, PB-$CD14^+$ cells have recently been investigated as a therapy for CLI[95]. The result of this study showed a clear therapeutic benefit to the addition of PB-$CD14^+$ cells; however, the therapeutic benefits were on par with those of PB-MNC treated patients. Multiple studies using PB-$CD14^+$ cells must be established before conclusions may be drawn.

In this study, we investigate the therapeutic efficacy of PB cells using in vivo mouse models of limb ischemia. Extrapolating from data found previously, we hypothesized that both the PB-$CD31^+CD14^+$ and PB-$CD31^+CD14^-$ populations would be therapeutically

beneficial toward cardiovascular repair. Our results demonstrate the potency of PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}CD14^{-}$ cells in limb reperfusion.

Methods

Isolation and culture of PB cells.

Human PB samples were collected from healthy male and female volunteers. Isolation of MNCs was performed using a modification of a previously reported method[147]. Briefly, each PB sample was diluted with one volume of calcium- and magnesium-free phosphate-buffered saline (PBS) containing 2% bovine serum albumin (Miltenyi Biotec, Auburn, California), layered on one volume of Histopaque-1077 (Sigma, St. Louis, Missouri), and centrifuged at 400 g for 35 min. The MNCs were harvested from the interface and washed with magnetic-activated cell sorting (MACS) buffer (Miltenyi Biotec). MNCs were centrifuged again at 400 g for 10 min to remove platelets. The cells were counted and resuspended in MACS buffer blocking reagent. Human CD14 microbeads (Miltenyi Biotec) were sequentially added and incubated at 4°C for 20 min. The cells were washed and resuspended in MACS buffer. $CD14^{+}$ and $CD14^{-}$ cells were isolated using magnetic LS columns (Miltenyi Biotec) according to the manufacturer's instructions. $CD14^{-}$ cells were then washed with MACS buffer and incubated with Human CD31 microbeads (Miltenyi Biotec) at 4°C for 20 min. $CD31^{+}CD14^{-}$ and $CD31^{-}$ cells were isolated using magnetic LS columns (Miltenyi Biotec) according to the manufacturer's instructions.

Hindlimb ischemia surgery and cell injection

Athymic BALB/c nude mice (Charles River, Wilmington, MA) were anesthetized with 1-3% isoflurane. Two ligations were made to the proximal region of the femoral artery near the groin. Two further ligations were made to the distal region of the femoral artery near the knee (Figure 19A). The femoral artery was removed beginning with at the distal region between the ligations and concluded between the proximal ligations (Figure 19B and Figure 19C). 1 x 10^6 CM-DiI labeled PB-CD31$^+$CD14$^+$, PB-CD31$^+$CD14$^-$, PB-CD31$^+$, PB-CD31$^-$ or PB-MNCs in 100 µl PBS were intramuscularly injected into the ischemic hindlimb of mice to compare their effects. 100 µl PBS was used as a negative/sham control.

Blood flow measurement in ischemic hindlimbs

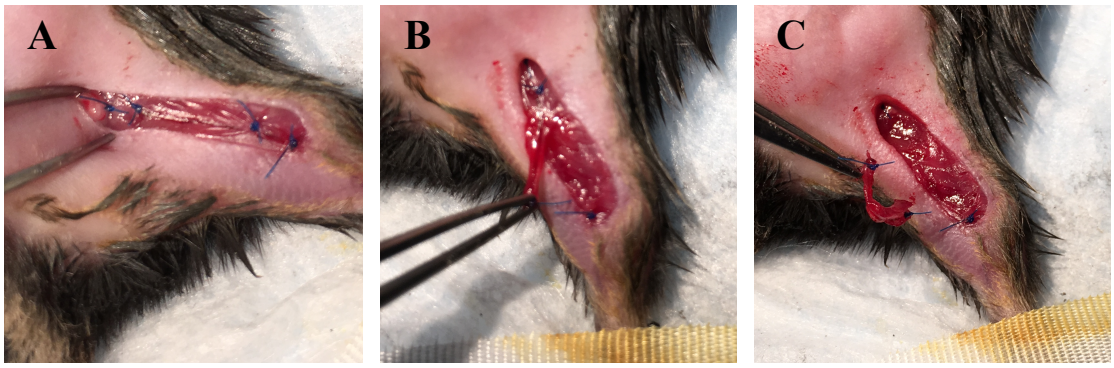

Figure 19. Procedural removal of the femoral artery.

Blood flow of the hindlimb was measured using a Laser Doppler perfusion imager (LDPI, Moor instrument, UK) at days 3, 7, 14 and 21. Mean values of perfusion were calculated from the stored digital color-coded images. The level of blood flow of the ischemic (left) limb was normalized to that of the non-ischemic (right) limb to avoid data variations caused by ambient light and temperature. Mice with > 50% perfusion recovery at day 3 were considered failed surgical procedures and removed from the study. Outliers were determined as LDPI readings that were greater than 2.2 times the interquartile range above the third quartile or below the first quartile. Given the need for statistical rigor in the

evaluation of therapeutic treatment and the high patient-to-patient variability that is typical with the LDPI assay, we determined ANOVA unsuitable as a statistical method for evaluating LDPI data.

Histological analysis: capillary density

7 , 21 and 28 days after the injection of cells into ischemic hindlimbs the adductor muscles were removed, fixed with 4% paraformaldehyde at 4°C overnight, snap frozen in liquid nitrogen and sectioned at 15 μm. To visualize capillaries, the sections were stained using FITC conjugated isolectin B4 and DAPI. The stained specimens were visualized under conventional inverted epifluorescence microscopy and the capillary density was calculated from at least 10 randomly selected fields.

Histologic analysis: confocal microscopy

Before euthanasia, mice were intravenously injected with FITC-conjugated isolectin B4 (Vector Laboratories). After 10 minutes, mice were euthanized using cervical dislocation. The hindlimb tissue was removed, fixed in 4% paraformaldehyde at 4°C for 16 hours, and incubated in 30% sucrose solution for 24 hours. Tissues were snap frozen in liquid nitrogen and imaged at 15 or 50 μm thickness. Nuclei were stained with DAPI (Invitrogen). ECs were identified by positive staining for isolectin B4 and their characteristic morphology within the vascular structure, and transplanted cells were identified by CM-DiI. For arteriogenesis assays, smooth muscle cells were stained with rabbit anti-alpha smooth muscle actin primary antibody (Abcam, Cambridge, United Kingdom) and Alexa fluor 647 goat anti-rabbit secondary antibody (Invitrogen). Incorporation of transplanted cells into the vasculature as ECs was determined using a

Zeiss LSM 510 Meta confocal laser scanning microscope and LSM 510 Image software (Carl Zeiss, Jena, Germany).

Statistical Analysis

All data are represented as means ± S.E.M and statistical analysis was conducted in R (Figure 26 and Figure 27). Krusker-Wallis one-way analysis of variance followed by pairwise Mann-Whitney U post hoc analysis was used to analyze LDPI data. Other data was analyzed using ANOVA followed by multiple comparison with Tukey's method for variables among more than 2 groups. A p value < 0.05 was considered statistically significant.

Results

PB-CD31$^+$CD14$^+$ and PB-CD31$^+$CD14$^-$ cells both increase limb perfusion.

In order to determine the therapeutic potential of PB- CD31$^+$CD14$^{+/-}$ cells, we treated the ischemic hindlimbs of BALB/c nude mice with each isolated cell fraction. By day 14, hindlimbs treated with PB-CD31$^+$ or PB-CD31$^+$CD14$^+$ cells had significantly higher perfusion compared to PBS, MNC and PB-CD31$^-$ cell treated hindlimbs. PB-CD31$^+$CD14$^-$ cell treated hindlimbs exhibited significantly more perfusion compared to PBS treated hindlimbs by day 14. After 21 days, both PB-CD31$^+$CD14$^-$ and PB-CD31$^+$CD14$^+$ cell treated hindlimbs showed significantly more perfusion compared to PBS, MNC, PB-CD31$^-$ and PB-CD31$^+$ cell treated hindlimbs. MNC, PB-CD31$^-$ and PB-CD31$^+$ cell treated hindlimbs were all significantly more perfused compared to PBS treated hindlimbs.

Interestingly, both the PB-CD31^{+}CD14^{-} and PB-CD31^{+}CD14^{+} cell treated hindlimbs were more effective at inducing limb perfusion than the parent PB-CD31^{+} cell treated hindlimbs by day 21 (Figure 20).

PB-CD31^{+}CD14^{+} cells increase collateral vessel growth

In order to determine if increased limb perfusion was due to increased collateral vessel growth, we performed a capillary density assay. Our results show that day 7 hindlimbs treated with PB-CD31^{+}CD14^{+} had significantly increased capillary density

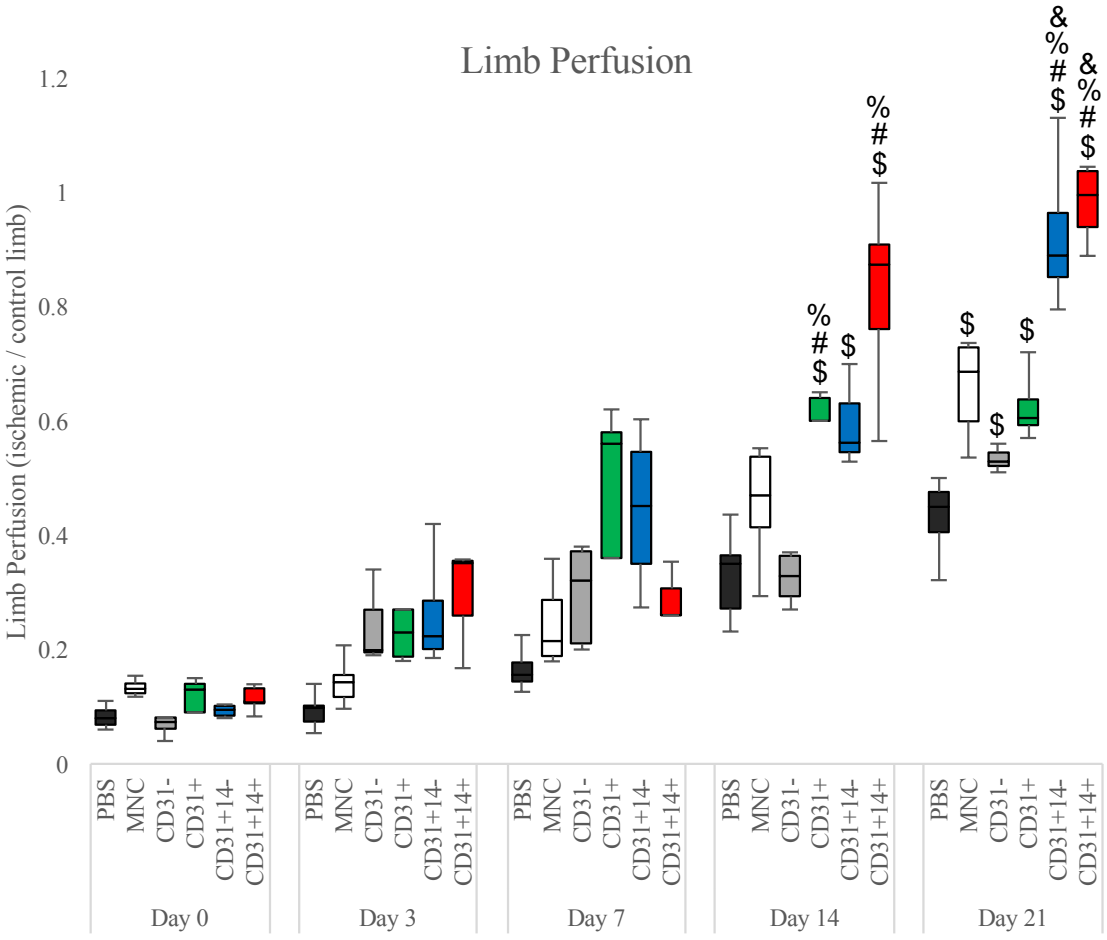

Figure 20. Limb perfusion in ischemic hindlimbs. Within each time point: $ = significance to PBS, # = significance to CD31^{-}, % = significance to MNC, & = significance to CD31^{+}. n = 6 - 15.

compared to PBS, MNC and $CD31^{+}CD14^{-}$ treatments. Interestingly, the increased capillary density observed in the PB-MNC treated limbs was completely ablated in the PB-$CD31^{+}CD14^{-}$ treated hindlimbs (Figure 21A and B). In order to determine if the observed difference in capillary density formation would persist, we performed a capillary density assay at day 28. We observed that not only had the total number of capillaries for each

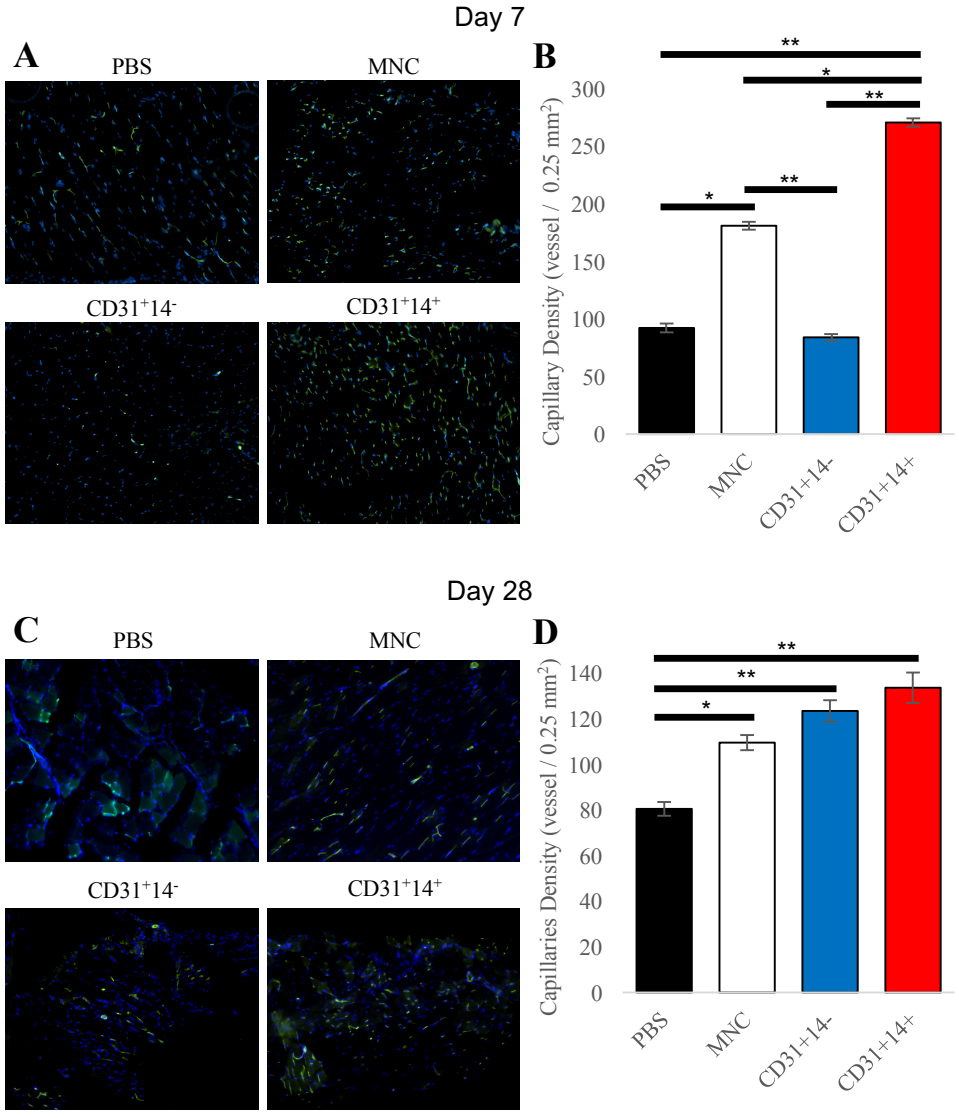

Figure 21. Capillary density in mouse ischemic hindlimbs. (A) Representative images of capillary density at day 7. (B) quantification of capillary density images at day 7. (A) Representative images of capillary density at day 28. (B) quantification of capillary density images at day 28. * $p < 0.05$, ** $p < 0.01$. n = 3.

group been reduced compared to those observed at day 7, but that there was also no longer any significance between the MNC, PB-CD31$^+$CD14$^-$ and PB-CD31$^+$CD14$^+$ cell treated hindlimbs. However, the capillary density in PBS treated hindlimbs remained at significantly lower levels compared to hindlimbs treated with cells (Figure 21C and D).

PB-CD31$^+$CD14$^-$ cells increase arteriole formation and encourage vessel muscularization

Given the expression patterns observed in the previous aim and effectiveness of PB-CD31$^+$CD14$^+$ cells at increasing limb perfusion, we hypothesized that PB-CD31$^+$CD14$^-$ cells must be increasing arteriogenesis in the hindlimbs. In order to measure the effect of arteriogenesis, we stained hindlimbs for alpha smooth muscle actin (aSMA). aSMA is a marker for smooth muscle cells. If arteriogenesis has been induced, there will be a difference in the number of vessels lined with aSMA$^+$ cells. Histological analysis of hindlimb tissue harvested at day 28 shows that tissues treated with PB-CD31$^+$CD14$^-$ cells had significantly increased vascular muscularization compared to PBS, MNC and PB-CD31$^+$CD14$^+$ cell treated hindlimbs. These data confirm the pro-arteriogenic nature of PB-CD31$^+$CD14$^-$ cells.

PB-CD31$^+$CD14$^+$ cells display limited vasculogenic potential

4-week old hindlimbs were imaged to determine cellular engraftment and examine vascular structure. Confocal imaging reveals that PB-CD31$^+$CD14$^+$ cells have limited potential for endothelial differentiation and incorporation into the host vasculature (Figure 23). This supports the hypothesis that circulating progenitor cells are a subset of the PB-CD31$^+$CD14$^+$ cell population. No such vascular integration was found in PB-CD31$^+$CD14$^-$ cell treated hindlimbs; however, engrafted PB-CD31$^+$CD14$^-$ cells were often found nestled

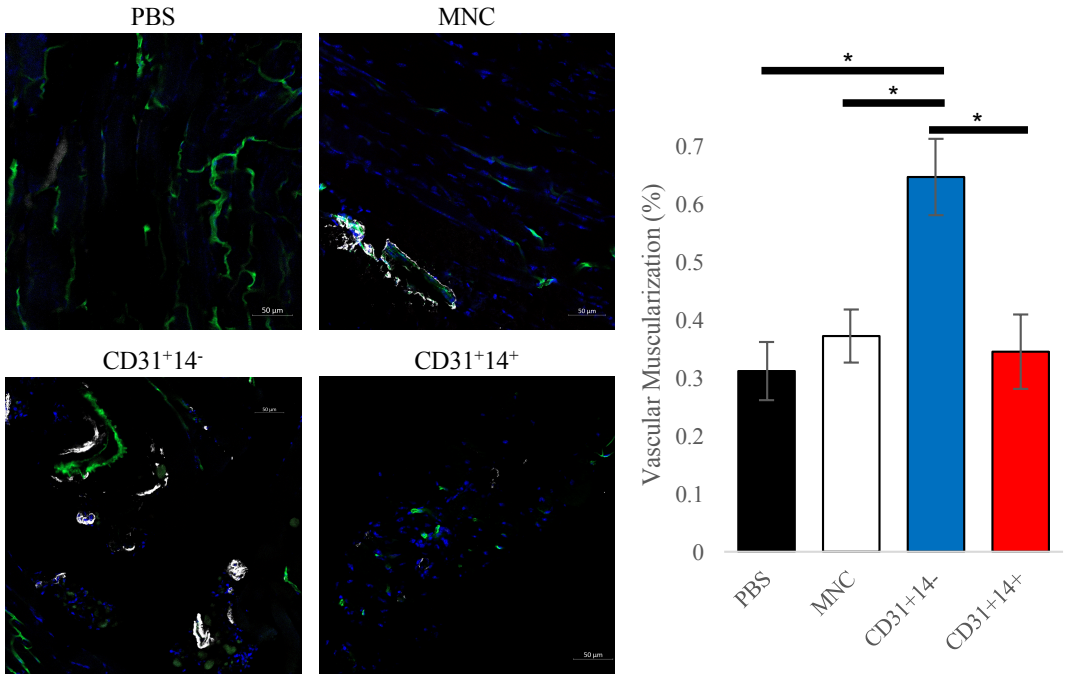

Figure 22. Histological analysis of vascular muscularization. White – aSMA, Green – lectin, Blue – DAPI. Data represented as mean ± SEM. * $p < 0.05$. n = 3.

against lectin perfused vessels (Figure 24). It is possible that PB-CD31$^+$CD14$^-$ cells may position themselves perivascularly in order to direct vascular ECM remodeling as has been implicated in their expression profiles.

Discussion

We used an athymic BALB/c nude hindlimb ischemia mouse model to determine the therapeutic efficacy of PB-CD31$^+$CD14$^+$ and PB-CD31$^+$CD14$^-$ cells. Our perfusion data showed that PB-CD31$^+$, PB-CD31$^+$CD14$^-$ and PB-CD31$^+$CD14$^+$ cell treated hindlimbs were capable of increasing limb perfusion at day 14. This support previous literature showing that PB-CD31$^+$ cells are angiogenic and are effective at rapidly increasing limb perfusion in mouse models of limb ischemia[10,11,124]. At day 21 we observed a significant increase of limb perfusion in both PB-CD31$^+$CD14$^+$ and PB-CD31$^+$CD14$^-$ cell

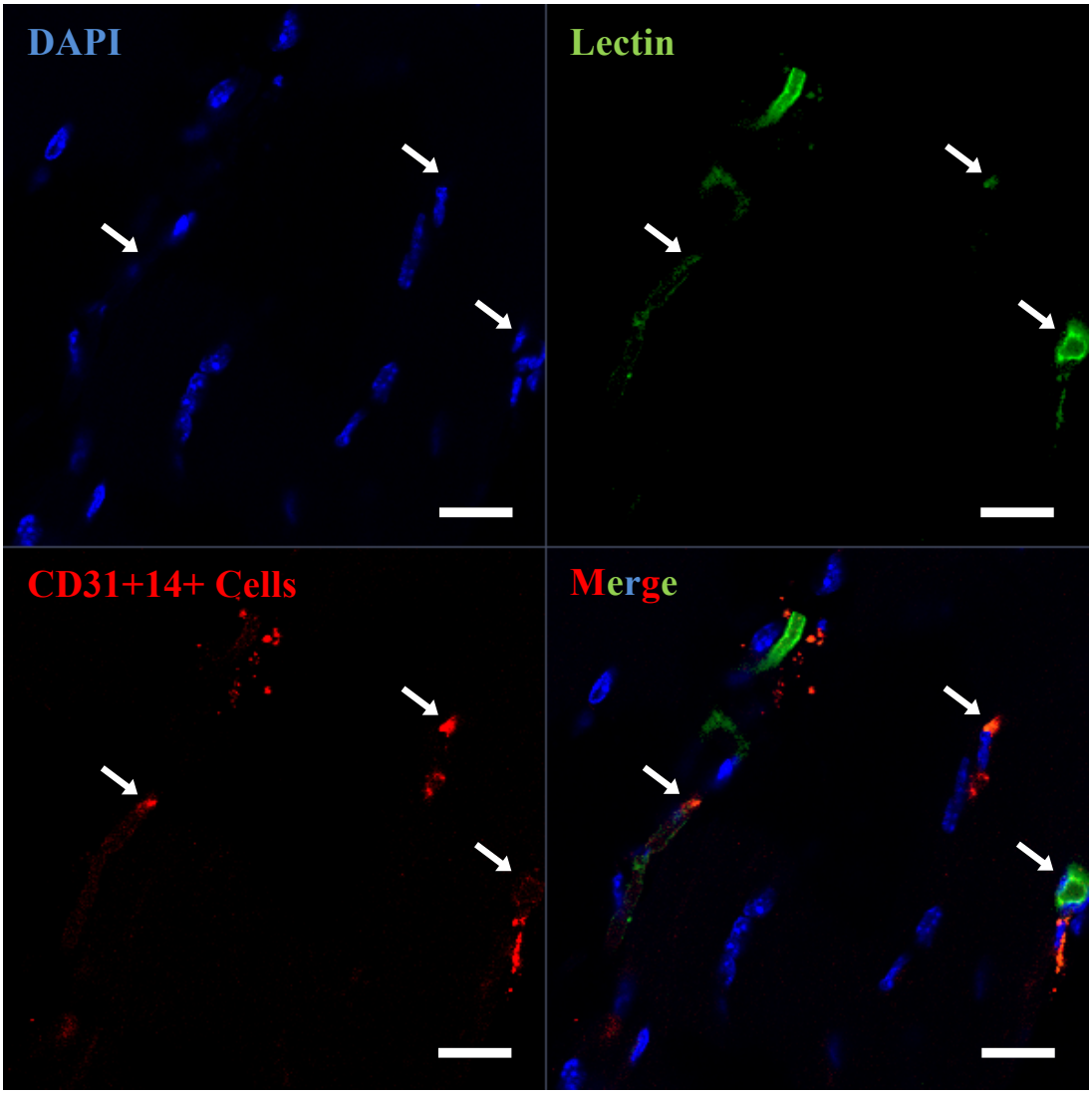

Figure 23. Confocal images of engrafted cells in mouse ischemic hindlimbs. $CD31^{+}CD14^{+}$ cells are shown double positive for lectin and CM-DiI. Red = CM-DiI, green = FITC-lectin, blue = DAPI. Scale bars = 25 µm.

treated hindlimbs compared to those of PBS, MNC, PB-$CD31^{-}$ and PB-$CD31^{+}$ cell injected mice by day 21 while MNC, PB-$CD31^{-}$ and PB-$CD31^{+}$ cell treated hindlimbs were only significantly increased compared to PBS treated hindlimbs. Interestingly, the perfusion from PB-$CD31^{+}CD14^{-}$ and PB-$CD31^{+}CD14^{+}$ cell treated groups individually was observed to be greater than that of the parent PB-$CD31^{+}$ population treated hindlimbs. Previous data of significantly expressed genes in the PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}CD14^{-}$ populations

show genes that function through varying pathways; pro-angiogenic and endothelial migration inducing for PB-CD31^{+}CD14^{+} cells and pro-vessel stabilization and maturation and for PB-CD31^{+}CD14^{-} cells. A number of these differentially expressed genes in the PB-CD31^{+}CD14^{-} cell population played active roles in the downregulation of genes highly expressed in the PB-CD31^{+}CD14^{+} cell population as is the case for ROBO4 downregulating UNC5B, PML repressing TGFB and EGR3 reducing the effectiveness of VEGF. This coupled with the inverse apoptosis regulatory functions of each cell population support the notion that co-injection of these two cell populations allows them to work antagonistically against each other.

As demonstrated in the capillary density assay, the PB-CD31^{+}CD14^{+} cells rapidly induce the formation of capillaries at early timepoints in limb ischemia; however, this bolus of vessel formation is lost by day 28 as is known be an issue in some angiogenic therapies such as the delivery of VEGF for peripheral artery disease[79]. The rapid induction of vessel formation often suffers from vascular leakiness and immaturity, lacking the ability to

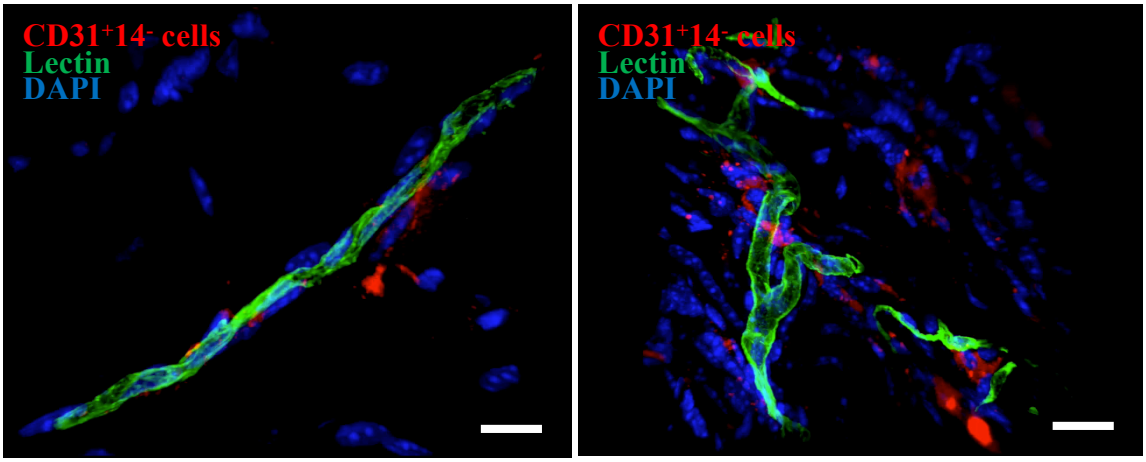

Figure 24. Confocal images of engrafted PB-CD31^{+}CD14^{-} cells in mouse ischemic hindlimbs. CD31^{+}CD14^{-} cells are shown adjacent to vessel. Red = CM-DiI, green = FITC-lectin, blue = DAPI. Scale bars = 25 µm.

persist long-term. It is necessary for other factors such as angiopoietins to subsequently begin the process of vascular maturation, strengthening the junctions between endothelial cells and reducing vascular permeability[79]. A key player in this process of vascular maturation is the SMC; however, SMCs typically arrive later in the neovascularization cascade. SMCs "muscularize" small capillaries developing them into arterioles. These arterioles have much more lasting potential and are the key to a successful long-term vascular therapy. PB-$CD31^{+}CD14^{-}$ cells displayed the ability to recruit SMCs to the ischemic region, causing muscularization of 65% of vessels compared to 35% of vessels in PB-$CD31^{+}CD14^{+}$ cell treated hindlimbs. It is known that pericytes and smooth muscle cells have a biologically relevant function in regulating endothelial cell growth and proliferation[240]. Furthermore, pathological conditions may encourage SMC mediated inhibition of endothelial migration[241]. An ideal therapy for limb ischemia would cause rapid induction of capillary formation in the ischemic region shortly followed by the recruitment of SMCs to mature and stabilize functional vessels. It is possible that the concentration and injection of cell populations that induce both processes simultaneously are counterproductive and the most efficient therapy would be a primary injection of PB-$CD31^{+}CD14^{+}$ cells followed by a secondary injection of PB-$CD31^{+}CD14^{+}$ cells.

Interestingly, PB-$CD31^{+}CD14^{-}$ cells were able to eventually induce limb perfusion despite the lack of rapid capillary network formation in the first 7 days. This implies that PB-$CD31^{+}CD14^{-}$ cells have some other compensatory mechanism that initiates tissue and vascular repair. It is possible that PB-$CD31^{+}CD14^{-}$ cells increase cell survival and take advantage of various ECM remodeling proteins and inflammatory cytokines to initiate debridement of the ischemic area. Histological images of engrafted PB-$CD31^{+}CD14^{-}$ cells

showed a number of cells positioned perivascularly implying that the injected cells are involved in the function or stability of the vasculature; however, such effects are not possible to ascertain with the current assays. Another interesting observation was the existence of engrafted PB-CD31^{+}CD14^{+} cells stained positive for perfused lectin. This implies that there is some subset of PB-CD31^{+}CD14^{+} cells with vasculogenic potential. This result coincides with data in the literature where CD14^{+} cells were shown to encompass the elusive highly angiogenic CD34^{+} cell population[94,132]. While it is possible that PB-CD31^{+}CD14^{+} cells are capable of contributing to the formation of new collateral vessels directly, this phenomenon was rarely observed and it is likely only a minor, secondary contributor to the efficacy of the cell therapy as a whole. The primary benefit of all injected cell populations is paracrine.

CHAPTER 5 SUMMARY AND FUTURE DIRECTIONS

Cardiovascular disease (CVD) continues to be the major cause of death in the developed world. Despite progress in the management of CVD, there is still a great need for new treatments and alternative therapies. In critical limb ischemia (CLI), a number of cell therapies have been investigated. However, there is a need to overcome the clinical limitations of cell therapies such as cell identity, therapeutic potency and isolation of adequate cell numbers. This dissertation explored the peripheral blood (PB)- $CD31^{+}CD14^{+}$ and PB-$CD31^{+}CD14^{-}$ populations of cells for their therapeutic potential to treat CLI.

Identification and Characterization of PB-MNC fractions

Summary

In this project, we characterized the phenotype and expression profile of PB-$CD31^{+}CD14^{+}$ and PB-$CD31^{+}CD14^{-}$ cells using flow cytometry, qRT-PCR and RNA-seq. Our data confirms the highly angiogenic expression profile of PB-$CD31^{+}CD14^{+}$ cells, strongly expressing potent endothelial stimulants such as FLT4, VEGF, HGF, TGFB1 and ICAM. Surprisingly, we discovered that PB-$CD31^{+}CD14^{-}$ cells strongly express ECM remodeling and vascular maturation proteins such as FGF2, PDGFB, MMP2, ROBO4, PML and ITGB1, implying a primarily arteriogenic, vascular stabilizing and maturing phenotype. PCA analysis, differential expression analysis and Gene set enrichment analysis (GSEA) further support a primarily angiogenic function of PB-$CD31^{+}CD14^{+}$ cells whereas PB-$CD31^{+}CD14^{-}$ cells appear to play a more prominent role in matrix remodeling such as SMC and monocytes and endothelial maturation. Assays using conditioned

medium work to further solidify the notion that PB-CD31^{+}CD14^{+} cells secrete angiogenic factors that stimulate endothelial movement and activity.

Limitations

The major limitation of this project is the lack of assays that evaluate the arteriogenic function of cells. Unfortunately, arteriogenesis is a complex process that is poorly understood and, as a result there are no recognized assays to decouple angiogenic from arteriogenic effects. Presently, the closest method is to examine expression and secretion of arteriogenic factors; however, in the case of high throughput systems like RNA-seq, there are no established gene ontology sets for arteriogenesis. The development of functional assays for arteriogenesis, similar to the tube forming assay for angiogenesis, will require greater understanding of the multiple processes involved in initiating and prolonging arteriogenesis. A second major limitation is the vague and controversial role of the immune response in the vascular repair process. Recruitment of immune cells such as monocytes, T-cells, natural killer cells and neutrophils to ischemic tissue to clear pathogens, debris and secrete growth factors is critical to the successful initiation of cardiovascular repair[63]. However, imbalances in monocyte/macrophage and T-cell polarization are known to aggravate injury despite the initial benefits that come from clearing debris[242-244]. As a result, it becomes difficult to assess the function of any particular cell population in cardiovascular repair outside of using animal models.

In this aim we used robust in vitro expression assays to gain insight into potential mechanisms PB cells fractions function through after transplantation into ischemic tissue. The limitation of this approach is that it does not take into account the dynamic nature of

viable cell in the tissue. PB cells are isolated from presumably healthy blood a are therefore expected to have an expression profile consistent with healthy in vivo conditions; however, leukocytes often undergo various stages of activation in diseased or ischemic tissue. As a result, potential mechanisms of therapeutic efficacy inferred from in vitro assays are only reliable regarding the initial stages after cell injection.

Future Directions

To overcome the limitations of our study, efforts must be made in the development of arteriogenic assays. Perhaps the simplest approach would be to compile a list of all known arteriogenic factors for use in RNA sequencing. While use of this list would not be able to decouple the angiogenic effects from the arteriogenic ones, such a list would still prove useful in the evaluation of a cell type or population's involvement in the process of vessel maturation.

Another future direction would be to determine the effects of PB cell population on other vascular cell types for example, pericytes, fibroblasts and smooth muscle cells. Expansion of the in vitro studies to include multiple cells would allow for more conclusions to be drawn on which aspects of the neovascularization process are most heavily influenced.

Evaluation of the Therapeutic Potential of PB-MNC fractions

Summary

In this aim we sought to determine the therapeutic efficacy of PB cell fractions on a mouse model of critical limb ischemia. Our results show that both the PB-$CD31^{+}CD14^{+}$

and PB-CD31^{+}CD14^{-} fractions of cells strongly induced vascular reperfusion in ischemic hindlimbs. Histologically, it was shown that the PB-CD31^{+}CD14^{+} cells increased limb perfusion in part due to the initial increase of collateral vessel growth. PB-CD31^{+}CD14^{-} cells were shown to increase SMC recruitment, implying a role in vascular maturity and stabilization.

Limitations

A major limitation of this study is its use of an acute hindlimb ischemia mouse model to evaluate a cell therapy for critical limb ischemia. In general, it is known that wildtype mice recover from ischemic injury much more quickly than humans. The use of BALB/c background mice helps to circumvent this issue due to their reduced collateral vessel growth; however, their innate neovascularization potential far exceeds that of humans. Furthermore, atherosclerosis and critical limb ischemia are diseases that do not naturally occur in wild type mice. This is compounded with the fact that limb ischemia in humans is typically a chronic disease that develops over a lifetime and presents in old age. The animal model we used is more indicative of acute limb ischemia. Secondly, we used athymic nude mice for our study in order to ablate the typical immune response from transplantation of xenogeneic cells. This ablation of the immune response completely removes the ability to evaluate the benefits of detriments that transplanted cells may have through their expression of inflammatory cytokines.

Another limitation of this study is the inclusion of so many cell populations that are so closely related to each other. We used five cell therapies, four of which originated from the first and two of those originating from the third. As a result, the statistical power of the

study may have been reduced. Furthermore, it is difficult to draw conclusions about cell behavior based on confocal imaging due to selection bias that is inherently introduced in the selection of timepoints, slides and regions for imaging. In essence, finding clusters of cells within a tissue has no bearing on the overall effects of the cells within that tissue.

Furthermore, our histology for capillary density and vessel muscularization were missing the PB-$CD31^-$ and PB-$CD31^+$ cell treatment data points. This limits our study's conclusions about how the PB-$CD31^+CD14^-$ and PB-$CD31^+CD14^+$ cell population interact when injected simultaneously. Unfortunately, our samples for the PB-$CD31^-$ and PB-$CD31^+$ treated hindlimbs were harvested at day 21 while our MNC, PB-$CD31^+CD14^-$ and PB-$CD31^+CD14^+$ treated hindlimbs were harvested at day 28. Presently, our IACUC protocols are more stringent on when animals must be sacrificed and our surgical procedures must be altered to accommodate these changes.

Future Directions

One way to overcome the limitations of the animal model is to use a chronic ischemia mouse model[245]. This model would more closely match the pathophysiology of those patients who are likely to receive our therapy in the future. For the second limitation, it was certainly possible to present the data without information regarding the PB-$CD31^+$ population as a combination of the PB-$CD31^-$, -$CD31^+CD14^-$ and –$CD31^+CD14^+$ cells completely reconstitute the parent PB-MNC population; however, given that previous research was done to show the neovasculogenic characteristics of bone marrow and PB-$CD31^+$ cells, we thought it necessary to include them as a pseudo positive control for functional studies. As such, a future study will include the addition of histological samples

from PB-CD31$^-$ and PB-CD31$^+$ treated hindlimbs in the capillary density and vascular muscularization assays.

Future work should be done to further investigate the arteriogenic function of the various cell therapies. One such method is to stain tissue sections at earlier timepoints with aSMA to build a temporal framework for when angiogenic and arteriogenic responses are initiated and how they progress. This method could be combined with the use of an image analysis software to determine vessel size. It may would also be useful to quantify the distribution of engrafted cells within the tissue as far as localization and general proximity to local vessels. A future direction of this study would be to use an in vivo model of arteriogenesis. For example, a recently developed rat model for measuring arteriogenesis was developed where the femoral artery and vein are sutured together to form an arteriovenous anastomosis. In this model, the hindlimb will experience reduced perfusion due to the high resistance of the capillaries. As such, vasodilation and increased vascular conductance become the endpoints for the study[246].

It would be insightful to harvest tissue from cell treated hindlimbs at days 7, 14 and 21 to examine mRNA expression of engrafted cell. This data would help to pinpoint the activation state of transplanted cells and elucidate some of the precise pathways that are initiated by the various cell therapies. This data could be combined with endogenous mRNA expression from the tissue to observe the host response to cell injection.

Lastly, a future direction for this study could include the use of these cells in other cardiovascular disease models such as myocardial infarction or diabetic retinopathy. These

cells may also be used in conjunction with biomaterials to aid in long-term engraftment, thereby bolstering therapeutic efficacy.

APPENDIX A. SUPPLEMENTARY INFORMATION

Table 3. Sequences of primers used for qRT-PCR

Gene	Orientation	Primer Sequence	Company
VEGFA	Forward	GTGCCCACTGAGGAGTCCA	Eurofins Genomics
	Reverse	TCCTATGTGCTGGCCTTGGT	
FGF2	Forward	AGCGACCCTCACATCAAGCTA	Eurofins Genomics
	Reverse	CCAGGTAACGGTTAGCACACACT	
ANGPT1	Forward	CAGAAAACAGTGGAGAAGATATAACC	Eurofins Genomics
	Reverse	TGCCATCGTGTTCTGGAAGA	
CXCL12	Forward	GAAGCGAAAAAACAGTGAATAAACC	Eurofins Genomics
	Reverse	TGGAACCTGAAACCCAGCTG	
HGF	Forward	CAATAGTCAATTTACCATCCCGTAAT	Eurofins Genomics
	Reverse	CGTGTTGGAATCCCATTTACAA	
PDGFB	Forward	CATCCCGAGGAGCTTTATGAG	Eurofins Genomics
	Reverse	TCCAACTCGGCCCCATCT	
IGF1	Forward	CCATGTCCTCCTCGCATCTC	Eurofins Genomics
	Reverse	CGTGGCAGAGCTGGTGAAG	
IL1	Forward	GCGACGGTCACCTTCATC	Eurofins Genomics
	Reverse	AGGCACGTGAGCCTCTCTTT	
IL6	Forward	GCTGCAGGCACAGAAC	Eurofins Genomics
	Reverse	GCTGCGCAGAATGAGATGAG	
IL8	Forward	CCTTTCCACCCCAAATTTATCA	MWG-Biotech AG
	Reverse	TTTCTGTGTTGGCGCAGTGT	
IL10	Forward	GAGGCTACGGCGCTGTCA	MWG-Biotech AG
	Reverse	TCCACGGCCTTGCTCTTG	
IFNG	Forward	CCAACGCAAAGCAATACATGA	MWG-Biotech AG
	Reverse	TTAGCTGCTGGCGACAGTTC	
TNFA	Forward	GGAGAAGGGTGACCGACTCA	Eurofins Genomics
	Reverse	CAGACTCGGCAAAGTCGAGATA	
TGFB	Forward	GGAAATTGAGGGCTTTCG	MWG-Biotech AG
	Reverse	ACCCGTTGATGTCCACTT	
CXCR4	Forward	TGGGTGGTTGTGTTCCAGTTT	MWG-Biotech AG
	Reverse	ATGCAATAGCAGGACAGGATGA	
MCP1	Forward	GCAATCAATGCCCCAGTCA	MWG-Biotech AG
	Reverse	GCCTCTGCACTGAGATCTTCCT	

```r
require(Biobase)
require(DESeq2)
require(graphics)
require(ggplot2)
require(ggfortify)
require(gplots)

# Import gene counts data from file on computer
CountFile  <- read.delim("/Users/Desktop/Research/Data/analysis in R/SeqData.txt", header=TRUE, row.names="gene_name")
# Import set containgin genes of interest and set the row names as gene names
GenesOfInterest <- read.delim("/Users/Beloved/Desktop/Research/Data/Sequencing/Gene Clustering/Hallmark Inflammatory Response.txt", header=TRUE)
rownames(GenesOfInterest) <- GenesOfInterst[,1]
# Extract interested genes from count file
InterestCountFile <- CountFile[rownames(GenesOfInterest),]
# Set Factors
Factors <- factor(c(rep("MNC",4),rep("CD31-",4),rep("CD31+",4),rep("CD31+14-",4),rep("CD31+14+",4)))
GOTable <- data.frame(cells = as.factor(cells))
rownames(GOTable) <- colnames(InterestCountFile)
# Run Deseq Analysis
deseqGO <- DESeqDataSetFromMatrix(countData = InterestCountFile, colData = GOTable, design = ~cells)
# Remove genes with < 10 counts
deseqGO <- deseqGO[rowSums(counts(deseqGO)) > 10, ]
d.deseqGO <- DESeq(deseqGO)

# Create PCA Plot
vsdBGO <- varianceStabilizingTransformation(d.deseqGO)
plotPCA(vsdBGO, intgroup=c("cells"))
# Create Heatmap
vsdB_tableGO <- as.data.frame(assay(vsdBGO))
heatmap.2(as.matrix(vsdB_tableGO)
```

Figure 25. Generalized R source code for RNA-Seq analysis.

```r
# Import data from file on computer
Data  <- read.delim("/Users/Desktop/Research/Data/for statistical analysis in R/data.txt", header = T)

# Perform Kruskal-Wallis test on imported data
kruskal.test(Observation ~ Treatment, data = Data)

# Perform pairwise Mann-Whitney U test on data if significance found in kruskal
pairwise.wilcox.test(Data$Observation, Data$Treatment, p.adjust.method = "BH")
```

Figure 26. General R source code for non-parametric statistical analysis.

```r
# Import gene counts data from file on computer
Data  <- read.delim("/Users/Desktop/Research/Data/for statistical analysis in R/Data.txt",
header=TRUE)

# Perform ANOVA on data and store information as object "anovaData
anovaData <- aov(Observation ~ Treatment, data = Data)

#Print results of ANOVA to console
summary(anovaData)

# Perform Tukey Post-hoc analysis on ANOVA data
TukeyHSD(anovaData, "Treatment")

# Perform Fischer Least Significant Difference test on data
LSDData <- LSD.test(anovaData, "Treatment", p.adj="holm")

# Output Fischer LSD groupings to console
LSDData$groups
```

Figure 27. General R source code for parametric statistical analysis.

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