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SYSTEMIC LEVELS OF G-CSF AND IL-6 DETERMINE THE ANGIOGENIC
POTENTIAL OF BONE MARROW RESIDENT MONOCYTES

by

Alyssa Diane Gregory

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

August 2009

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

Systemic Levels of G-CSF and IL-6 Determine the Angiogenic Potential of Bone Marrow Resident Monocytes

by

Alyssa Diane Gregory

Doctor of Philosophy in Biology and Biomedical Sciences (Immunology)

Washington University in St. Louis, 2009

Professor Daniel C. Link, Chairperson

Recent studies have demonstrated the efficacy of hematopoietic cell-based therapies in promoting therapeutic angiogenesis for a wide variety of vascular syndromes, however the cell populations responsible and the mechanisms involved are poorly understood. Using a mouse model of hindlimb ischemia, we previously showed that an adoptive transfer of donor monocytes significantly enhanced revascularization. Monocytes are a widely heterogeneous cell population and differences in the ability of various monocyte subsets to mediate revascularization have not been previously investigated. Using the hindlimb ischemia model we demonstrate that an adoptive transfer of inflammatory ($CX3CR1^{lo}Gr-1^{+}$), but not resident ($CX3CR1^{hi}Gr-1^{-}$) monocytes, significantly enhances revascularization post-ischemia. Additionally, we show that the inflammatory subset of monocytes is selectively recruited from the bone marrow to the blood and that these cells accumulate at the ischemic lesion. These findings demonstrate that the adoptive transfer of only a small proportion of monocytes from a non-ischemic donor significantly enhances revascularization despite the presence of a far greater

proportion of endogenous (ischemia-conditioned) monocytes. Herein, we provide data suggesting that upon induction of distant ischemia, systemic signals are generated which reduce the angiogenic capacity of bone marrow resident monocytes. We provide evidence that granulocyte-colony stimulating factor (G-CSF) and interleukin-6 (IL-6) provide these “conditioning” signals. Systemic levels of G-CSF and IL-6 are significantly increased following induction of hindlimb ischemia, and accordingly, bone marrow resident monocytes from ischemic mice exhibited increased STAT3 phosphorylation and STAT3 target gene expression. Finally, G-CSF receptor^{-/-} and IL-6^{-/-} mice were resistant to the deleterious effects of ischemic conditioning on monocyte angiogenic potential. The mechanism by which this ischemia-driven signals limit the angiogenic potential of monocytes was examined using RNA expression profiling which suggested that ischemia-conditioned monocytes in the bone marrow are polarized towards expression of M2-associated genes. Consistent with this observation, M2-skewed monocytes from SHIP^{-/-} mice also had impaired angiogenic capacity. Lastly we demonstrate that the efficacy of an adoptive transfer of non-ischemic donor monocytes may be due, at least in part, to increased expression of the fractalkine receptor CX3CR1 as well as to increases in local concentrations of the angiogenic factors MCP-1, VEGF, MMP-9 and ApoA1.

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TABLE OF CONTENTS

Abstract of Dissertation	ii
Acknowledgements	iv
List of Figures and Tables	ix
Chapter 1 Introduction	
1.1 Angiogenesis	2
1.1.1 Types of angiogenesis	2
1.1.2. Hindlimb ischemia model of angiogenesis	3
1.2 Monocyte regulation of angiogenesis	5
1.2.1. In vitro and animal model findings	5
1.2.2. Clinical trials utilizing BM-MNCs	7
1.3 Monocyte biology	9
1.3.1 Development and functions	9
1.3.2. M1/M2 polarization	10
1.3.3. CX3CR1 monocyte subsets	12
1.3.4. Tie2 monocyte subsets	14
1.4 CX3CR1/fractalkine receptor	16
1.5 Apolipoprotein A1	19
1.6 Summary	21
1.8 References	22

Chapter 2 The inflammatory subset of monocytes potently stimulates post-ischemic angiogenesis

2.1 Abstract	31
2.2 Introduction	31
2.3 Materials and Methods	35
2.4 Results	
2.4.1 Monocytes are recruited to ischemic tissue post-HLI	37
2.4.2 An adoptive transfer of the inflammatory, but not resident, subset of monocytes significantly improves revascularization post-HLI	38
2.4.3 The inflammatory subset of monocytes is selectively mobilized from the bone marrow into the blood post-HLI	39
2.4.4 Monocytes of the inflammatory subset accumulate at the ischemic lesion post-HLI	39
2.4.5 Bone marrow resident monocytes decrease expression of CX3CR1 upon induction of ischemia and CX3CR1 ^{-/-} monocytes exhibit a reduced ability to improve angiogenesis.	40
2.4.6 Local production of VEGF, MCP-1, MMP-9, and apolipoprotein A1 is increased post-adoptive transfer.	41
2.4.7. An adoptive transfer of apolipoprotein A1-deficient monocytes does not improve revascularization post-HLI.	41
2.5 Discussion	42
2.6 Figures and Figure Legends	47

2.7 References	58
----------------	----

**Chapter 3 Systemic Signals Generated by IL-6 and G-CSF Influence the
Angiogenic Capacity of Bone Marrow Resident Monocytes**

3.1 Abstract	63
3.2 Introduction	64
3.3 Materials and Methods	66
3.4 Results	
3.4.1 The addition of a small proportion of macrophages obtained from a non-ischemic donor significantly improves revascularization post-HLI	70
3.4.2 An adoptive transfer of BM-MNCs obtained from an ischemic donor does not improve revascularization post-HLI	71
3.4.3 Ischemia-conditioned monocytes are not competent to mediate revascularization	72
3.4.4. Adoptively transferred ischemia-conditioned monocytes are competent to home to ischemic tissue	73
3.4.5 Increased STAT3 phosphorylation is observed in ischemia-conditioned bone marrow monocytes	73
3.4.6 IL-6 and G-CSF are produced upon induction of ischemia and loss of either of these signals is sufficient to restore the ability of ischemic adoptively transferred	74

monocytes to promote angiogenesis	
3.4.7 Ischemia-conditioned bone marrow monocytes	75
demonstrate a shift toward expression of M2 genes and	
M2 skewed monocytes do not accelerate revascularization	
3.5 Discussion	76
3.6 Figures and Figure Legends	80
3.7 References	91
Chapter 4 Summary and Future Directions	
4.1 Summary	96
4.2 Application to other ischemic models	97
4.3 Role of type I interferon	99
4.4 Clinical trial using mobilizing agents to recruit monocytes	100
4.5 References	102

LIST OF FIGURES AND TABLES

Chapter 1: Introduction

Figure 1	Schematic of femoral artery excision and laser Doppler imaging	4
Table 1	Clinical trials utilizing BM-MNC transplantation	8
Table 2	CX3CR1/Gr-1 monocyte subsets in mice	14
Table 3	CD14/CD16 monocytes subsets in humans	14

Chapter 2: The Inflammatory Subset of Monocytes Potently Stimulates Post-Ischemic Angiogenesis

Figure 2.1	Adoptively transferred donor monocytes home to the site of ischemia and a concomitant recruitment of additional endogenous monocytes is observed.	51
Figure 2.2	An adoptive transfer of the inflammatory, but not resident, subset of monocytes significantly improves revascularization post-HLI.	52
Figure 2.3	The inflammatory subset of monocytes is selectively mobilized from the bone marrow into the blood post-HLI	53
Figure 2.4	Monocytes of the inflammatory subset accumulate at the ischemic lesion post-HLI	54
Figure 2.5	Expression of CX3CR1 is decreased upon induction	55

of ischemia and CX3CR1^{-/-} monocytes demonstrate a reduced capacity to stimulate angiogenesis.

- Figure 2.6 Local production of VEGF, MCP-1, MMP-9, and apolipoprotein A1 is increased post-adoptive transfer 56
- Figure 2.7 An adoptive transfer of apolipoprotein A1-deficient monocytes does not improve revascularization post-HLI 57

Chapter 3: Systemic Signals Generated by IL-6 and G-CSF Influence the Angiogenic Capacity of Bone Marrow Resident Monocytes

- Figure 3.1 The addition of a small proportion of macrophages obtained from a non-ischemic donor significantly improves revascularization post-hindlimb ischemia. 80
- Figure 3.2 An adoptive transfer of BM-MNCs obtained from an ischemic donor does not improve revascularization post-HLI. 81
- Figure 3.3 Ischemia-conditioned monocytes are not competent to mediate revascularization. 82
- Figure 3.4 Adoptively transferred ischemia-conditioned monocytes are competent to home to ischemic tissue. 83
- Figure 3.5 Increased STAT3 phosphorylation is observed in ischemia-conditioned bone marrow monocytes. 84

Figure 3.6	IL-6 and G-CSF are produced upon induction of ischemia and loss of either of these signals is sufficient to restore the ability of ischemic adoptively transferred monocytes to promote angiogenesis.	85
Figure 3.7	Differential M1/M2 gene expression post-ischemic conditioning, and reduced revascularization capacity of SHIP ^{-/-} M2-skewed monocytes.	86
Table 3.1	Genes upregulated in ischemia-conditioned bone marrow monocytes	87
Table 3.2	Genes downregulated in ischemia-conditioned bone marrow monocytes	88
Table 3.3	M1-associated genes	89
Table 3.4	M2-associated genes	90

Chapter 1

Introduction

1.1 Angiogenesis

1.1.1 Types of neovascularization

Neovascularization is a critical biological process in a variety of developmental, repair, and pathological settings. The general term neovascularization describes three distinct processes that result in the formation of blood vessels: vasculogenesis, arteriogenesis, and angiogenesis. Vasculogenesis refers to the de novo formation of vascular structures from mesenchymal angioblasts (Risau, 1995). Postnatally, some vasculogenesis mediated by endothelial progenitor cells may occur, however the contribution of this process to adult neovascularization is controversial (Asahara, 1999; Simons, 2003). In adults, blood vessel formation occurs by two distinct mechanisms referred to as angiogenesis and arteriogenesis. Arteriogenesis refers to the enlargement of pre-existing vessels to form arteries which have 3 distinct wall layers as well as vasomotor activity (Helisch, 2003). In contrast, during angiogenesis new capillary networks are formed due to the recruitment of local endothelial cells from nearby vessels which assemble into vascular structures in a process referred to as tube formation (Folkman, 2005). The two processes by which angiogenesis can occur are referred to as “sprouting” in which a new branch is formed from an existing vessel, or “intussusception” which refers to the splitting of a pre-existing capillary. Each of these processes results in the formation of a thin-walled endothelial cell-lined capillary. The studies outlined below will focus exclusively on post-ischemic neovascularization. Angiogenesis is a major contributor to post-ischemic neovascularization as only this process, and not arteriogenesis, is induced in response to hypoxia-inducible factor-1 α (HIF-1 α) expression which is

induced under conditions of ischemia/hypoxia (Madeddu, 2005; Schaper, 2003).

However, both angiogenic and arteriogenic mechanisms are triggered by shear stress which is generated in ischemic tissues and thus both processes are involved in recovery from ischemic insult.

1.1.2 Hindlimb ischemia model of angiogenesis

In order to study post-ischemic neovascularization, we will utilize a mouse model first described by Couffinhal, et al. for femoral artery excision (FAE) which results in hindlimb ischemia (HLI) (1998). In this model, the right hindlimb is dissected to allow visualization of the femoral artery. The obturator nerve which runs directly parallel and in close proximity to the femoral artery is dissected away from the artery taking care that it remains intact. The femoral artery and all of its side branches, as well as the proximal saphenous artery are then ligated and the portions of the vessels located between these points of ligation are excised. Laser Doppler imaging is used to monitor the disruption and subsequent restoration of blood flow to the ischemic hindlimb. Imaging software assigns a “flux value” describing the surface blood flow observed. As ischemia is induced in a unilateral fashion, the amount of blood flow present in the non-ischemic leg is used as an internal control to minimize variations among individual mice. Blood flow recovery is thus reported as the ratio of the flux value observed in ischemic hindlimb divided by the flux value observed in the non-ischemic hindlimb abbreviated as (ischemic:non-ischemic). Prior to femoral artery excision, the flux values obtained for the right and left hindlimbs are nearly identical, resulting in a flux ratio of approximately 1.0. In contrast, post-HLI,

blood flow to limb undergoing femoral artery excision is nearly absent, resulting in a flux ratio of approximately 0.1. Mice are monitored over a 14-day time course to assess revascularization. Of note, in our studies laser Doppler quantitation is performed only on the foot region of the mouse, excluding the adductor and calf muscle regions. Histological analyses suggest that angiogenesis is the major repair mechanism present in the ischemic calf muscle and foot while arteriogenesis is responsible for repair of the ischemic adductor muscle (Wahlberg, 2006; Shireman, 2007). Thus angiogenesis, and not arteriogenesis, is the major neovascularization process reflected in this model.

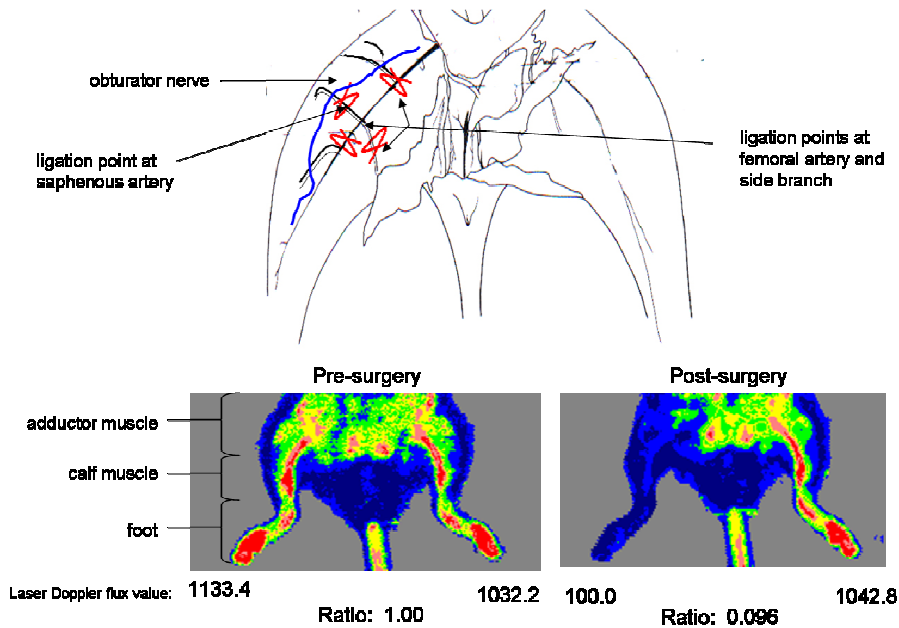


Figure 1.1

1.2 Monocyte regulation of angiogenesis

1.2.1 In vitro and animal model findings

Monocytes are known to play a critical role in promoting angiogenesis in a wide variety of settings including wound repair, rheumatoid arthritis, tumor development, and ischemic injury (Sunderkotter, 1994). Monocytes play a variety of distinct roles in regulating the angiogenic process. First, they exhibit proteolytic and phagocytic activity which allows for the degradation and removal of damaged tissue, thus generating an appropriate scaffolding upon which new blood vessels can be formed. Secondly, macrophages generate a wide range of cytokines which recruit and/or activate additional inflammatory cells (MCP-1, IL-8) or endothelial cells (VEGF). Lastly, several studies have reported that monocytes may, in fact, be able to incorporate into developing vasculature. Recent evidence however, has demonstrated that CD45⁺ hematopoietic cells do not incorporate into developing endothelium (Capoccia, 2006). Monocytes are recruited to hypoxic areas and produce angiogenic factors which stimulate endothelial cells located in nearby areas to migrate, proliferate, and differentiate into new vessels (Lewis, 2005). In response to hypoxia, macrophages produce VEGF, a mitogen specific to endothelial cells which is potently angiogenic (Leung, 1989). Additionally, macrophages have been shown to express the inflammatory mediators IL-1, TNF, IL-6, and arginase-1 (Arg1) in response to hypoxia (Bosco, 2008).

In addition to the phagocytic, proteolytic, and proinflammatory properties described above, monocyte/macrophages have also been ascribed two unique functions outlined in recent studies. First, although macrophages are largely thought

to be non-dividing, several studies have indicated a limited degree of proliferation in response to co-culture with endothelial cells and M-CSF in vitro (Cheung, 1992; Munn, 1993; Antonov, 1997). However, the contribution of local proliferation of macrophages under inflammatory conditions in vivo is unclear. Second, an additional study using a mouse transgenic model of ischemic cardiomyopathy has indicated that macrophages can drill endothelial cell-free “tunnels” using macrophage metalloelastase which penetrate the ischemic myocardium (Moldovan, 2000). Whether or not these tunnels are subsequently colonized by endothelial cells to form capillaries is unknown.

In light of the well-established important roles played by monocytes in mediating angiogenesis, several groups have explored the possibility that a local injection or intravenous adoptive transfer of monocytes may serve as an efficacious treatment for a variety of angiogenic disorders. Recent studies by Capoccia et al. have demonstrated that an adoptive transfer of bone marrow mononuclear cells, and in particular the monocyte fraction contained within this population, significantly enhanced blood flow recovery post-hindlimb ischemia in mice (2006, 2008). These findings were corroborated by (Zhang, 2008) and extended to myocardial infarction models (Burchfield, 2008; Ziebart, 2008). Studies such as these provided the rationale for the clinical trials described below.

1.2.2 Clinical trials utilizing monocytes

Promising reports using mouse modeling have prompted a variety of clinical trials in which bone marrow-derived mononuclear cells are used as a treatment for ischemic disorders including myocardial infarction and critical limb ischemia. Critical limb ischemia (CLI) is a severe manifestation of peripheral arterial disease (PAD). The estimated annual incidence of CLI ranges between 500 and 1,000 new cases per 1 million, and occurs most frequently in patients with diabetes or severe atherosclerosis (Minar, 2009). The primary goals of treatment in patients with CLI are to relieve ischemic pain and to treat ischemic ulcers, and in the most severe cases to prevent loss of the ischemic limb. In an effort to treat CLI, the growth factor proteins VEGF and bFGF have been administered locally, however results to this type of treatment have been mixed (Al Sabti, 2007). These mixed results are not entirely surprising as these proteins have a short serum half-life and a sole factor is unlikely to recapitulate all the signals necessary to induce angiogenesis.

As a means of circumventing the limitations of single protein therapies, recent studies have focused on cell-based approaches to therapeutic angiogenesis. Studies in animal models demonstrating the efficacy of hematopoietic cells, in promoting angiogenesis led to the rapid development of clinical trials in which autologous hematopoietic cells were injected intramuscularly into the ischemic limb. These studies utilize autologous bone marrow mononuclear cells (BM-MNCs) obtained directly from bone marrow aspirates or G-CSF-mobilized peripheral blood-mononuclear cells (PB-MNCs). In one group of trials, summarized in Table 1, autologous BM-MNCs were injected intramuscularly into the ischemic limb of

patients with critical limb ischemia. In each of these trials therapeutic benefits were observed in the treatment group as measured by a variety of indicators including improvements in ankle-brachial index, mean walking distance, and collateral formation. While these promising findings have been reported for critical limb ischemia, similar myocardial infarction trials have yielded disappointing results. Little efficacy has been observed using either BM-MNC or PB-MNC therapies (Dimmeler, 2008). Therefore an understanding of the specific pro-angiogenic populations contained within the mononuclear cell compartment and the mechanism by which these cells promote angiogenesis may reveal important information about how to improve the design of these therapies for myocardial infarction patients.

1.3 Monocyte biology

1.3.1 Development and functions

Monocytes are mononuclear cells that develop from a common myeloid progenitor in the bone marrow and are released into the blood under both basal and pathogenic conditions (Fogg, 2006). Monocytes comprise approximately 5-10% of the total leukocyte population in the blood where they exhibit a relatively brief lifespan. Monocytes in mice and humans demonstrate a half-life of 17 hours (van Furth, 1989) and 71 hours (Whitelaw, 1972), respectively. Upon recruitment to peripheral tissues, monocytes can follow one of two differentiation pathways generating either macrophages or dendritic cells (DCs). Macrophages engulf dead cells and debris, proteolytically degrade this cellular material, and release cytokines

which further promote the clearance of dead cells. After processing this cellular debris, macrophages do not process or present antigens or generate a T cell response (Zammit, 2005). DCs likewise phagocytose cellular debris but in addition they process and present them to T cells via MHC molecules (Trombetta, 2003). The migration properties of these cell types likewise differ: while macrophages remain largely confined to tissues, DCs can traffick from the tissues to peripheral lymphoid organs. In addition to deriving from bone marrow/blood precursors, some macrophage populations, such as the pulmonary macrophages are known to self-renew from local precursors (Tarling, 1987). After emigrating from the blood to tissues, macrophages can exhibit any of a wide array of phenotypes depending on local signals as outlined below.

1.3.2 M1/M2 polarization

In response to a variety of stimuli, monocytes are recruited from the bone marrow to the blood and subsequently to peripheral tissues where they can differentiate into several effector macrophage subsets. Infectious and pro-inflammatory stimuli generate signals which drive macrophages down the classical activation or M1 pathway. In vitro, cells exhibiting an M1 phenotype can be generated by treating with interferon- γ (IFN- γ) or lipopolysaccharide (LPS). M1 polarized macrophages produce a panoply of inflammatory mediators and utilize the nitric oxide synthase pathway to generate large amounts of nitric oxide (NO). Accordingly, nitric oxide measurements are frequently used as an assessment of polarization to the M1 phenotype (Mills, 2000). Conversely, macrophages can

undergo alternative activation to polarize along the M2 pathway. Alternative activation occurs biologically upon infection with parasitic helminths or in response to allergens. This subset of macrophages is referred to as M2a. In addition, M2 polarization can occur in response to activation by immune complexes, generating a subset called M2b. Lastly, the M2 phenotype can be generated in response to IL-10 production at the resolution of inflammation (Kreider, 2007). In keeping with *in vivo* observations, polarization towards the M2 phenotype can be driven *in vitro* by treating with IL-4, IL-13, or IL-10. M2 polarized macrophages utilize the arginase pathway and expression of arginase 1 (Arg1) is a distinct marker for this cell type. Additionally, expression of the markers Ym1/chitinase and FIZZ1 are widely accepted as M2 markers across all M2 subsets and are upregulated in response to the vast majority of helminth infections (Martinez, 2009).

Genetic mouse models for M1 or M2 polarization have been described, but are wrought with important caveats. Mice lacking Src homology inositol phosphatase (SHIP) demonstrate a skewing towards increased M2 polarization as SHIP has been found to repress alternative activation in macrophages *in vitro* (Rauh, 2005). In addition, PPAR-gamma deficiency has been shown to result in preferential M1 macrophage polarization (Odegaard, 2007). Interestingly, these phenotypes are highly dependent upon mouse strain. For example, in the Th1-permissive Balb/c mouse strain, PPAR-gamma deficiency results in increased M1 polarization, however in the Th1-resistant C57Bl/6 mouse strain this phenotype is not observed.

While M1-polarized macrophages clearly play a role in inflammatory processes, the role of M2-polarized macrophages is less clear. M2a macrophages in

concert with Th2 cells to elicit antibody-mediated immunity which allows antibodies to coat invading parasites such that they can be cleared via a process referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). Monocytes exhibiting an M2 phenotype have been extensively reported in a variety of tumor models (Nardin, 2008).

The ability of M1 or M2 macrophage subsets to influence angiogenesis outside of the tumor microenvironment has not been extensively studied, however one report indicates that IL-4-induced M2 macrophages elicited a 3-fold greater proliferation in endothelial cells in vitro compared to IFN-g-elicited M1 macrophages (Kodelja, 1997). The studies described in Chapter 3 will assess M1 and M2 polarization phenotypes in an ischemic setting.

1.3.3 CX3CR1 monocyte subsets

Monocytes are a widely heterogeneous cell population in which the full extent of existing subsets and the plasticity between subsets is largely unknown. One major subset designation in monocytes has been recently described based upon differential expression of the cell surface markers CX3CR1 and Gr-1 (Table 2). In the bone marrow, monocytes can be divided into two distinct populations based upon these markers, namely CX3CR1^{hi}Gr-1⁻ and CX3CR1^{lo}Gr-1⁺ subsets. The CX3CR1^{lo}Gr-1⁺ subset is referred to as the inflammatory subset, due to the ability of these cells to migrate to the peritoneum in response to intraperitoneal thioglycollate. In addition, these cells express the adhesion markers VCAM-1 and L-selectin, as well as the

chemokine receptor CCR2. In contrast, cells of the CX3CR1^{hi}Gr-1⁻ subset do not traffic to the peritoneum in response to thioglycollate treatment and do not express the adhesion markers or chemokine receptors observed in the inflammatory subset (Geissmann, 2008). Based on these observations, this subset is referred to as the resident subset. While the precise function of this subset is unclear, a recent study has suggested that the resident monocyte subset patrols blood vessels, allowing rapid tissue invasion and subsequently give rise to macrophages while inflammatory monocytes reach the inflammatory site at a later time and differentiate into inflammatory DCs (Auffray, 2007). Subsets analogous to the mouse inflammatory and resident subsets have been described in humans in which a CD14^{hi}CD16⁻ population resembles the inflammatory monocyte population while CD14⁺CD16⁺ is functionally comparable to the resident subset (Table 3) (Zeigler, 2007).

The bone marrow is made up largely of the CX3CR1^{lo}Gr-1⁺ inflammatory subset while the resident subset dominates in the peripheral blood. Several studies have indicated that the inflammatory population in the bone marrow may convert into the resident population in the peripheral blood based upon labeling and tracking studies (Tacke, 2006; Arnold, 2007). Another study utilizing a mouse model of myocardial infarction has indicated that the inflammatory subset is selectively recruited first and that these cells exhibit phagocytic and proinflammatory functions and that subsequently the healing myocardium recruits monocytes of the resident subset which mediate angiogenesis and the deposition of collagen (Nahrendorf, 2007). While no studies have directly examined the contribution of the inflammatory and resident subsets to angiogenesis, in the ApoE^{-/-} atherosclerosis-prone mouse

model, a skewing towards the CX3CR1^{lo}Gr-1⁺ inflammatory monocyte subset was observed in the blood and these cells also preferentially accumulated in the atherosclerotic lesion (Tacke, 2007). Further studies are required to determine if this subset is responsive to specifically responsive to ischemic signals or if the accumulation of the cells is in response to inflammatory signals.

	inflammatory (CX₃CR1^{lo}Gr-1⁺)	resident (CX₃CR1^{hi}Gr-1⁻)
size	10-14- μ M	8-12 μ M
localization post-adoptive transfer	Spleen	blood, spleen, lung, liver, brain
response to i.p. thioglycollate	Recruited to peritoneum	No recruitment to peritoneum
expression of adhesion molecules	VLA2 ⁺ L-selectin ⁺	VLA2 ⁻ L-selectin ⁻
expression of chemokine receptors	CCR2 ⁺	CCR2 ⁻
differentiation into DCs	population of CD11c ⁺ I-A ⁺ found at inflammatory site	small population of CD11c ⁺ I-A ⁺ found in spleen

Table 2. Properties of mouse CX₃CR1 monocyte subsets

	inflammatory (Cx₃CR1^{lo}CD14^{hi}CD16⁻)	resident (CX₃CR1^{hi}CD14^{lo}CD16⁺)
expression of adhesion molecules	L-selectin ⁺	L-selectin ⁻
expression of chemokine receptors	CCR2 ⁺	CCR2 ⁻
Fc receptor expression	Fc γ RI (CD64)	Fc γ RII (CD32)
MHC class II expression	MHC class II ^E	MHC class II ^H

Table 3. Properties of human CX₃CR1 monocyte subsets

Modified from Geissmann, 2003

1.3.4 Tie2 monocyte subset

Recent studies have revealed a potentially angiogenic monocyte subset that expresses the receptor tyrosine kinase Tie2. The Tie2 receptor binds two major ligands: the agonist ligand angiopoietin-1 (Ang1) and the antagonist ligand angiopoietin-2 (Ang2). Binding of Ang1 to Tie2 receptor strengthens interactions

between endothelial cells and extracellular matrix (Thurston, 2000). Ang2 competes for binding with Tie2 preventing the binding of Ang1, thus leading to destabilization of vessels (Maisonpierre, 1997). However, in vitro, Ang-2 stimulates endothelial cells migration and tube cell formation (Teichert-Kuliszewska, 2001). Roughly 2-5% percent of bone marrow cells have been shown to express Tie2. Flow cytometric analysis revealed that these Tie2⁺ cells present in the bone marrow were comprised of both endothelial cells (<5% CD31⁺CD45⁻) and hematopoietic cells (>95% CD45⁺). Tie2-expressing CD45⁺ hematopoietic cells were found to be enriched for Sca-1⁺ (~30%) and c-kit⁺ (~50%) progenitors (De Palma, 2003). In the peripheral blood, Tie2-expressing monocytes (TEMs) have been shown to comprise 2-7% of the total circulating monocytes in healthy human subjects (Venneri, 2007) as well as in mice (Lewis, 2007). TEMs in the peripheral blood largely did not express the HSC/HPC markers c-kit (95% c-kit⁻) and Sca-1 (>70% Sca-1⁻). Interestingly, circulating TEMs were found to exhibit a CCR2⁻, L-selectin⁻, CCR5⁺ phenotype—a surface profile previously associated with resident monocytes (Gordon, 2005).

While TEMs comprise only a small fraction of blood monocytes, they are have been found to be enriched in a variety of tumor environments. 55% and 70% of the CD14⁺ monocytes present in both a colorectal and lung carcinoma expressed Tie2. It is thought that these TEMs play an important role in vascularizing tumors based on several lines of evidence. For example, U87 tumor cells coinjected with human CD14⁺Tie2⁺ monocytes led to larger and more highly vascularized tumors than those observed in mice receiving U87 cells alone (De Palma, 2009). In addition, in a knockout mouse model lacking TEMs, there was a complete prevention of human

glioma neovascularization in the mouse brain as well as substantial tumor regression (De Palma, 2005). It is currently unknown whether TEMs represent a unique monocyte lineage or if this phenotype is induced by the tumor microenvironment.

1.4 CX3CR1/fractalkine receptor

Fractalkine or CX3CL1 is CX3C motif chemokine which is produced by macrophages, activated vascular endothelial cells, smooth muscle cells, epithelial cells, dendritic cells, and neurons (Landsman, 2009). CX3CR1 is the sole known receptor for fractalkine ligand and is expressed on blood monocytes, T cells, DC subsets, and natural killer (NK) cell subsets. Fractalkine ligand is a transmembrane mucin-like stalk which mediates integrin independent adhesion to leukocytes expressing CX3CR1 (Bazan, 1997). CX3CL1 can also be cleaved and secreted, and in its secreted form it acts as a chemoattractant for cells bearing its cognate receptor (Garton, 2001; Hundhausen, 2003).

Expression of fractalkine is induced in inflammatory conditions downstream of TNF- α and IL-1 production (Bazan, 1997). IFN-g has been shown to increase fractalkine expression in epithelial cells and dermal fibroblasts (Fujimoto, 2001; Fahy, 2003) acting through the STAT1 signaling pathway (Lombardi, 2008). A recent study has demonstrated that in the absence of either CX3CR1 or CX3CL1 there is a reduction in circulating monocyte levels at steady state as well as in the inflammatory setting present in ApoE^{-/-} atherosclerosis-prone mice (Landsman, 2009). This study also reported that overexpression of the cell survival factor Bcl2 in CX3CR1^{-/-} or CX3CL1^{-/-} mice restored monocyte recruitment, suggesting that the

CX3CR1 pathway promotes cell survival. In a model for wound healing, CX3CR1 was found to play a critical role as CX3CR1^{-/-} mice exhibited defects in macrophage infiltration, VEGF production, collagen deposition, and neovascularization (Ishida, 2008).

A variety of studies have indicated that fractalkine and its receptor may be induced in response to hypoxia and may play an important role in blood vessel growth. Specifically, hypoxia has been shown to inhibit the IFN- γ -induced expression of fractalkine in HUVEC; however this inhibition is reversible as the reintroduction of oxygen increases fractalkine levels (Yamashita, 2003). The addition of exogenous fractalkine ligand has been shown to induce new vessel formation in vitro using the rat aortic ring and chick chorioallantoic membrane in vitro assays (Ryu, 2008). This vessel formation was associated with upregulation of HIF-1 α and VEGF-A. The role of CX3CR1 in mediating post-ischemic angiogenesis varies according to the models tested. In a mouse model for retinal vascular repair, CX3CR1 signaling was shown to have no role in revascularizing injured retinal tissue (Zhao, 2009). In contrast CX3CR1^{-/-} mice demonstrated reduced macrophage accumulation as well as decreased injury in models for kidney ischemia/reperfusion (Li, 2008) and focal cerebral ischemia (Denes, 2008). Interestingly, bone marrow transplantation experiments demonstrated that mice lacking CX3CR1 in the hematopoietic compartment were able to promote blood vessel growth within an implanted Matrigel/fractalkine matrix whereas mice lacking CX3CR1 in all tissues demonstrated no blood vessel growth (Ryu, 2008). This finding suggests that CX3CR1 signaling by endothelial cells may be a greater contributor to angiogenesis

than signaling by this receptor on hematopoietic cells, however data from other angiogenic models is currently lacking.

In addition to an accumulating body of evidence that fractalkine may play an important role in angiogenesis, several studies have linked fractalkine and its receptor to atherosclerosis susceptibility. Polymorphisms in this gene which reduce the ability of CX3CR1 to bind to its ligand are associated with a reduced risk for atherosclerosis in humans (Lesnik, 2003). In addition CXCL1 expression has been observed in both macrophages and coronary artery smooth muscle cells in the vessels of atherosclerotic but not normal patients (Wong 2003, Lucas 2002). While the role of CX3CR1/CX3CL1 in leading to atherosclerosis is unclear a recent study has demonstrated that the lipid components of low density lipoprotein (LDL) activate peroxisome proliferator-activated receptor- γ (PPAR- γ) in macrophages leading to decreased CCR2 expression (and therefore reduced CCR2-dependent migration) and increased CX3CR1 expression leading to adhesion to the vessel wall (Barlic, 2006). CX3CL1^{-/-} ApoE^{-/-} mice demonstrated no reduction in the number of circulating monocytes, however there was a significant reduction in the number of macrophages which infiltrated the atherosclerotic lesion (Saederup, 2007). This finding suggests a role for CX3CL1 in the capture and retention of monocytes by the endothelium and not in their ability to traffick from the bone marrow to the periphery. Taken together the aforementioned studies indicate a clear role for the CX3CR1/CX3CL1 interaction in the retention of monocytes at inflammatory and ischemic lesions.

1.5 Apolipoprotein A1

ApoA1 is the major protein component of high density lipoprotein (HDL). ApoA1 is synthesized in the liver and small intestine, exported and associates with lipids extracellularly. The primary function of HDL is to promote reverse cholesterol transport from the tissues to bile. HDL-associated ApoA1 is classically regarded as a negative acute-phase protein, a term assigned to serum proteins whose levels are decreased by at least 25% during acute inflammation (Gabay, 1999). In keeping with its anti-inflammatory designation, ApoA1 has been shown to inhibit monocyte activation in vitro (Murphy, 2008). Additionally, gene transfer of ApoA1 led to reductions in macrophage accumulation in several mouse models of atherosclerosis (Tangirala, 1999; Paszty, 1994). Likewise, administration of ApoA1 significantly limited macrophage-related pathology in mouse models of kidney ischemia/reperfusion injury (Shin, 1998), myocardial infarction (Gu, 2007), and stroke (Paterno, 2004).

While the above studies indicate an anti-inflammatory role for ApoA1, recent evidence suggests that the precise relationship of HDL/ApoA1 to the regulation of inflammation may, in fact, be more complex. HDL has been shown to be both pro-inflammatory and anti-inflammatory depending on the model tested. On the whole it is accepted that HDL prevents inflammation in the absence of systemic inflammation but becomes pro-inflammatory in response to systemic inflammation or an acute phase response (Navab, 2005; van Lenten, 1995). For example, when mice were subjected to a viral pneumonia, HDL became pro-inflammatory and resulted in a marked increase in IL-6 production and in macrophage trafficking to the infected areas (van Lenten, 2002). In this same study, when mice were treated with an ApoA1

mimetic peptide, a dramatic reduction in macrophage accumulation and IL-6 production was observed. Interestingly, increased levels of ApoA1 have been observed in the synovial fluid of rheumatoid arthritis patients concomitant with a reduction in serum levels of this protein (Oliviero, 2009; Ananth, 1993). It is unclear whether the redistribution of this protein from the circulation to the inflamed joint is a mechanism to reduce inflammation or if it is, in fact, contributing to the pathology.

With respect to vascular models, one study has demonstrated that ApoA1 stimulates endothelial cell migration in vitro (Seetharam, 2006). Additionally, mice lacking ApoA1 demonstrate impaired revascularization in response to carotid artery injury and this defect is rescued by reconstitution of ApoA1 expression by gene transfer (Seetharam, 2006). Another study revealed that treatment of mice with recombinant HDL post-hindlimb ischemia significantly improves blood flow recovery in an endothelial nitric oxide synthase (eNOS)-dependent fashion (Sumi, 2007). Based on these studies and findings described above, ApoA1 clearly plays a role in the regulation of revascularization as well as macrophage recruitment. In Chapter 2 we will examine the contribution of this protein to the recovery from hindlimb ischemia.

1.6 Summary

This study will center on defining the proangiogenic subsets of monocytes and also exploring the mechanism by which these cells improve angiogenesis. Three main questions will be addressed:

1. What subset of monocytes is angiogenic?
2. What factors released by monocytes accelerate angiogenesis?
3. How do signals generated by ischemia impact the phenotype of endogenous, bone marrow resident monocytes?

In **Chapter 2** we will define the subset of monocytes responsible for angiogenesis and examine the importance of two factors with a previously undetermined role in post-ischemic angiogenesis. In **Chapter 3** we will assess differences between non-ischemic donor and ischemia-conditioned bone marrow monocytes. Finally, in **Chapter 4** we will summarize our results, discuss the significance of this work, and outline future studies.

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Chapter 2

The Inflammatory Subset of Monocytes Potently Stimulates Post-Ischemic Angiogenesis

Portions of this chapter are contained in the following published article:

Capoccia, B.J., Gregory, A.D., Link, D.C. *Recruitment of the Inflammatory Subset of Monocytes to Sites of Ischemia Induces Angiogenesis in an Monocyte Chemoattractant Protein-1 Dependent Fashion.* J Leukoc Biol. 2008 Sep;84(3):760-8.

2.1 ABSTRACT

Monocytes are a widely heterogeneous cell population which has been shown to significantly accelerate angiogenesis in a variety of models. Differences in the ability of various monocyte subsets to mediate revascularization have not been previously investigated. Herein, we address the two subsets of monocytes designated based on differential expression of CX3CR1 and Gr-1, the CX3CR1^{lo}Gr-1⁺ inflammatory subset and the CX3CR1^{hi}Gr-1⁻ resident subset. Using a mouse model for hindlimb ischemia, we demonstrate that an adoptive transfer of the inflammatory subset, but not the resident subset, significantly enhances revascularization post-ischemia. Additionally, we show that the inflammatory subset of monocytes is selectively recruited from the bone marrow to the blood upon induction of ischemia. Accordingly an accumulation of monocytes of the inflammatory subset is observed at the ischemic lesion at all analyzed timepoints post-hindlimb ischemia. Lastly we demonstrate that the efficacy of an adoptive transfer of inflammatory monocytes may be due, at least in part, to increased expression of the fractalkine receptor CX3CR1 as well as to increases in local concentrations of the angiogenic factors MCP-1, VEGF, MMP-9 and ApoA1.

2.2 INTRODUCTION

Peripheral arterial disease is an atherosclerosis-associated syndrome which affects nearly 15% of the United States population over the age of 55 (Weitz, 1996). Critical limb ischemia is a severe manifestation of peripheral arterial disease and is a

leading cause of limb amputation. Various strategies have been used in an effort to restore angiogenesis in the setting of critical limb ischemia, including cytokine and cell therapies. Treatment with growth factors alone, namely VEGF (Rajagopalan, 2003) and rFGF (Lederman, 2002) has demonstrated little efficacy in clinical trials. Based on the assumption that treatment with a single growth factor is unlikely to recapitulate all the signals necessary for angiogenesis, recent studies have focused on the ability of hematopoietic cell-based therapies to mediate revascularization. Notably, several studies have demonstrated that intramuscular injections of bone marrow mononuclear cells (BM-MNCs) or apheresed, G-CSF-mobilized peripheral blood-mononuclear cells (PB-MNCs) can improve revascularization as assessed by several indicators including improvement of ischemic ulcers, increase in maximum walking distance, and a reduction in amputations (Al Mheid, 2008). Mobilization of the patients' own mononuclear cells via G-CSF (Arai, 2006) or GM-CSF (van Royen, 2005) has likewise shown promise as a treatment for critical limb ischemia. However, as a non-specific and widespread mobilization or local injection of a heterogeneous population of inflammatory cells may have deleterious side effects, particularly within the context of atherosclerosis, there exists a demand for cell-based therapies in which a small population of highly angiogenic cells is targeted to the ischemic tissue.

Recent studies by our laboratory have demonstrated that of the major cell types contained within the mononuclear cell fraction, monocytes exhibit the most potent angiogenic activity (Capoccia, 2006). Monocytes play a variety of roles in the angiogenic process, including the secretion of a variety of factors including VEGF,

bFGF, TGF- α , GM-CSF, IL-8, and PDGF. These factors promote proliferation and migration of endothelial cells as well as tube formation (Sunderkotter, 1994). Activation of endothelial cells by these cytokines induces production of VEGF and MCP-1, providing a positive amplification loop leading to the recruitment of additional monocytes (Shireman, 2007). Additionally, monocytes express metalloproteases and serine proteases which play a critical role in degradation of the extracellular matrix, thus generating an appropriate scaffolding upon which new vessels can form (Moldovan, 2005). Mice subjected to ischemia exhibit a marked endogenous monocytic response, consisting of increased production in the bone marrow and trafficking to the lesion (Capoccia, 2008). Additionally, ablation of macrophages is associated with a significant reduction in post-ischemic angiogenesis (Heil, 2002; DiPietro, 1993; Brechot, 2008). Therefore a determination of the mechanisms governing monocyte recruitment and retention at ischemic sites as well as an understanding of what constitutes a proangiogenic phenotype may reveal important therapeutic strategies for the treatment of ischemic disorders.

Geissmann and colleagues described two unique monocytic populations present in the bone marrow and blood based on differential expression of the fractalkine receptor, CX3CR1, and Gr-1 (2003). The fractalkine receptor CX3CR1 binds to fractalkine ligand (CX3CL1) and this interaction promotes adhesion. Both CX3CR1 and its ligand are expressed on hematopoietic cells, including monocytes, as well as endothelial cells. Cells with a CX3CR1^{lo}Gr-1⁺ phenotype were named “inflammatory” based on the observation that these cells traffick from the bone marrow to the peritoneum in response to intraperitoneal injections of thioglycolate.

In contrast, cells exhibiting the CX3CR1^{hi}Gr-1⁻ phenotype, did not respond to challenge with thioglycolate and were thus referred to as the “resident” subset. A recent study has indicated that cells of the inflammatory subset may convert into resident cells in the periphery, however the precise relationship between these subsets is not fully defined (Tacke, 2006).

Herein, we provide evidence that the inflammatory subset of monocytes is preferentially recruited from the bone marrow to the peripheral blood to the site of ischemia. In accordance with this finding, an adoptive transfer inflammatory monocytes significantly improves revascularization post-hindlimb ischemia (HLI). This improvement is associated with an increased influx of endogenous monocytes to the ischemic lesion as well as increased local production of the known angiogenic factors vascular endothelial growth factor-A (VEGF-A), monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-9 (MMP-9), as well as of apolipoprotein A1 (ApoA1). Additionally, we show that an adoptive transfer of ApoA1^{-/-} inflammatory monocytes does not improve revascularization post-HLI, demonstrating for the first time an important role for this protein in mediating post-ischemic angiogenesis.

2.3 MATERIALS AND METHODS

Mice. ApoA1^{-/-} mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). CX3CR1^{GFP/+} and CX3CR1^{GFP/GFP} mice on a C57BL/6 background were a generous gift from Dr. Dan Littman (Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY, USA). Mice were housed in a specific pathogen-free environment. The Washington University Animal Studies Committee (St. Louis, MO, USA) approved all experiments.

Murine hindlimb ischemia model. The hindlimb ischemia surgical procedure was performed as described previously (Couffinal, 1998). In brief, an incision was made in the skin at the mid-portion of the right hindlimb overlying the femoral artery, and the femoral artery and vein were then dissected free from the nerve, and the proximal portion of the femoral artery and vein ligated with 6-0 silk sutures. The distal portion of the saphenous artery and vein and remaining arterial and venous side branches were ligated, followed by their complete excision from the hindlimb. The overlying skin was then closed using Nexaband veterinary glue (Abbott Animal Health, Abbott Park, IL, USA).

Laser Doppler perfusion imaging. Blood perfusion in the hindlimb was monitored by laser Doppler imaging (MoorLDI-2, Moor Instruments, UK). Before initiating scanning, mice were anesthetized with ketamine/xylazine and placed on a heating plate at 37°C to minimize temperature variations. For each time-point, the laser

Doppler image obtained was analyzed by averaging the perfusion, expressed as the relative unit of flux as determined by Moor Instruments, over the surface of the ischemic and nonischemic foot. To control for ambient light and temperature, calculated perfusion was expressed as the flux ratio between the ischemic and nonischemic limbs.

Inflammatory and resident monocyte subset isolation. Bone marrow mononuclear cells from CX3CR1^{GFP/+} mice were incubated at 4°C with PE-conjugated Gr-1 antibody (PharMingen). CX3CR1^{lo}Gr-1⁺ and CX3CR1^{hi}Gr-1⁻ monocytes were isolated using a MoFlo high-speed flow cytometer (Dako Cytomation, Fort Collins, CO, USA).

Adoptive transfer of CFSE-labeled cells. Bone marrow was harvested from the femurs of donor mice, and mononuclear cells were isolated by centrifugation across a 1.011 density gradient (Histopaque, Sigma-Aldrich, St. Louis, MO, USA) at 1700 x g for 30 min. Mononuclear cells were then incubated with 2.5µM CFSE in PBS for 10 min at 37°C. CFSE-labeled mononuclear cells (1x10⁶) were administered i.v. into recipient mice 24 h after the induction of hindlimb ischemia; this cell dose is the minimum number that consistently stimulated angiogenesis in the hindlimb ischemia model.

Flow cytometry. The adductor muscle from ischemic and nonischemic hindlimbs was surgically isolated after hindlimb ischemia and then treated with 3 mg/ml Type I collagenase (Worthington Biomedical Corp., Lakewood, NJ, USA) for 40 min at 37°C. After filtering through a 50-µm cell strainer (Partec, Munster, Germany), cells

were incubated with Fc block (Miltenyi Biotec, Auburn, CA, USA) for 10 min at 4°C, followed by incubation with PE-conjugated antibodies to Gr-1, F4/80, CD3, B220, or NK1.1 (PharMingen, San Diego, CA, USA). Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data are reported as the total number of the indicated cell type recovered from an entire adductor muscle.

Unbiased screen for soluble factors. Following digestion of adductor muscle with collagenase to generate a single-cell suspension, cells and debris were removed by centrifugation at 500 x g for 5 min. The cell-free tissue supernatant was recovered and analyzed using a multiplexed ELISA assay for 58 unique soluble factors (Rules Based Medicine, Austin, TX, USA).

Statistical analysis. Statistical significance was determined by a two-way ANOVA analysis or by a two-sided Student's *t*-test.

2.4 RESULTS

2.4.1 Monocytes are recruited to ischemic tissue post-HLI. In order to determine if adoptively transferred monocytes home to the ischemic lesion, donor BM-MNCs were labeled with CFSE and were injected intravenously into ischemic recipients at 24 hours post-HLI. The ischemic adductor muscle was harvested at several time points post-ischemia, homogenized, and cell populations contained in this fraction were analyzed by flow cytometry. As shown in Figure 2.1A, adoptively transferred

monocytes to home to ischemic tissue, with a peak influx of 2.5×10^4 cells observed at 24 hours post-adoptive transfer. Likewise, contaminating neutrophils present in BM-MNC preparations were observed to home to the ischemic adductor muscle (Figure 2.1B). Interestingly, the adoptive transfer of BM-MNCs lead to increased recruitment of CFSE- endogenous monocytes to the ischemic adductor muscle with peak numbers observed at 24 hours post-adoptive transfer (Figure 2.1.C). These data indicate that both monocytes and neutrophils contained within the adoptively transferred BM-MNC fraction home to the site of ischemia, and that a specific recruitment of additional endogenous monocytes is observed.

2.4.2 An adoptive transfer of the inflammatory, but not resident, subset of

monocytes significantly improves revascularization post-HLI. The recent designation of the inflammatory and resident subsets of monocytes is based on the ability of these subsets to traffick in response to thioglycollate. The contribution of these particular subsets in responding to alternative signals, such as ischemia, has not been previously examined. In order to determine if the inflammatory and resident monocyte subsets differ in their ability to promote post-ischemic angiogenesis, we sort-purified these populations and adoptively transferred them into ischemic recipients. Revascularization of ischemic recipients was assessed over a 14-day time course using laser Doppler imaging. While an adoptive transfer of resident monocytes showed no improvement over PBS-treated controls, an adoptive transfer of inflammatory monocytes lead to a significant acceleration of revascularization (Figure 2.2). This finding indicates that the $CX3CR1^{lo}Gr-1^{+}$ inflammatory subset is

uniquely responsible for the enhanced revascularization observed upon an adoptive transfer of unfractionated monocytes.

2.4.3 The inflammatory subset of monocytes is selectively mobilized from the bone marrow into the blood post-HLI. Having determined that an adoptive transfer of inflammatory monocytes significantly increases revascularization post-HLI, we next assessed the contribution of inflammatory and resident monocyte subsets to the endogenous response to ischemia. At baseline, the resident monocyte subset dominates in the peripheral blood while, the inflammatory subset comprises the majority of bone marrow monocytes. However, in response to hindlimb ischemia, an increase in the number of inflammatory cells in the blood is observed (Figure 2.3A) concomitant with an increase in the production of this subset in the bone marrow (Figure 2.3B). These data indicate that the inflammatory subset of monocytes is selectively mobilized from the bone marrow into the peripheral blood in response to hindlimb ischemia.

2.4.4 Monocytes of the inflammatory subset accumulate at the ischemic lesion post-HLI. As monocytes of the inflammatory subset preferentially mobilize from the bone marrow to the blood in response to hindlimb ischemia, we next asked if this subset likewise accumulates at the ischemic lesion. In fact, the monocytic infiltrate in the ischemic adductor muscle was largely comprised of the inflammatory subset. Around 10^6 inflammatory monocytes were observed at the peak time of influx, whereas levels of resident monocytes peaked at only around 10^5 cells (Figure 2.4).

This data suggests that inflammatory monocytes are the major subset mediating repair at the site of ischemia in this model. This finding contrasts with chronic models of ischemia in which a bimodal “switch” from inflammatory to resident monocyte infiltrate is observed (Nahrendorf, 2007; Arnold, 2007).

2.4.5 Bone marrow resident monocytes decrease expression of CX3CR1 upon induction of ischemia and CX3CR1^{-/-} monocytes exhibit a reduced ability to improve angiogenesis. In order to understand why the trafficking of a small number of adoptively-transferred donor monocytes, representing only 0.1-1% of the total monocytes present in the ischemic lesion, so potently accelerates revascularization we set out to assess phenotypic differences between donor monocytes and endogenous monocytes. Interestingly, we observed that upon induction of ischemia, expression of the fractalkine receptor CX3CR1 is markedly reduced (Figure 2.5A). As CX3CR1 is known to play a role in adhesion of monocytes to endothelium and also in stimulating endothelium to produce a variety of angiogenic factors, we set out to assess if the downregulation of CX3CR1 which occurs as monocytes leave the bone marrow impairs the downstream angiogenic capacity of these cells. To test this, we sort-purified monocytes from a CX3CR1^{-/-} donor and adoptively transferred these cells into ischemic recipients. In fact, these cells did exhibit a slight, but statistically significant, defect in accelerating revascularization compared to wild type controls (Figure 2.5B). This finding suggests that the loss of CX3CR1 expression that occurs as monocytes are mobilized from the bone marrow to the periphery may partially explain the loss of angiogenic capacity of these cells compared to donor cells which express high levels of this receptor.

2.4.6. Local production of VEGF, MCP-1, MMP-9, and apolipoprotein A1 is increased post-adoptive transfer. As CX3CR1 expression in endogenous monocytes appears to play only a partial role in mediating the proangiogenic effects of monocytes, we next performed an unbiased screen to determine what factors are produced locally which may impact revascularization. An unbiased ELISA-based screen for 58 unique soluble factors was performed on cell-free muscle supernatants obtained from the ischemic adductor muscles of mice undergoing HLI only (poor blood flow recovery) and from mice receiving HLI plus an adoptive transfer of BM-MNCs (improved blood flow recovery). The screen indicated that levels of the proangiogenic factors VEGF (Figure 2.6A), MCP-1 (Figure 2.6B), and MMP-9 (Figure 2.6C) as well as the major protein component of HDL, apolipoprotein A1 (Figure 2.6D) were increased upon adoptive transfer. As VEGF, MCP-1, and MMP-9 have well-described roles in post-ischemic angiogenesis, we decided to examine the importance of apolipoprotein A1 in mediating the potent pro-angiogenic effect of a monocytic adoptive transfer.

2.4.7. An adoptive transfer of apolipoprotein A1-deficient monocytes does not improve revascularization post-HLI. ApoA1 is the major protein component of HDL known to be largely synthesized by hepatocytes in the liver and then exported into the circulation where it assembles with the lipid components of HDL (ref). The ApoA1 lipid complex classically is known to play an important role in reverse cholesterol transport. Synthesis of ApoA1 by monocytes and its role in post-ischemic

angiogenesis has not been previously described. In order to determine if production of ApoA1 by monocytes is necessary for monocytes to mediate their angiogenic effect, we sort-purified inflammatory monocytes from ApoA1^{-/-} mice and adoptively transferred them into ischemic recipients. Interestingly, an adoptive transfer of monocytes lacking ApoA1 was not sufficient to enhance revascularization compared to wild type controls (Figure 2.7). This data indicates an essential role for ApoA1 in the angiogenic repair capacity of monocytes.

2.5 DISCUSSION

BM-MNCs have demonstrated efficacy in the treatment of a variety of ischemic disorders in both mice and humans. Many of these studies have centered on the role of progenitor populations present in the BM-MNC fraction and their ability to differentiate into endothelial cells. However, recent work by our laboratory has demonstrated that the monocyte fraction contained within BM-MNCs potently enhances revascularization following hindlimb ischemia (Capoccia, 2006). We observed that adoptively transferred monocytes home to the site of ischemia and also recruit additional endogenous monocytes to the lesion (Figure 2.1).

A further examination of monocyte subsets revealed that only the inflammatory and not the resident subset of monocytes is competent to accelerate revascularization (Figure 2.2). Additionally, the inflammatory subset of monocytes likewise is selectively recruited from the bone marrow into the blood upon induction of ischemia and this is the major subset that accumulates at the ischemic lesion at all time points post-HLI. In several other models including myocardial infarction

(Nahrendorf, 2007) as well as a muscle injury model (Arnold, 2007), inflammatory monocytes dominate the early time points while the resident population comprises the majority of the infiltrate at late timepoints. Interestingly, Tie2-expressing monocytes (TEMs), thought to be potently angiogenic in tumor models, are thought to fall within the resident Gr-1⁺ subset of monocytes (Venneri, 2007). Our finding underscores the potential differences in tumor versus ischemia-driven angiogenesis. These data provide the first evidence that the CX3CR1^{lo}Gr-1⁺ inflammatory subset is the major contributor to angiogenic repair in response to acute ischemia.

CX3CR1 is the receptor for the fractalkine ligand CX3CL1 which is expressed as a transmembrane protein on a mucin-like stalk. Interaction of CX3CR1 with this membrane-bound ligand allows for integrin-independent adhesion. The membrane-bound form of CX3CL1 can also be cleaved by metalloproteases generating free CX3CL1 which can then act as a chemoattractant. CX3CR1^{-/-} and CX3CL1^{-/-} mice both demonstrate a reduction in circulating resident monocytes and normal numbers of inflammatory monocytes. A recent study has demonstrated that this is due to the critical Bcl2-dependent survival signals generated by the CX3CR1/CX3CL1 interaction in the resident monocytes (Landsman, 2009). Interestingly, local injections of CX3CL1 lead to improved blood flow recovery in a rat model of hindlimb ischemia (Ryu, 2008). As CX3CR1 expression was markedly decreased in bone marrow monocytes post-HLI, but intact in donor monocytes from mice that did not receive surgery, we examined the possible contribution of this receptor in mounting the angiogenic response. An adoptive transfer of CX3CR1^{-/-} monocytes showed a modest, but significant, decrease in revascularization compared

to CX3CR1-sufficient controls (Figure 2.5). Further studies will be necessary to determine if this defect in revascularization is due to a decreased ability of CX3CR1^{-/-} cells to home to the ischemic lesion, to attach to damaged endothelium via the CX3CR1/CX3CL1 interaction, or to survive at the inflammatory site.

As the loss of CX3CR1 only appeared to have a minor effect on the revascularization potential of monocytes, we looked for other ways in which non-ischemic donor monocytes may have such a profound impact on the revascularization of ischemic recipients. To accomplish this, an unbiased screen for 58 unique soluble factors was performed on adductor muscle supernatant from mice undergoing HLI with and without an adoptive transfer. Interestingly, the potentially angiogenic factors VEGF, MCP-1, and MMP-9 were all found to be upregulated post-adoptive transfer. The role of VEGF in recruiting macrophages which contribute to angiogenesis has been extensively described. For example a host of experiments using neutralizing antibodies or soluble receptors demonstrated that VEGF is a major contributor to post-ischemic as well as tumor angiogenesis (Cursifen, 2004; Frank, 1995; Adamis, 1996). Based upon the critical role of this factor in angiogenesis, the increased production of VEGF observed upon adoptive transfer likely is a large contributor to the accelerated angiogenesis observed in our model.

Like VEGF, MCP-1 is likewise a well-known regulator of angiogenesis. MCP-1 is secreted from monocyte/macrophages, smooth muscle cells, and endothelial cells within ischemic tissue (Goede, 1999; Lakshminarayanan, 2001). In addition, MCP-1 has been shown to directly induce VEGFA expression from endothelial cells (Hong, 2005). The addition of recombinant MCP-1 or its overexpression by gene

transfer has been shown to significantly enhance revascularization in ischemic tissues which is associated with increased macrophage recruitment (Ito, 1997; Muhs, 2004; Schwarz, 2004). MCP-1^{-/-} mice had impaired monocyte recruitment and blood flow recovery after hindlimb ischemia, which could be reversed by local treatment with purified MCP-1 protein (Vosukil, 2004).

MMP-9 belongs to a family of zinc-containing endopeptidases which play a role in vascular remodeling by degrading the extracellular matrix (Heissig, 2003). MMP-9 has been shown to mobilize endothelial progenitor cells (EPCs) from the bone marrow niche (Hessig, 2002) and to recruit bone marrow-derived leukocytes to tumors (Jodele, 2005). Secretion of MMP-9 by tumor associated macrophages (TAMs) is a major signal governing tumor angiogenesis in several models (Coussens, 2004; Giraudo, 2004; Dirkx, 2006). The increased levels of MMP-9 observed post-adoptive transfer demonstrate that in addition to its importance in tumor models, this proteinase likewise plays a critical role in repairing post-ischemic injury.

Apolipoprotein A1 (ApoA1) is the major protein component of the high density lipoprotein (HDL) complex. While largely described as an anti-inflammatory regulator, recent evidence suggests that ApoA1 may exhibit anti-inflammatory functions at the steady state but pro-inflammatory functions under conditions of systemic inflammation (Navab, 2005). In our model of hindlimb ischemia, ApoA1 is clearly protective as markedly increased levels are observed post-adoptive transfer and an adoptive transfer of ApoA1^{-/-} monocytes does not improve revascularization. Interestingly, ApoA1 is known to be secreted by hepatocytes and some cells of the small intestine, and its production by macrophages has not been previously described.

Further studies will be required to determine if monocyte/macrophages do, in fact, secrete ApoA1, or if alternatively, macrophage development in an ApoA1-deficient setting leads to phenotypic changes which limits their pro-angiogenic capacity.

Taken together, these data identify a specific subset of monocytes which potently enhances angiogenesis in an adoptive transfer setting and describe several factors which are significantly increased in association with this adoptive transfer. Future studies will be required to delineate the specific mechanism by which an adoptive transfer of a small proportion of monocytes leads to significantly increased blood flow recovery. A better understanding of this process may unveil promising therapeutic strategies for a variety of ischemic disorders.

Figure 2.1

Adoptively transferred donor monocytes home to the site of ischemia and a concomitant recruitment of additional endogenous monocytes is observed. Ficoll-purified BM-MNCs were harvested from nonischemic donor mice, labeled with CFSE, and 2×10^6 cells were adoptively transferred into ischemic recipients at 24 hours post-HLI. The ischemic adductor muscle was harvested at the times indicated, subjected to collagenase digestion and homogenization, and analyzed by flow cytometry. The absolute number of CFSE⁺F4/80⁺ donor monocytes (A), CFSE⁻F4/80⁺ endogenous monocytes (B), CFSE⁺Gr-1^{hi} donor neutrophils (C), and CFSE⁻Gr-1^{hi} endogenous neutrophils (D) was determined by multiplying the percentages of cells obtained by flow cytometry by hemacytometer counts of whole adductor muscle.

Figure 2.2

An adoptive transfer of the inflammatory, but not resident, subset of monocytes significantly improves revascularization post-HLI. Monocytes of the CX3CR1^{lo}Gr-1⁺ inflammatory and CX3CR1^{hi}Gr-1⁻ resident subsets were sort-purified from CX3CR1^{+/GFP} mice. 2×10^5 sorted monocytes were adoptively transferred via a tail vein injection into ischemic recipients at 24 hours post-HLI. The blood flow ratio of the ischemic versus nonischemic leg was assessed using laser Doppler imaging over a 14 day time course.

Figure 2.3.

The inflammatory subset of monocytes is selectively mobilized from the bone marrow into the blood post-HLI. Hindlimb ischemia surgeries were performed in CX3CR1^{GFP/+} mice and peripheral blood (A) and bone marrow (B) were harvested at the times indicated. Percentages of the inflammatory (GFP^{lo}Gr-1⁺) or resident (GFP^{hi}Gr-1⁻) subsets were identified by flow cytometry and multiplied by the total white blood count in order to determine the absolute number of monocytes present.

Figure 2.4

Monocytes of the inflammatory subset accumulate at the ischemic lesion post-HLI. CX3CR1^{GFP/+} mice were subjected to hindlimb ischemia and the ischemic adductor muscles were harvested at the indicated time points. Muscles were homogenized and the total number of inflammatory (GFP^{lo}Gr-1⁺) and resident (GFP^{lo}Gr-1⁻) monocytes were quantified using percentages obtained using flow cytometry multiplied by absolute cell numbers obtained by hemacytometer counts (n=3-5).

Figure 2.5

Expression of CX3CR1 is decreased upon induction of ischemia and CX3CR1^{-/-} monocytes demonstrate a reduced capacity to stimulate angiogenesis. (A) Whole bone marrow was prepared from CX3CR1^{+/GFP} mice at baseline or at 24 hours post-HLI. Cells were stained for Gr-1 and analyzed using flow cytometry. (B) The CX3CR1^{lo}Gr-1⁺ inflammatory monocyte subset was sort purified from the bone marrow of a CX3CR1^{-/-} donor and 2x10⁵ cells were adoptively transferred into ischemic recipients at 24 hours post-HLI. Mice were analyzed using laser Doppler scanning over a 14-day time course.

Figure 2.6.

Local production of VEGF, MCP-1, MMP-9, and apolipoprotein A1 is increased post-adoptive transfer. Ischemic adductor muscles from ischemic mice with (dashed bars) or without (solid bars) an adoptive transfer of 2x10⁶ BM-MNCs were harvested. Muscles were treated with collagenase and homogenized as described in Methods and the cell free supernatant was obtained. Cell free muscle supernatants were subjected to a multiplex ELISA-based screen for 58 unique soluble factors. Levels of VEGF (A), MCP-1 (B), MMP-9 (C) and apolipoprotein A1 (D) were upregulated locally in mice receiving adoptive transfer. (n=1-2 pooled samples, representing 3-6 mice, respectively).

Figure 2.7

An adoptive transfer of apolipoprotein A1-deficient monocytes does not improve revascularization post-HLI The inflammatory subset of monocytes was sorted from ApoA1^{-/-} mice based on CD115⁺Gr-1⁺ expression. 2x10⁵ of these sorted cells were adoptively transferred into ischemic recipients at 24 hours post-HLI. The blood flow ratio of the ischemic versus nonischemic leg was assessed over a 14 day time course using laser Doppler imaging.

Figure 2.1

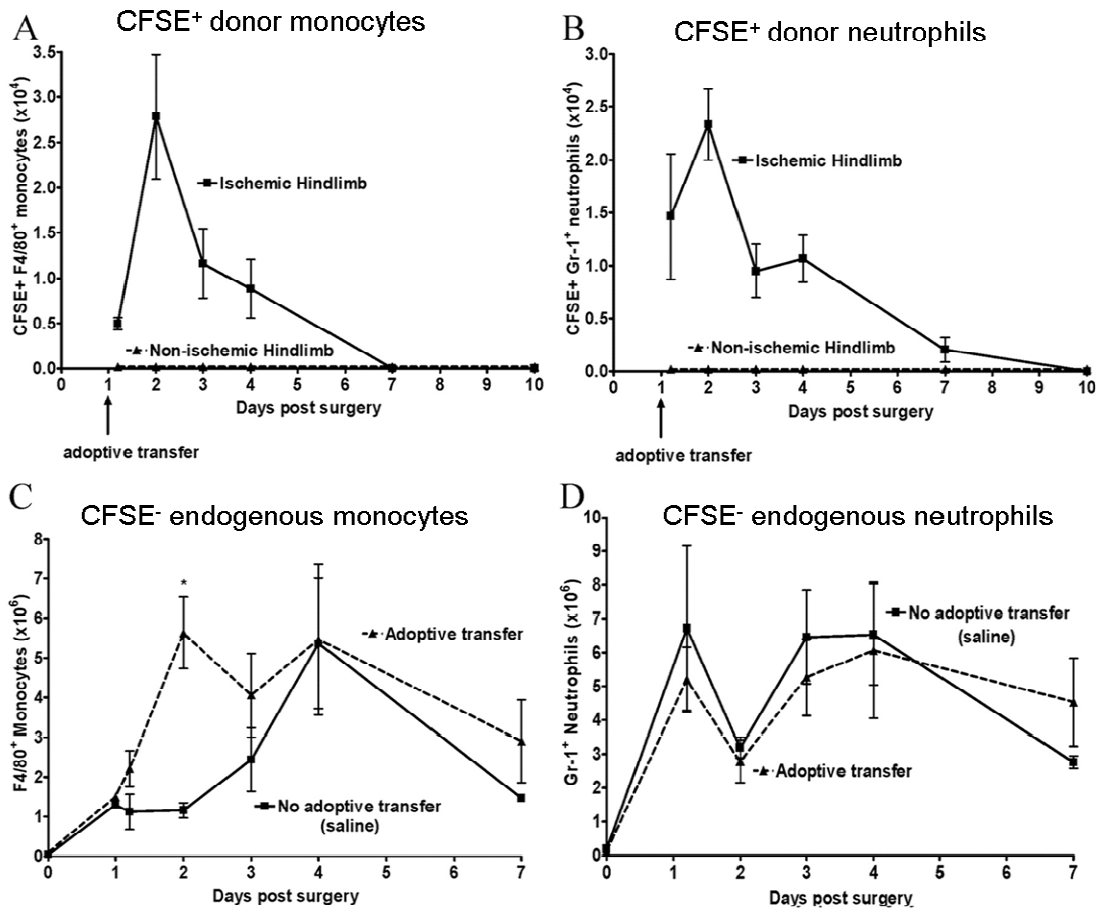


Figure 2.2

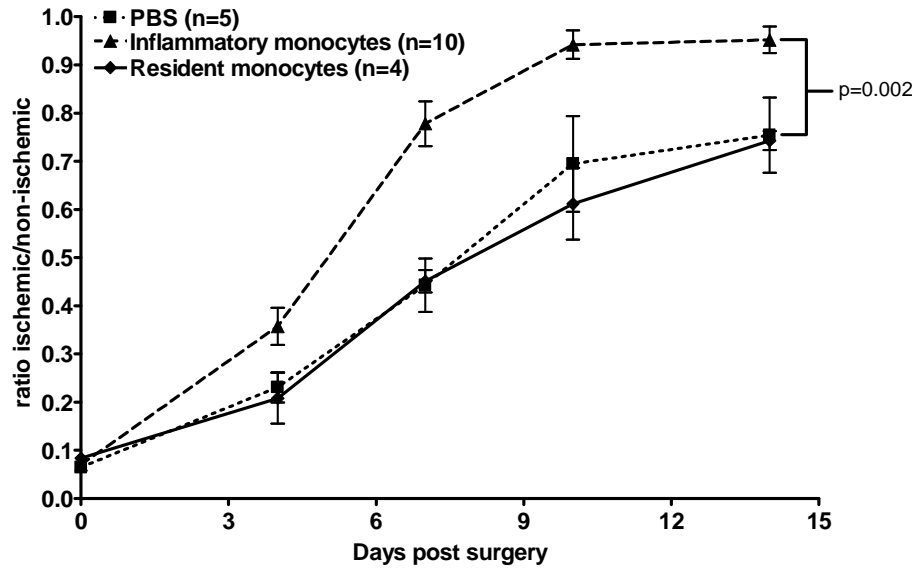


Figure 2.3

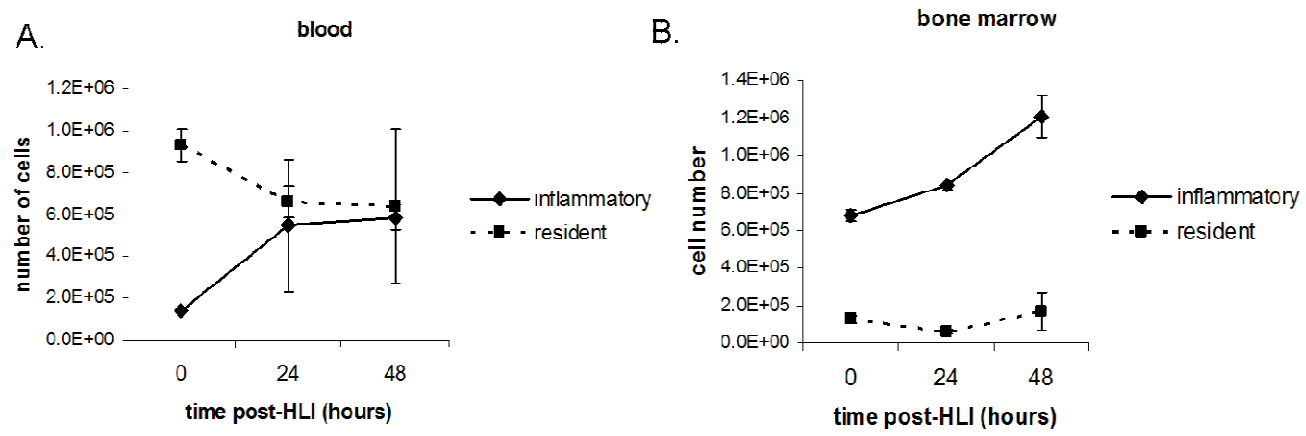


Figure 2.4

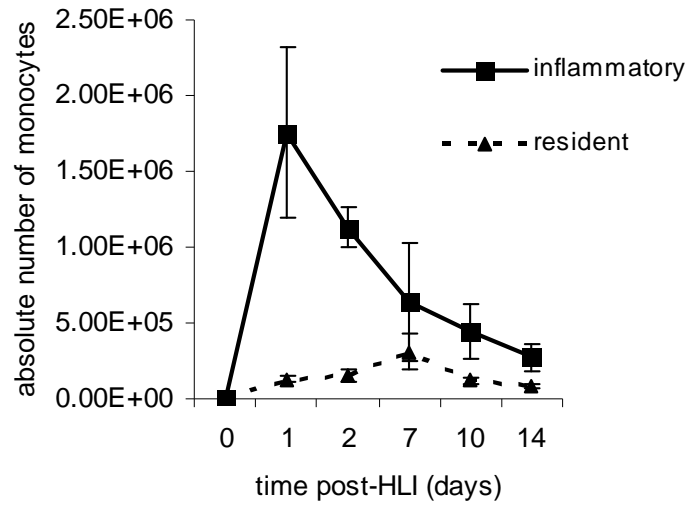
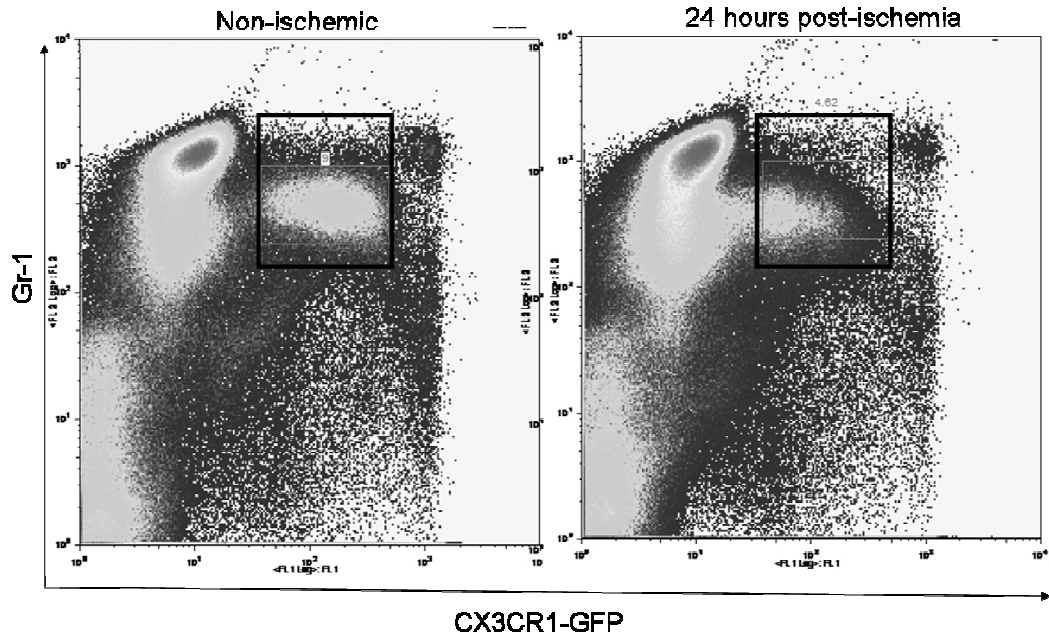


Figure 2.5

A



B.

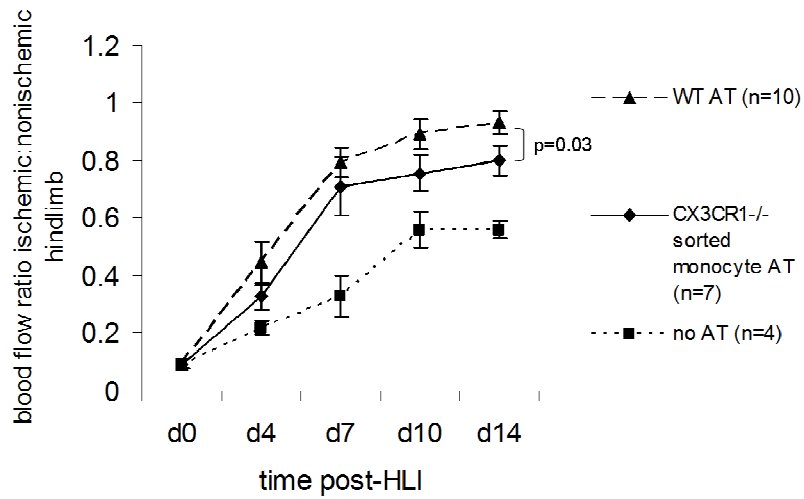


Figure 2.6

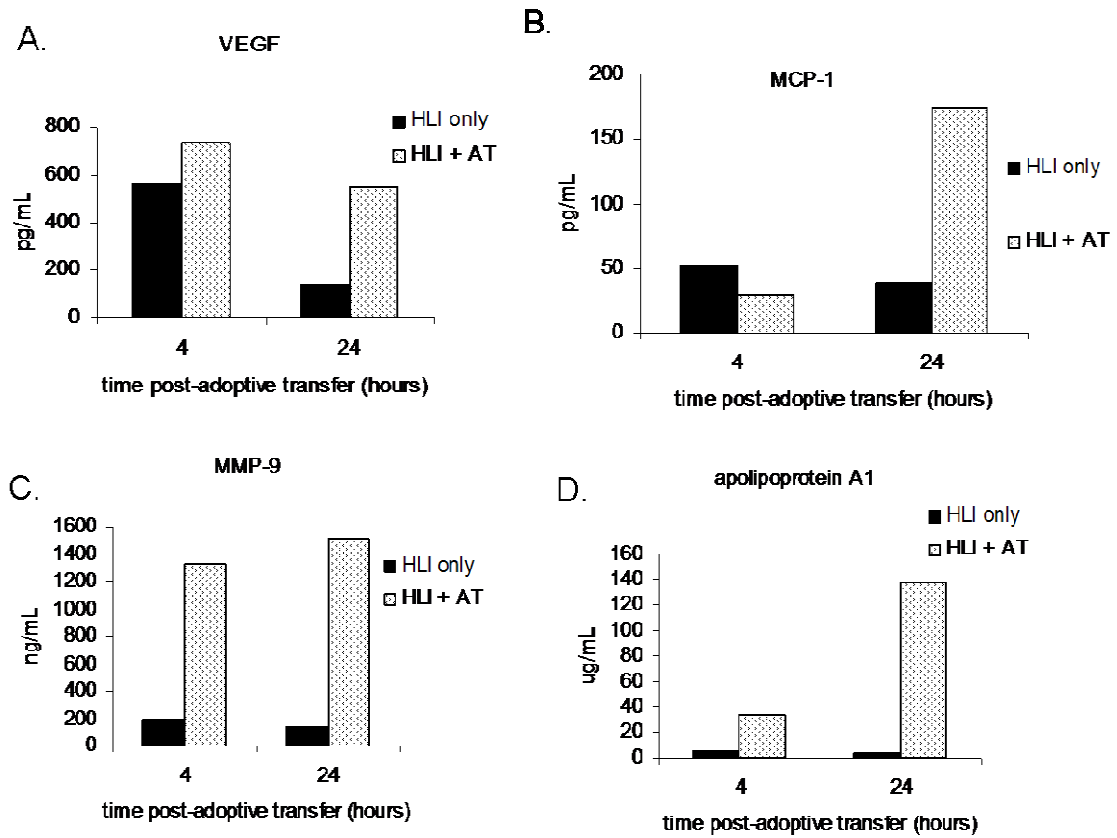
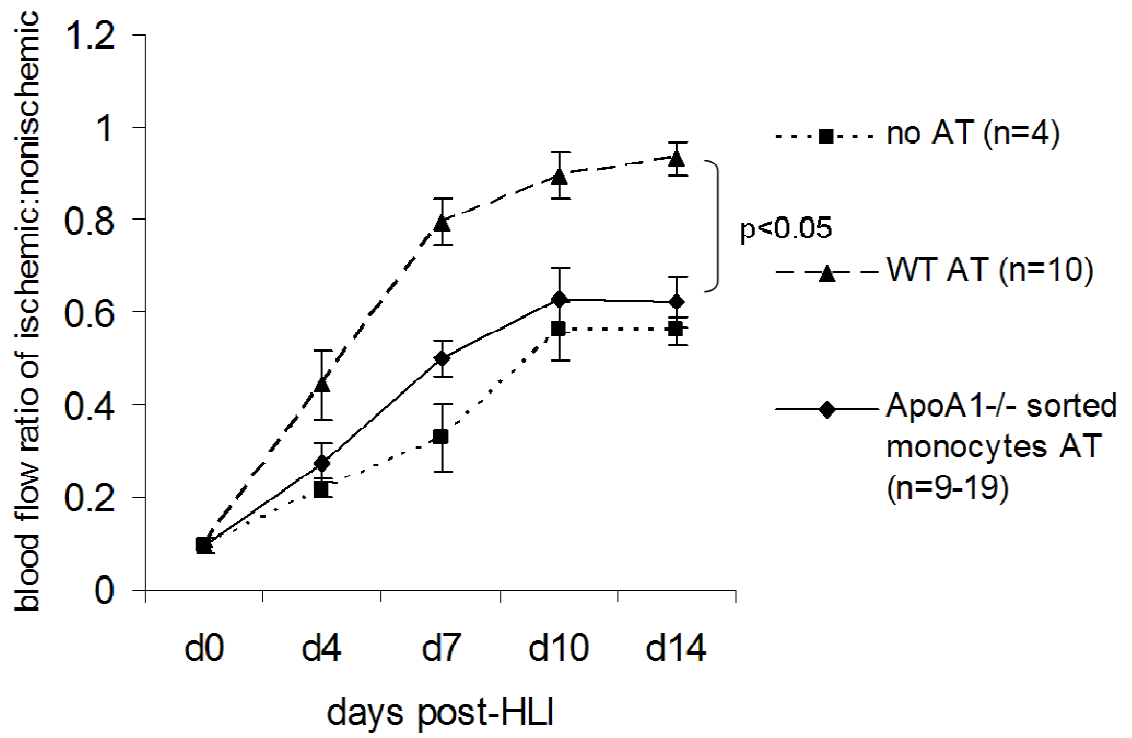


Figure 2.7



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Chapter 3

Systemic Signals Generated by Interleukin-6 (IL-6) and Granulocyte-Colony Stimulating Factor (G-CSF) Influence the Angiogenic Capacity of Bone Marrow Resident Monocytes

This work has been submitted in the following manuscript:

Gregory, A.D., Capoccia, B. J., Link, D.C. *Systemic Signals Generated by IL-6 and G-CSF Influence the Angiogenic Capacity of Bone Marrow Resident Monocytes.*
Submitted.

3.1 ABSTRACT

There is considerable interest in the potential of cell-based approaches to mediate therapeutic angiogenesis for acute and chronic vascular syndromes. Using a mouse model of hindlimb ischemia, we previously showed that adoptive transfer of a small number of donor monocytes significantly enhanced revascularization. Herein, we provide data suggesting the bone marrow resident monocytes sense systemic signals that influence their future functional capacity. Specifically, following induction of distant ischemia, the angiogenic capacity of bone marrow resident monocytes is markedly reduced. We provide evidence that granulocyte-colony stimulating factor (G-CSF) and interleukin-6 (IL-6) represent such “conditioning” signals. Systemic levels of G-CSF and IL-6 are significantly increased following induction of hindlimb ischemia. Accordingly, bone marrow resident monocytes from ischemic mice exhibited increased STAT3 phosphorylation and STAT3 target gene expression. Finally, G-CSF receptor^{-/-} and IL-6^{-/-} mice were resistant to the deleterious effects of ischemic conditioning on monocyte angiogenic potential. RNA expression profiling suggested that ischemia-conditioned monocytes in the bone marrow are polarized towards expression of M2-associated genes. Consistent with this observation, M2-skewed monocytes from SHIP^{-/-} mice also had impaired angiogenic capacity. Collectively, these data show that G-CSF and IL-6 provide signals that determine the angiogenic potential of bone marrow resident monocytes.

3.2 INTRODUCTION

The bone marrow represents a rich reservoir of cells that are able to stimulate angiogenesis. Delivery of bone marrow cells to sites of ischemia, either by direct injection into ischemic tissue (1) or by mobilization of bone marrow cells into the blood (2), has been shown to stimulate angiogenesis in animal models. Moreover, several clinical trials have utilized direct injection of bone marrow cells to stimulate revascularization. Though preliminary, there is evidence that this approach may have modest clinical benefit in patients with acute myocardial infarction (3) or peripheral vascular disease (4). Importantly, the bone marrow is comprised of many cell types, and the cell population(s) that stimulate angiogenesis have not been fully defined. The identification of the relevant cell populations and a better understanding of the signals that regulate their angiogenic activity may lead to improved strategies for cell-based therapeutic angiogenesis.

Within the bone marrow, cell populations with known angiogenic activity include monocytes, endothelial cells and natural killer cells (5). Accumulating evidence suggests that monocytes may be the key bone marrow cell type mediating angiogenesis upon delivery to sites of ischemia. Mice subjected to ischemia exhibit a marked endogenous monocytic response, consisting of increased production in the bone marrow and trafficking to the lesion (6). Monocytes are thought to stimulate angiogenesis through secretion of angiogenic growth factors, degradation of extracellular matrix by the release of proteases, and increases in vascular permeability by the deposition of fibrin. Accordingly, ablation of macrophages is associated with a significant reduction in post-ischemic angiogenesis (7-9). Conversely, we recently

showed that early delivery of bone marrow monocytes to sites of ischemia markedly enhanced reperfusion in a murine model of acute hindlimb ischemia (6).

The cytokines granulocyte-colony stimulating factor (G-CSF) and interleukin-6 (IL-6) are known to provide important survival and activation signals to monocyte/macrophages, acting through signal transducer and activator of transcription 3 (STAT3) signaling intermediates (10). IL-6 and G-CSF are induced in several ischemic settings including a murine model of ischemic acute kidney injury (11) and a rat model of gut ischemia/reperfusion (12). Likewise, levels of these cytokines have been shown to be elevated in a variety of ischemic disorders in patients, including severe heart failure patients (13, 14) and vascular surgery-associated ischemia (15). The effect of these cytokines on the bone marrow compartment has not previously been investigated.

Herein, we demonstrate that the addition of only a small proportion of adoptively transferred monocytes from a non-ischemic donor significantly enhances revascularization post-hindlimb ischemia (HLI). Based on this finding we set out to assess differences in proangiogenic capacity between endogenous ischemia-conditioned monocytes and donor basal monocytes. We demonstrate that ischemia-conditioned monocytes are unable to enhance revascularization post-HLI and that this defect is dependent upon signaling by G-CSF and IL-6. Additionally, bone marrow resident monocytes subjected to ischemic signals exhibit reduced expression of classical M1 genes and increased expression of M2 genes. Accordingly, an adoptive transfer of M2-skewed monocytes obtained from Src homology 2 domain-containing inositol 5'-phosphatase 1^{-/-} (SHIP^{-/-}) mice does not improve blood flow recovery

post-HLI. This study provides the first evidence that systemic signals generated by ischemia can lead to functional alterations in bone marrow resident monocytes and has important implications for cell-based therapeutic angiogenesis approaches.

3.3 MATERIALS AND METHODS

Mice. IL-6^{-/-} on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). CX3CR1^{GFP/+} mice on a C57BL/6 background were a generous gift from Dr. Dan Littman (Skirball Institute of Biomolecular Medicine, New York School of Medicine, New York, NY). SHIP^{-/-} mice on a C57BL/6 background were a generous gift from Dr. F. Patrick Ross (Washington University School of Medicine, St. Louis, MO). G-CSFR^{-/-} mice on a C57BL/6 background were generated as previously described (43). Mice were housed in a specific pathogen-free environment. The Washington University Animal Studies Committee (St. Louis, MO) approved all experiments.

Murine hindlimb ischemia model. Mice were anesthetized by an intraperitoneal injection of a solution of 87 mg/kg ketamine; 13 mg/kg xylazine. An incision was made at the mid-portion of the right hindlimb proximal to the femoral artery and the femoral artery and vein were dissected free from the nerve. The proximal portion of the femoral artery and vein were ligated with Perma-Hand taper point (6-0) nonabsorbable silk surgical sutures (Ethicon, Summerville, NJ). The distal portion of the saphenous artery and vein and remaining arterial and venous side branches were

ligated and then excised from the hindlimb. The incision was then closed using Nexaband veterinary glue (Abbott Animal Health, Abbott Park, IL).

Laser Doppler perfusion imaging. Blood perfusion in the hindlimb was monitored by laser Doppler imaging (MoorLDI-2, Moor Instruments, UK). Prior to imaging, mice were anesthetized with ketamine/xylazine, and placed on a heating plate at 37°C for 10 minutes in order to minimize temperature variations. The laser Doppler image was analyzed by averaging the perfusion, expressed as the relative unit of flux as determined by Moor Instruments, over the surface of the ischemic and non-ischemic foot. To control for ambient light and temperature, calculated perfusion was expressed as the flux ratio between the ischemic and non-ischemic limbs.

Isolation and adoptive transfer of bone marrow mononuclear cells (BM-MNCs) or purified monocytes. Bone marrow was harvested from the femurs of donor mice and mononuclear cells were isolated by centrifugation across a 1.011 density gradient (Histopaque, Sigma-Aldrich, St. Louis, MO) at 1700xg for 30 minutes. Pure monocyte populations were obtained from CX3CR1^{GFP/+} mice using a MoFlo high speed cell sorter (Dako, Fort Collins, CO) based on CX3CR1^{lo}Gr-1⁺ expression or CD115⁺Gr-1⁺ expression in mice which do not express CX3CR1-GFP. 2x10⁶ BM-MNCs or 2x10⁵ purified monocytes were administered intravenously into recipient mice 24 hours after the induction of hindlimb ischemia. For trafficking experiments, cells were incubated with 2.5uM carboxyfluorescein succinimidyl ester (CFSE)

(Invitrogen, Eugene, OR) in PBS for 10 minutes at 37°C prior to intravenous injection.

Homogenization of muscle tissue. The adductor muscle was dissected free of the ischemic hindlimb, subjected to mechanical disruption, and incubated 3 mg/mL collagenase type I (Worthington Biochemical, Lakewood, NJ) for one hour on a 37° shaking incubator. Cells were further homogenized by drawing through a 18-gauge and 22-gauge needle and then filtered through a 50 µm cell filter (Partec, Mt. Laurel, NJ). The resulting suspension was spun at 8000xg for 5 minutes, and the cell-free supernatant analyzed by ELISA.

Histological analyses. Ischemic adductor muscles were excised and fixed overnight in 10% formalin. Tissue was then paraffin-embedded, sectioned, and stained with hematoxylin/eosin (H&E). The number of newly regenerated myofibrils containing centrally-localized nuclei was scored by counting 100 cells in three distinct fields of each section (300 total myofibrils scored). Leukocyte infiltration present in the entire section area was given a histological score as follows: 1=0-25% affected area; 2=25-50% affected area; 3=50-75% affected area; 4=75-100% affected area; n=3, p<0.05. All scoring was conducted in a blinded fashion by two independent investigators.

Unbiased screen for secreted factors. Cell free supernatants were obtained from homogenized adductor muscle as described above. Supernatants were analyzed using

the RodentMap v2.0 multiplex analysis for 58 unique soluble factors (Rules Based Medicine, Austin, TX).

ELISA. ELISA analysis for G-CSF and IL-6 (R&D Systems, Minneapolis, MN) were performed according to manufacturer's instructions.

Flow cytometry. Cells were incubated with the indicated primary antibody for 1 hour at 4°C in PBS containing 0.2% BSA and 0.1% sodium azide. The following primary antibodies were used: Gr-1, B220, CD3, F4/80, CD115 (eBioscience, San Diego, CA). Cells were analyzed on a FACScan flow cytometer (BD Bioscience, San Jose, CA).

Flow cytometric phosphorylation assay. Femurs obtained from ischemic or non-ischemic CX3CR1^{GFP/+} transgenic mice were flushed with PBS containing 0.2% bovine serum albumin (BSA). Cells were immediately fixed and permeabilized using BD Cytofix/Cytoperm reagent according to manufacturer's instructions (BD Biosciences, San Jose, CA). Permeabilized cells were stained using BD Phosflow anti-STAT3 or anti-STAT5 antibodies (BD Biosciences). Cells were then gated on the CX3CR1^{lo} inflammatory monocyte population and the mean fluorescent intensity measured.

Microarray analysis. CX3CR1-GFP^{lo}Gr-1⁺ inflammatory monocytes were sorted from the bone marrow of CX3CR1^{GFP/+} transgenic mice, with or without hindlimb

ischemia, directly into Trizol (Invitrogen, Carlsbad, CA). Three independent replicates were performed. Total RNA was prepared according to manufacturer's instructions, amplified, labeled, and hybridized to a MouseRef-6 BeadChip (Illumina, San Diego, CA, USA). Expression profiles from basal inflammatory monocytes were compared to those of ischemia-conditioned monocytes using the Spotfire analysis program (TIBCO, Somerville, MA, USA). After normalization, mean signal intensities for each probeset were calculated across the three experiments. Probesets upregulated or downregulated in ischemia-conditioned monocytes versus basal monocytes were selected based upon the following selection criteria: (i) mean signal intensity > 40 in at least two of three probesets corresponding to the upregulated condition; (ii) greater than threefold changes in mean signal intensity (hereafter referred to as "fold-induction ratio"). (iii) Statistical significance ($P < 0.03$) by t-test/Anova. In cases where multiple probesets corresponded to the same gene, the probeset with the highest average signal intensity and greatest number of present calls was used.

Statistics. Statistical significance between curves in time courses was determined by two-way ANOVA with Bonferroni post-testing. Unpaired students' t-test was performed on single observation data. Significance was determined as $p < 0.05$.

3.4 RESULTS

3.4.1 Adoptive transfer of bone marrow monocytes significantly improves revascularization in mice after induction of hindlimb ischemia

Monocytes were sort-purified from the bone marrow of donor mice, labeled with carboxyfluorescein succinimidyl ester (CFSE), and adoptively transferred into recipient mice 24 hours after surgical induction of unilateral hindlimb ischemia. Consistent with a previous report by our laboratory (6), infusion of as few as 2×10^5 bone marrow monocytes significantly enhances blood flow recovery as assessed by laser Doppler analysis (Figure 1A). The number of donor (CFSE⁺) and recipient (CFSE⁻) monocytes recruited to the ischemic adductor muscle was assessed by flow cytometry (Figure 1B-C). Interestingly, though donor monocytes clearly home to and are retained in the ischemic tissue, they make up only a small fraction (0.1% to 1%) of the total monocytes/macrophages present in the lesion. Moreover, in control mice not receiving donor monocytes, blood flow recovery was significantly delayed despite the recruitment of a large number of CFSE⁻ host monocytes (Figure 1B-C). Together, these data suggest dramatic differences in the ability of donor versus host monocytes to stimulate revascularization.

3.4.2 Adoptive transfer of BM-MNCs from ischemic donors does not improve revascularization after induction of hindlimb ischemia

Local ischemia/inflammation can induce systemic production of cytokines, such as IL-6, G-CSF, tumor necrosis factor-alpha (TNF- α), interferon-gamma (IFN- γ), and transforming growth factor-beta (TGF- β), that regulate immune cell function (16). We hypothesized that systemic factors induced by hindlimb ischemia may alter

the ability of bone marrow resident monocytes to stimulate angiogenesis. To test this hypothesis, we harvested BM-MNCs from mice 24 hours after induction of hindlimb ischemia. Of note, cells were obtained only from the non-ischemic hindlimb of the ischemic donor, as cells present in the femur of the ischemic hindlimb are subject to local effects of hypoxia and inflammation (Figure 2A). In this study, we refer to these cells as ischemia-conditioned BM-MNCs and cells from non-ischemic mice as basal BM-MNCs. Basal or ischemia-conditioned BM-MNCs were adoptively transferred into recipient mice 24 hours after induction of hindlimb ischemia and revascularization assessed by laser Doppler imaging (Figure 2B). In contrast to basal cells, ischemia-conditioned BM-MNCs did not improve blood flow recovery (Figure 2B).

Accelerated restoration of blood flow should result in improved tissue preservation. To assess the integrity of muscle tissue post-ischemia, we examined histological sections of ischemic adductor muscle 21 days after induction of HLI (Figure C-E). Adoptive transfer of basal bone marrow cells resulted in a significant increase in myofibrils with centrally-located nuclei (Figure 2D), a feature associated with regenerating myofibrils (17). In contrast, muscle sections obtained from mice receiving ischemia-conditioned BM-MNCs had reduced myofiber regeneration, as measured by this assay (Figure 2D). Interestingly, however, an adoptive transfer of either basal or ischemia-conditioned BM-MNC significantly reduced leukocyte infiltration present in the ischemic lesion (Figure 2E). Collectively, these data suggest that the tissue reparative capacity of ischemia-conditioned BM-MNCs is reduced compared with basal BM-MNCs.

3.4.3 Ischemia conditioned bone marrow monocytes demonstrate a reduced capacity to stimulate revascularization

The inability of ischemia-conditioned bone marrow cells to improve blood flow recovery may be due directly to a reduction in their ability to stimulate angiogenesis or may be secondary to the loss of angiogenic cells from the bone marrow. We have previously used sort-fractionation experiments to show that monocytes of the CX3CR1^{lo}Gr-1⁺ inflammatory subset represent the angiogenic cell type within the BM-MNC fraction (6). In order to assess if this pro-angiogenic population is, in fact, reduced, we quantified the number of inflammatory monocytes in the bone marrow 24 hours after induction of ischemia in the contralateral limb. Compared with baseline bone marrow, no differences in the percentage or absolute number of inflammatory monocytes in the ischemia-conditioned bone marrow were observed (Figure 3A & B). We next asked whether ischemic conditioning leads to a defect in monocyte-promoted angiogenesis on a per cell basis. Inflammatory monocytes were sorted from the bone marrow of mice at baseline or at 24 hours after induction of hindlimb ischemia and adoptively transferred into ischemic recipients. Consistent with our previous data, adoptive transfer of basal inflammatory monocytes was associated with significant improvement in blood flow recovery (Figure 3C). In contrast, adoptive transfer of ischemia-conditioned inflammatory monocytes had no effect on blood flow recovery (Figure 3C). These data suggest that ischemia-conditioned bone marrow resident monocytes have reduced capacity to stimulate revascularization.

3.4.4 Ischemia-conditioned monocytes home to sites of ischemia normally

We next assessed whether ischemia-conditioned monocytes are competent to home to sites of ischemia. Basal or ischemia-conditioned bone marrow monocytes were labeled with CFSE and adoptively transferred into recipient mice 24 hours after induction of hindlimb ischemia. The magnitude and kinetics of the accumulation of ischemia-conditioned monocytes in the ischemic muscle was similar to basal monocytes (Figure 4), suggesting that homing of ischemia-conditioned monocytes was normal.

In addition, there was no significant difference in the number of recipient (CFSE⁻) monocytes, neutrophils, T cells, or B cells that infiltrated the ischemic tissue (data not shown).

3.4.5 Ischemia-conditioned bone marrow resident monocytes have increased STAT3 but not STAT5 activation.

To begin to elucidate the molecular mechanisms responsible for the reduced angiogenic capacity of ischemia-conditioned monocytes, we sorted basal or ischemia-conditioned monocytes from the bone marrow and performed RNA expression profiling. Data from three independent experiments were pooled; genes that were consistently upregulated or downregulated in ischemia-conditioned monocytes are summarized in Supplemental Tables 1 and 2. Since several well known STAT3 target genes were found to be significantly upregulated in ischemia-conditioned monocytes, including SOCS3, we next assessed STAT3 activation in these cells.

Bone marrow cells were harvested from mice at baseline or 24 hours after induction of hindlimb ischemia and STAT3 and STAT5 phosphorylation in monocytes assessed using a phospho-flow assay in which CX3CR1^{lo} inflammatory monocytes were gated (Figure 5A) and mean fluorescence intensity of pSTAT3 or pSTAT5 was assessed (Figure 5B). Compared with basal monocytes, a modest but significant increase in STAT3 phosphorylation in ischemia-conditioned monocytes was observed (Figure 5C). In contrast, no difference in STAT5 phosphorylation levels was observed (Figure 5C) suggesting specific induction of the STAT3 pathway post-ischemia.

3.4.6 The impaired angiogenic capacity of ischemia-conditioned monocytes is dependent on IL-6 and G-CSF signals

Localized ischemia can lead to the production of inflammatory cytokines that might contribute to STAT3 activation in bone marrow resident monocytes. To address this possibility, we performed an unbiased screen for 58 biomarkers, including many cytokines and chemokines, in the serum of mice at baseline or 24 hours after induction of hindlimb ischemia. Interestingly, the only factor which was significantly elevated from baseline was IL-6 (data not shown). ELISA confirmed increased IL-6 levels (Figure 6A). We also assayed for G-CSF by ELISA, as G-CSF likewise activates STAT3 in monocytes (18), and which was not included in the unbiased screen. Like IL-6, this cytokine was significantly upregulated in the serum of ischemic mice (Figure 6B).

We next asked if the signals generated by G-CSF and IL-6 are responsible for the impaired ability of ischemia-conditioned monocytes to improve revascularization. To test this possibility, we assessed the angiogenic capacity of monocytes harvested from the bone marrow of IL-6^{-/-} or G-CSFR^{-/-} mice at baseline or 24 hours after induction of hindlimb ischemia. Similar to wild type mice, induction of contralateral ischemia did not alter the number or percentage of monocytes in the bone marrow of IL-6^{-/-} or G-CSFR^{-/-} mice (data not shown). Remarkably, ischemic conditioning of IL-6^{-/-} or G-CSFR^{-/-} monocytes had no effect on their ability to promote revascularization (Figure 6C & D). These data suggest that signaling by G-CSF and IL-6 in bone marrow monocytes is a key determinant of their ability to stimulate revascularization.

3.4.7 Ischemia-conditioned monocytes demonstrate a shift towards the expression of M2 genes and M2-skewed SHIP^{-/-} monocytes do not improve blood flow recovery

In response to local environmental signals, tissue macrophages may be polarized into two broad activation states: classical (M1) and alternative (M2). Classically activated macrophages are induced by proinflammatory signals such as IFN- γ and have enhanced cytotoxic and anti-microbial activities. In contrast, alternative activated macrophages are polarized towards tissue repair and angiogenesis (19). Though this classification has been designated based on tissue macrophages, we asked whether bone marrow resident monocytes were undergoing similar polarization following induction of distant ischemia. Specifically, we

analyzed the RNA profiling data from basal and ischemia conditioned bone marrow monocytes for expression of a curated list of well-defined M1- or M2-associated genes (Supplemental Table 3 & 4) (20-22). Expression of the majority of the M1-associated genes was similar, with the exception of 3 genes that were significantly reduced in ischemia-conditioned monocytes (Figure 7A). Conversely, 7 of 32 M2-associated genes were increased in ischemia conditioned bone marrow monocytes (Figure 7B). Thus, the RNA expression pattern is suggestive of at least partial polarization towards alternative (M2) activation.

Alternative activation of macrophages is classically associated with tissue repair and angiogenesis, not the decreased repair that was observed with ischemia-conditioned (M2-polarized) monocytes. Thus, we assessed the capacity of monocytes from SHIP^{-/-} mice to stimulate revascularization in the hindlimb ischemia model; macrophages in SHIP^{-/-} mice are reported to be preferentially polarized towards M2 activation (23). Bone marrow monocytes of the inflammatory subset were sort-purified from SHIP^{-/-} mice and adoptively transferred into ischemic recipients (Figure 7C). Consistent with our results with ischemia-conditioned monocytes, M2-skewed monocytes from SHIP^{-/-} mice had markedly reduced capacity to stimulate revascularization in this model.

3.5 DISCUSSION

In this study, we show that bone marrow monocytes harvested from mice with distant ischemia have reduced capacity to stimulate angiogenesis upon adoptive

transfer. Interestingly, this finding is consistent with a recent study in which BM-MNCs obtained from patients with chronic ischemic cardiomyopathy were defective in improving angiogenesis post-HLI in nude mice (24). The bone marrow is recognized as the major site of monocyte production and provides a reservoir of monocytes that can be mobilized into the blood in response to stress (25). Our data suggest that bone marrow resident monocytes also may sense systemic signals that influence their future functional capacity. This “conditioning” of monocytes in the bone marrow represents another level of regulation of monocyte activation.

We show that in the hindlimb ischemia model, G-CSF and IL-6 provide such “conditioning” signals to bone marrow resident monocytes. Systemic expression of G-CSF and IL-6 were significantly increased after induction of hindlimb ischemia, reaching levels in the serum that are in seen in other inflammatory conditions (26-28). Consistent with G-CSF and IL-6 signaling, a modest but consistent increase in STAT3 phosphorylation and induction of several STAT3 target genes was observed in ischemia-conditioned bone marrow resident monocytes. Finally, we show that G-CSF and IL-6 signaling in monocytes is relevant, since ischemia-conditioned IL-6^{-/-} or G-CSFR^{-/-} bone marrow monocytes are able to stimulate revascularization. Interestingly, systemic levels of G-CSF and/or IL-6 are increased in many inflammatory conditions (29-31), suggesting that these findings may have broad relevance for monocyte biology.

G-CSF has been shown to improve tissue recovery in animal models of hindlimb ischemia (2), myocardial infarction (32), focal cerebral ischemia injuries (33, 34), and renal ischemia-reperfusion (35, 36). Moreover, clinical trials utilizing

G-CSF to mobilize cells for adoptive transfer have demonstrated efficacy in the treatment of peripheral arterial disease in humans (4). While at first glance, these studies would seem at odds with the current findings that G-CSF inhibits the angiogenic capacity of monocytes, G-CSF also is a potent mobilizer of monocytes, endothelial progenitors, and other cells with potential angiogenesis-promoting activity. Thus, the increased delivery of these cells to sites of ischemia/tissue injury may overcome the negative effect that G-CSF signaling has on the function of recruited monocytes. On the other hand, trials of stem cell/leukocyte mobilization by G-CSF after myocardial infarction in humans have been disappointing (37). It is possible that monocytes mobilized by alternative agents, such as AMD3100, may have better efficacy.

Macrophages are known to polarize along the classical M1 activation pathway or conversely to exhibit an M2 alternative activation phenotype, however emerging data suggest that macrophage activation is quite heterogeneous and that these classifications may, in fact, be oversimplified. M1 macrophages are generated in response to infectious or inflammatory stimuli and function to clear intracellular pathogens and also play a role in tumor resistance. In contrast, M2 macrophages are elicited in response to IL-4 and IL-13 (M2a subset) or IL-10 (M2c subset) signaling and exhibit diverse functions including immunity to extracellular parasites, the evolution of an allergic response, matrix remodeling, and tumor promotion (38). M2 macrophages have been associated with angiogenesis *in vitro* (39) and in a wide variety of tumor models (40). The role that the M2 macrophage subset plays in post-ischemic angiogenesis, however, has not previously been examined.

The M1 and M2 classification of macrophage activation is based on data from tissue macrophages. Thus, its relevance to monocyte activation in blood or bone marrow is unclear. With this caveat in mind, our RNA profiling data suggest that ischemia-conditioned bone marrow monocytes are polarized towards a M2 phenotype. Expression of hallmark M2 genes, Arg1 and Ym1, is increased, while a trend of decreased expression of several M1-associated genes was observed in ischemia-conditioned bone marrow monocytes (Supplemental Tables 3 & 4). At first glance, these results may be counter-intuitive, since M2 activation is associated with enhanced angiogenesis and tissue repair and not the impaired angiogenic capacity that was observed. However, these results are consistent with our finding that monocytes obtained from SHIP^{-/-} mice, known to exhibit preferential M2 polarization, also do not improve blood flow recovery in ischemic recipients. Likewise, two recent studies have demonstrated that mice deficient in IL-10, a critical determinant of M2c polarization, have improved blood flow recovery post-HLI compared to wild type controls due, at least in part, to the increased levels of VEGF and active matrix metalloproteinases in these mice (41, 42). Though it will require further study, it is possible that functions associated with classically activated (M1) macrophages, including secretion of proinflammatory cytokines and degradation of extracellular matrix, are advantageous early during the course of tissue reparation following acute ischemic injury.

In summary, our data demonstrate that monocytes derived from an ischemic donor have a markedly reduced capacity to improve revascularization compared to non-ischemic controls. We demonstrate that G-CSF and IL-6 are generated upon

induction of ischemia and that signaling by these cytokines plays an essential role in altering the phenotype of bone marrow-resident monocytes, including the induction of STAT3 target genes and increased expression of well-defined M2-associated genes. These data provide the first evidence that systemic inflammatory signals lead to significant functional changes in bone marrow-resident monocytes. A specific delineation of the ways in which inflammation- and ischemia-driven signals impact the angiogenic capacity of bone marrow-resident monocytes may have important implications for the treatment of a variety of ischemic disorders.

3.6 FIGURE AND FIGURE LEGENDS

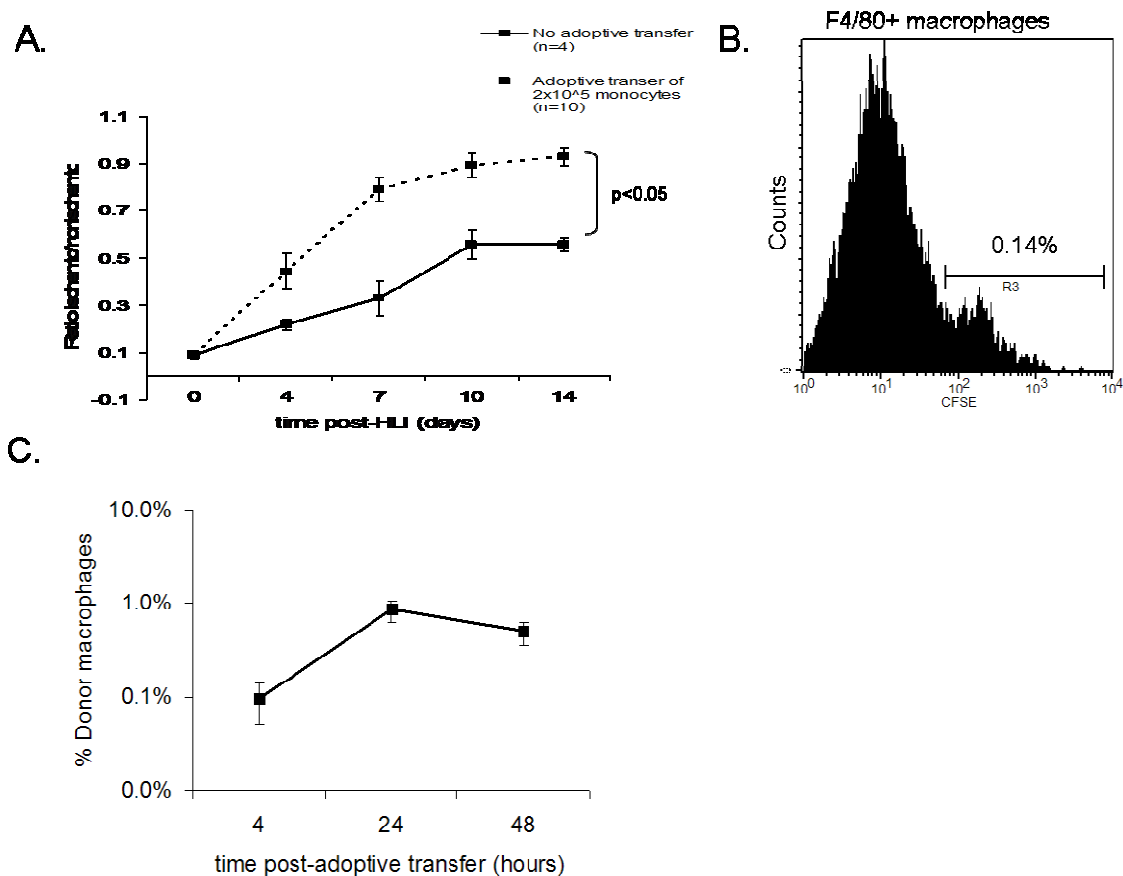


Figure 1. The addition of a small proportion of macrophages obtained from a non-ischemic donor significantly improves revascularization post-hindlimb ischemia. (A) Monocytes were sort-purified from non-ischemic donors, labeled with CFSE and adoptively transferred into ischemic recipients at 24 hours post-HLI. Revascularization of recipients was assessed over 14 days using laser Doppler imaging. (B) F4/80⁺ macrophages present in homogenized, ischemic adductor muscle tissue were gated and assessed for CFSE expression as shown in this representative histogram plot. (C) The ratio of (CFSE⁺ donor)/(CFSE⁻ endogenous) F4/80⁺ macrophages is shown at several time points post-adoptive transfer (n=3 at 4 and 72 hours; n=10 at 48 hours). Data represent mean \pm SEM.

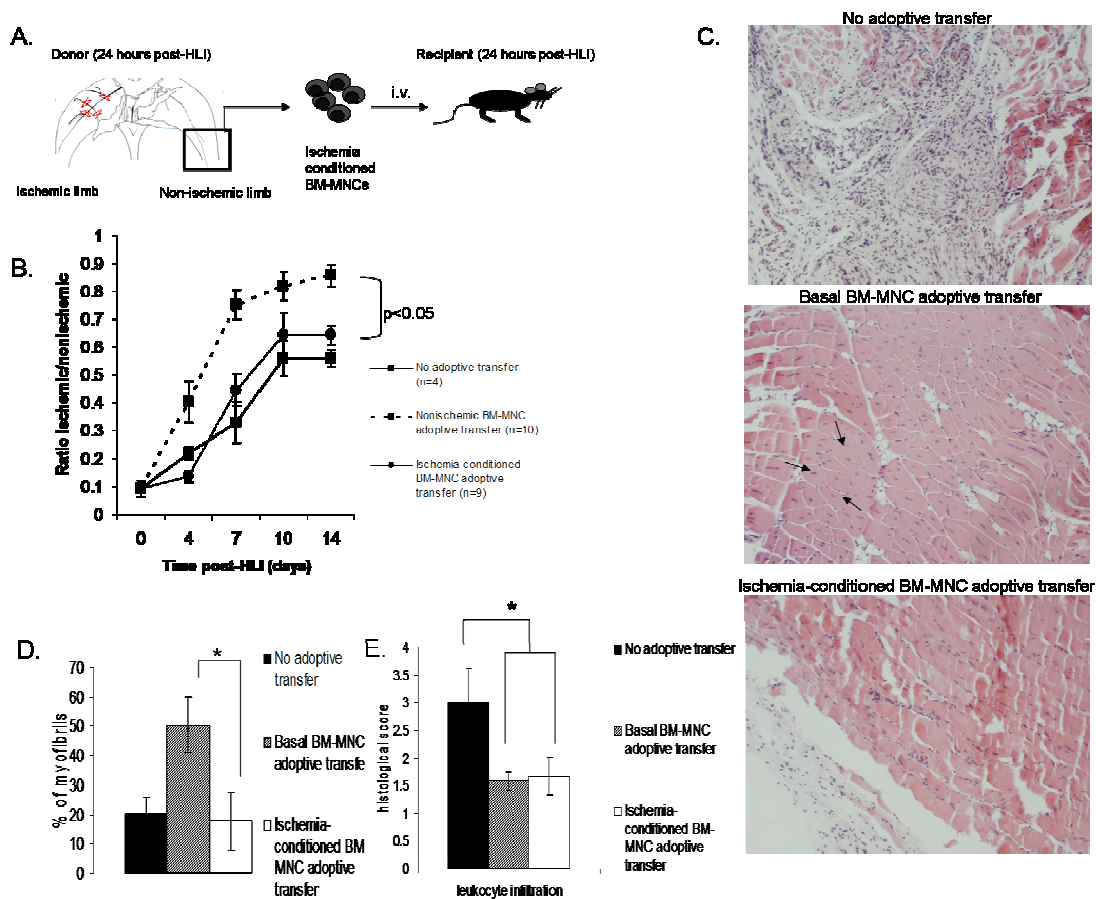


Figure 2. An adoptive transfer of BM-MNCs obtained from an ischemic donor does not improve revascularization post-HLI. (A) BM-MNCs were obtained from the non-ischemic limb of a 24-hour post-HLI donor using Ficoll purification and injected intravenously into an ischemic recipient at 24 hours post-HLI according to the scheme illustrated. (B) Blood flow recovery in recipient mice was assessed by laser Doppler imaging over a 14-day time course; n=6. (C) Ischemic adductor muscles were obtained at 21 days post-HLI and were sectioned and stained with H&E. Original magnification x 40. (D) The percentage of newly regenerated myofibrils containing centrally-localized nuclei, indicated in panel (C) with arrowheads, was scored by counting 300 cells in three distinct fields of each section; n=3, p<0.05. (E) Leukocyte infiltration present in the entire section area was given a histological score as follows: 1=0-25% affected area; 2=25-50% affected area; 3=50-

75% affected area; 4=75-100% affected area; n=3 p<0.05. Data represent the mean \pm SEM.

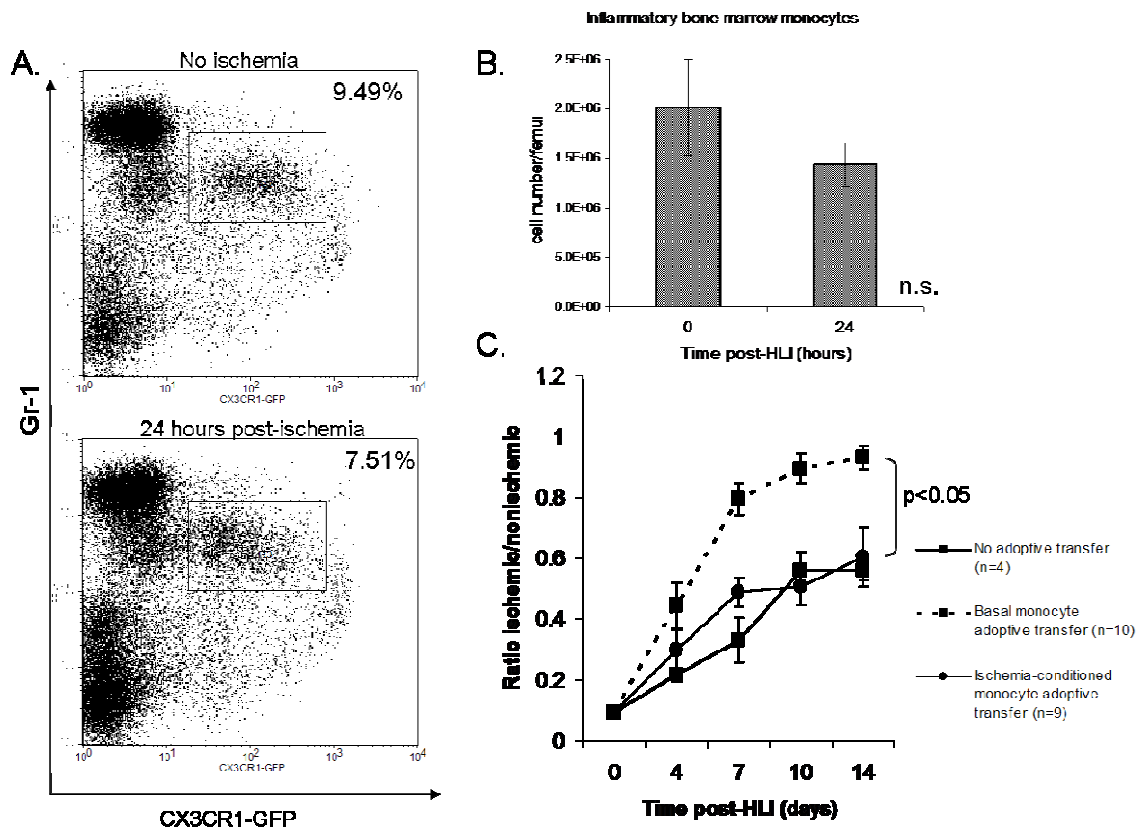


Figure 3. Ischemia-conditioned monocytes are not competent to mediate revascularization. (A) Bone marrow cells were obtained from CX3CR1^{GFP/+} mice at baseline (upper panel) or at 24 hours post-ischemia (lower panel) and analyzed by flow cytometry. The percentages of CX3CR1^{lo}Gr-1⁺ inflammatory monocytes are noted. (B) The absolute number of inflammatory bone marrow monocytes was quantified by multiplying the percentage obtained by flow cytometry by the total white blood count per femur (n=5). (C) Laser Doppler analysis of mice receiving 2×10^5 sort-purified inflammatory monocytes from non-ischemic donors (squares,

dashed line) or ischemia-conditioned donors (circles, solid line). Data represent the mean \pm SEM, $p < 0.05$.

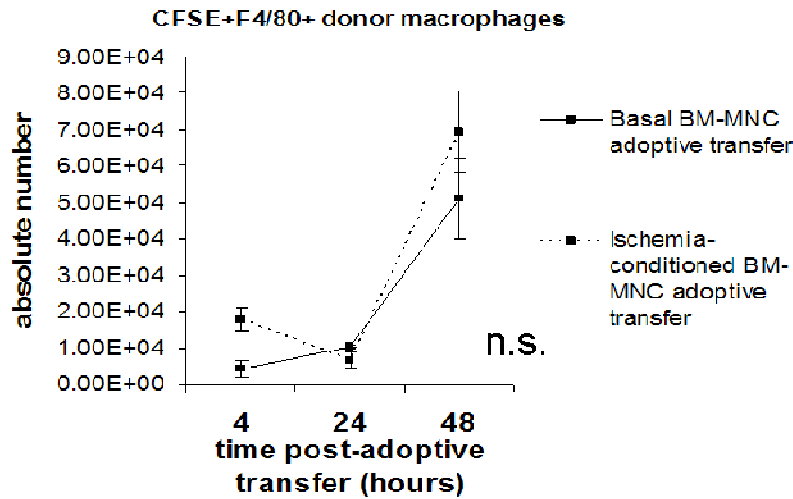


Figure 4. Adoptively transferred ischemia-conditioned monocytes are competent to home to ischemic tissue. BM-MNCs were harvested from wild type or 24 hour-post HLI donors, labeled with CFSE, and intravenously adoptively transferred into ischemic recipients. At the time points indicated, the absolute number of CFSE⁺ donor macrophages present in the ischemic adductor muscle was quantified using cell percentages obtained by flow cytometry multiplied by the total cell number obtained by hemacytometer counts; $n=3$ at 4 and 48 hours; $n=10$ at 24 hours. Data represent the mean \pm SEM.

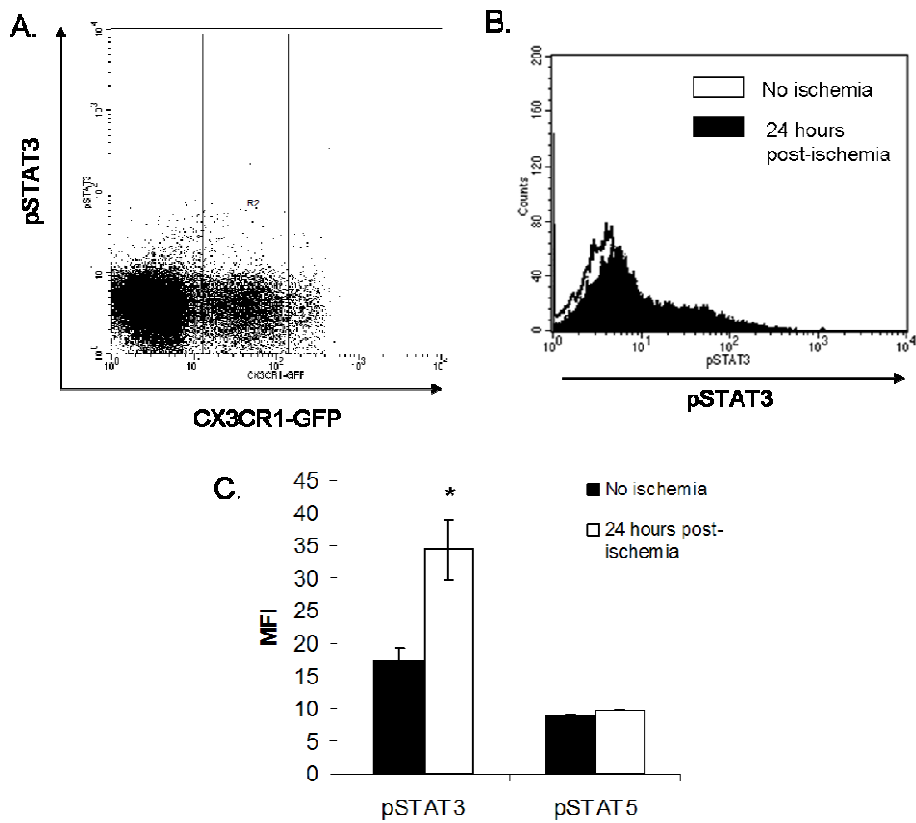


Figure 5. Increased STAT3 phosphorylation is observed in ischemia-conditioned bone marrow monocytes. Bone marrow cells were harvested from non-ischemic or ischemic mice, immediately subjected to fixation/permeabilization, and stained with a phospho-specific anti-STAT3 or anti-STAT5 antibodies for flow cytometric analysis. Inflammatory (CX3CR1-GFP^{lo}) monocytes were gated (A) and mean fluorescent

intensity of pSTAT3 staining was quantitated (B-C). Data are n=3, representative of four independent experiments. Data represent the mean \pm SEM, $p < 0.05$.

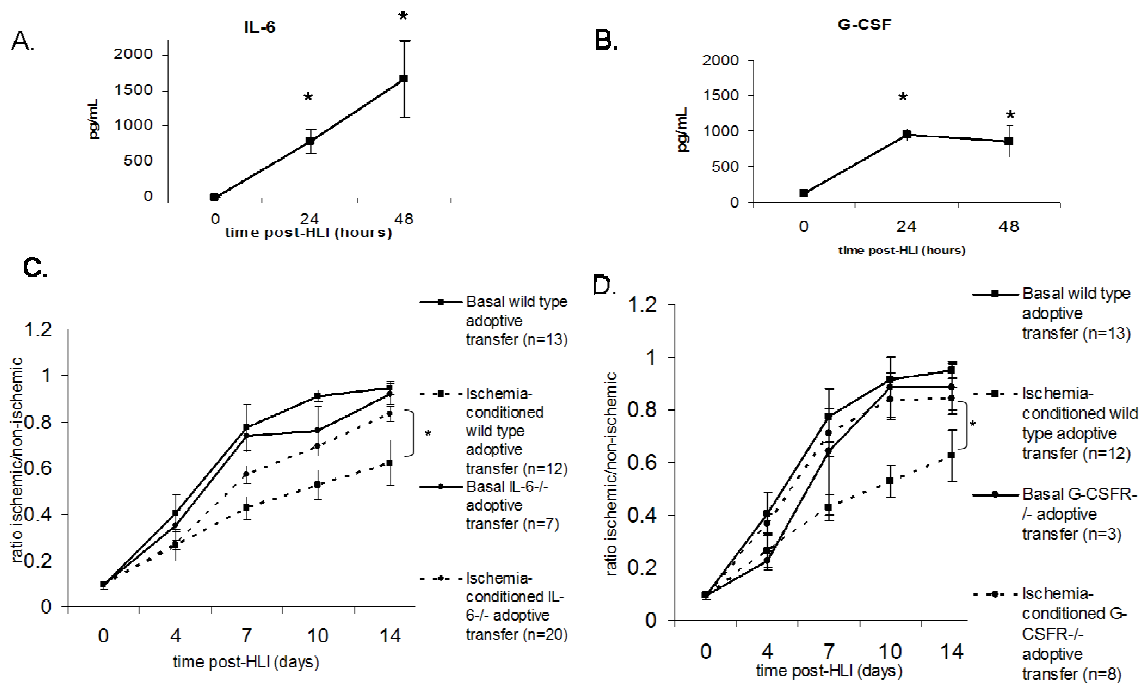


Figure 6. IL-6 and G-CSF are produced upon induction of ischemia and loss of either of these signals is sufficient to restore the ability of ischemic adoptively transferred monocytes to promote angiogenesis. (A-B) Serum levels of IL-6 (A) and G-CSF (B) were assessed at the indicated time points post-HLI. Asterisks denote a

significant change from baseline, n=4-9. (C-D) Bone marrow monocytes were purified using cell sorting from non-ischemic (solid line) or 24-hour ischemic (dotted line) wild type, IL-6^{-/-} (C), or G-CSFR^{-/-} (D) donors and 2x10⁵ cells were adoptively transferred into ischemic recipients at 24 hours post-HLI. Revascularization of recipients was then assessed for 14 days using laser Doppler imaging. Data represent the mean ± SEM, p<0.05.

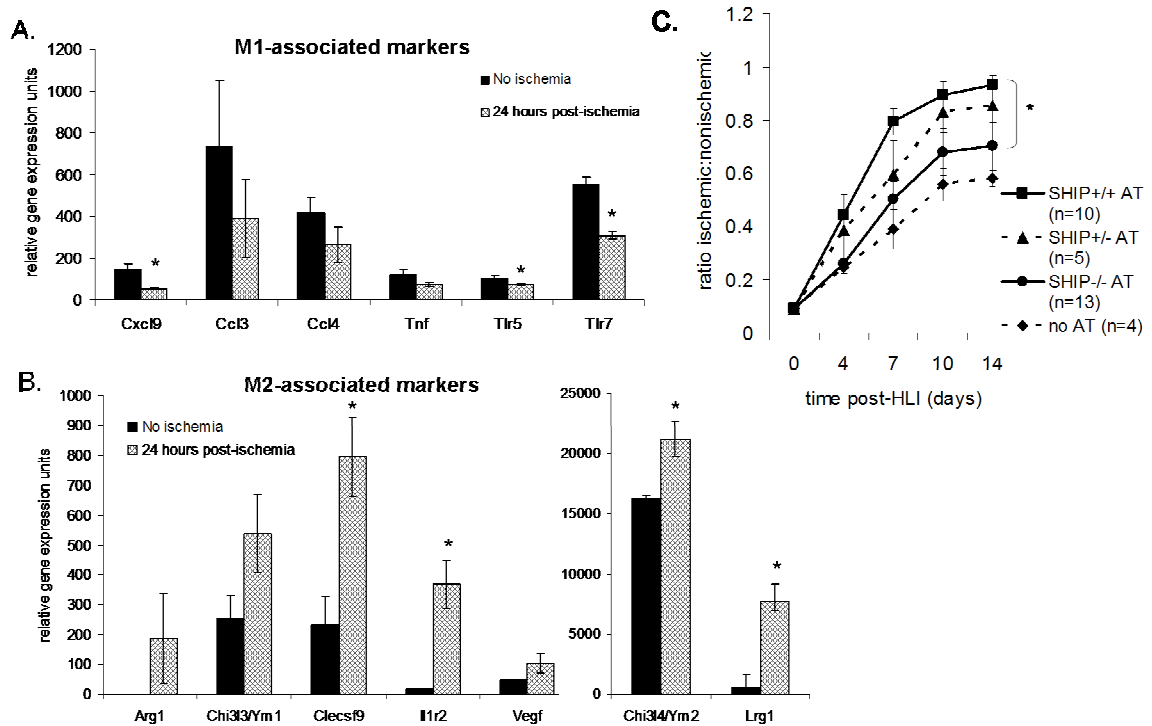


Figure 7. Differential M1/M2 gene expression post-ischemic conditioning, and reduced revascularization capacity of SHIP^{-/-} M2-skewed monocytes.

(A-B) CX3CR1^{lo}Gr-1⁺ inflammatory bone marrow monocytes were sort-purified from mice at baseline or at 24 hours post-HLI and RNA expression profiling performed on three independent replicates. Shown are normalized signals for all M1-associated genes (A) or M2-associated genes that displayed consistent changes (increased or decreased) from control cells. (C) CD115⁺Gr-1⁺ monocytes were sorted from SHIP^{+/+} (squares, solid line), SHIP^{+/-} (triangles, dashed line) and SHIP^{-/-} (circles, solid line) mice and adoptively transferred into ischemic recipients at 24 hours post-HLI. Blood flow recovery was assessed by laser Doppler imaging over a 14-day time course. Data represent the mean \pm SEM, $p < 0.05$.

Supplemental Table 1. Genes upregulated in ischemia-conditioned bone marrow monocytes

Gene	Symbol	Baseline	Ischemia-conditioned	Fold-Change	t-test/ Anova
serum amyloid A 3	Saa3*	124 ± 50	7425 ± 1183	59.9	0.00
interleukin 1 receptor, type II	Il1r2	17 ± 1	368 ± 82	20.6	0.01
leucine-rich alpha-2-glycoprotein 1	Lrg1	618 ± 257	7712 ± 1477	12.5	0.01
suppressor of cytokine signaling 3	Socs3*	137 ± 79	1191 ± 315	8.7	0.03
sterol O-acyltransferase 2	Soat2	36 ± 6	296 ± 54	8.2	0.01
glycosylphosphatidylinositol specific phospholipase D1	Gpld1	22 ± 10	164 ± 11	7.2	0.00
interferon induced transmembrane protein 1	Ifitm1	366 ± 54	2334 ± 576	6.4	0.03
chondroitin sulfate proteoglycan 2	Cspg2	48 ± 8	290 ± 13	6.4	0.01
potassium channel, subfamily K, member 13	Kcnk13	37 ± 4	217 ± 38	6.0	0.01
serine protease inhibitor, Kunitz type 1	Spint1	47 ± 2	269 ± 72	5.7	0.03
insulin-like growth factor binding protein 6	Igfbp6	44 ± 5	240 ± 23	5.5	0.00
DNA (cytosine-5-)-methyltransferase 3-like	Dnmt3l	36 ± 12	174 ± 19	4.8	0.00
ribosomal protein S19	Rps19	11 ± 3	52 ± 8	4.7	0.01
guanine nucleotide binding protein, beta 5	Gnb5	27 ± 7	125 ± 8	4.6	0.01
spermidine synthase	Srm	303 ± 173	1054 ± 80	3.5	0.03
a disintegrin and metalloprotease domain 8	Adam8	17 ± 7	62 ± 7	3.5	0.00
complement component 1, r subcomponent-like	C1rl	193 ± 35	610 ± 28	3.2	0.02
heat shock protein 1	Hspd1	159 ± 84	488 ± 38	3.1	0.02
cytidine 5-triphosphate synthase	Ctps	59 ± 28	154 ± 15	2.6	0.04
ribonucleotide reductase M2	Rrm2	107 ± 49	266 ± 18	2.5	0.04

The average (+ SEM) raw signal of triplicate experiments is shown.

Genes denoted with * indicate STAT3 target genes.

Supplemental Table 2. Genes downregulated in ischemia-conditioned bone marrow monocytes

Gene	Symbol	Baseline	Ischemia-conditioned	Fold-Change	t-test/Anova
ureidopropionase, beta	Upb1	349 ± 24	51 ± 15	0.152	0.00
solute carrier family 14 (urea transporter), member 1	Slc14a1	90 ± 24	12 ± 2	0.159	0.03
myxovirus (influenza virus) resistance 2	Mx2	209 ± 28	46 ± 11	0.217	0.01
reticulon 1	Rtn1	78 ± 14	19 ± 12	0.221	0.03
toll-like receptor 11	Tlr11	68 ± 11	15 ± 2	0.229	0.01
2-5 oligoadenylate synthetase-like 2	Oasl2	142 ± 33	30 ± 3	0.231	0.03
2-5 oligoadenylate synthetase-like 1	Oasl1	195 ± 9	47 ± 10	0.241	0.00
interferon regulatory factor 4	Irf4	303 ± 32	73 ± 8	0.244	0.00
similar to Ifi204 protein	LOC240921	268 ± 58	59 ± 10	0.244	0.02
interferon-induced protein with tetratricopeptide repeats 2	Ifit2	538 ± 67	141 ± 23	0.260	0.00
interferon regulatory factor 7	Irf7	670 ± 42	175 ± 7	0.265	0.00
PHD finger protein 11	Phf11	454 ± 105	116 ± 42	0.277	0.04
angiotensin receptor-like 1	Agtr1	76 ± 9	20 ± 2	0.278	0.00
peptidyl arginine deiminase, type II	Padi2	2136 ± 227	605 ± 22	0.288	0.00
Otoferlin	Otof	212 ± 26	59 ± 1	0.288	0.00
interferon activated gene 203	Ifi203	287 ± 42	83 ± 13	0.290	0.01
ubiquitin specific protease 18	Usp18	816 ± 147	230 ± 8	0.295	0.02
D site albumin promoter binding protein	Dbp	179 ± 28	52 ± 4	0.304	0.01
angiotensin converting enzyme	Ace	218 ± 21	70 ± 47	0.306	0.04
keratin complex 2, basic, gene 7	Krt2-7	139 ± 16	46 ± 10	0.321	0.01

The average (+ SEM) raw signal of triplicate experiments is shown.

Supplemental Table 3. M1-associated genes

M1 Genes which change* upon induction of ischemia:

Gene	Symbol	Baseline A	Ischemia A	Baseline B	Ischemia B	Baseline C	Ischemia C	Fold Δ	p-value
chemokine (C-X-C motif) ligand 9; MIG	Cxcl9	133	62	197	47	107	47	0.38	0.03
chemokine (C-C motif) ligand 3; Mip1alpha	Ccl3	1111	395	984	711	106	65	0.56	0.40
chemokine (C-C motif) ligand 4; Mip1beta	Ccl4	522	368	450	332	281	88	0.59	0.24
tumor necrosis factor	Tnf	117	77	167	86	74	55	0.64	0.17
toll-like receptor 5	Tlr5	120	81	98	71	98	69	0.70	0.02
toll-like receptor 7	Tlr7	621	344	502	280	535	303	0.56	0.00

M1 Genes unchanged** upon induction of ischemia:

Gene	Symbol	Baseline A	Ischemia A	Baseline B	Ischemia B	Baseline C	Ischemia C	Fold Δ	p-value
chemokine (C-C motif) ligand 5	Ccl5	88	100	56	20	43	38	0.79	0.75
CD80 antigen	Cd80	315	323	302	281	298	310	1.00	1.00
CD86 antigen	Cd86	751	866	796	721	802	883	1.05	0.50
Fc receptor, IgG, low affinity III	Fcgr3	5474	4899	3387	4016	3109	3634	1.08	0.83
Fc receptor, IgG, low affinity IIB	Fcgr2b	3180	2525	1903	1816	2557	2241	0.87	0.45
Fc receptor, IgG, high affinity I	Fcgr1	1837	901	795	801	636	617	0.82	0.46
interleukin 12a	Il12a	43	67	36	33	87	48	1.02	0.77
interleukin 1 beta	Il1b	204	177	67	86	103	302	1.69	0.44
lymphocyte antigen 64	Ly64	69	82	44	37	60	48	0.94	0.90
toll-like receptor 1	Tlr1	61	57	55	56	74	57	0.90	0.28
toll-like receptor 2	Tlr2	4135	3730	2323	2365	2373	3339	1.11	0.80
toll-like receptor 4	Tlr4	313	374	204	365	275	300	1.36	0.10
toll-like receptor 6	Tlr6	518	507	412	485	401	434	1.08	0.50

M1 genes not expressed:***

Gene	Symbol	Baseline A	Ischemia A	Baseline B	Ischemia B	Baseline C	Ischemia C	Fold Δ	p-value
chemokine (C-C motif) receptor 7	Ccr7	9	17	12	14	33	21	N/A	N/A
nitric oxide synthase 2, inducible, macrophage	Nos2	-4	8	8	1	8	4	N/A	N/A
chemokine (C-C motif) ligand 8	Ccl8	10	-2	-5	-2	16	0	N/A	N/A
interferon alpha family, gene 1	Ifna1	12	5	7	21	15	15	N/A	N/A
interferon alpha family, gene B	Ifnab	-7	-7	-2	-6	4	-7	N/A	N/A
interleukin 1 receptor, type I	Il1r1	2	23	22	13	18	25	N/A	N/A
chemokine (C-X-C motif) ligand 10	Cxcl10	27	10	35	-3	27	-5	N/A	N/A
chemokine (C-X-C motif) ligand 11	Cxcl11	5	7	2	4	9	2	N/A	N/A
chemokine (C-C motif) ligand 2	Ccl2	43	43	14	11	21	12	N/A	N/A
interleukin 6	Il6	1	1	-2	-3	5	-4	N/A	N/A

*All genes which were expressed at levels greater than 40 relative expression units and which showed consistent induction or reduction across all three data sets are reported as "changed."

**Genes which did not show consistent induction or reduction across all three data sets are reported as "unchanged."

***Genes with a relative expression value <40 are reported as "not expressed."

Supplemental Table 4. M2-associated genes

M2 Genes which change* upon induction of ischemia:

Gene	Symbol	Baseline A	Ischemia A	Baseline B	Ischemia B	Baseline C	Ischemia C	Fold Δ	p-value
arginase 1, liver (Arg1)	Arg1	-5	490	1	49	1	23	562.00	0.28
chitinase 3-like 3 (Chi3l3)	Chi3l3	407	803	194	422	164	391	2.00	0.14
interleukin 1 receptor, type II (Il1r2)	Il1r2	17	419	20	211	17	475	21.00	0.01
C-type lectin, superfamily member 9	Clecsf9	425	993	159	542	110	849	34.00	0.03
leucine-rich alpha-2-glycoprotein 1	Lrg1	1080	9427	566	8880	211	4830	13.00	0.01
vascular endothelial growth factor A	Vegfa	48	169	46	77	44	61	2.00	0.17
chitinase 3-like 4	Chi3l4	10308	13183	5603	9085	2169	10190	1.80	0.02

M2 Genes unchanged** upon induction of ischemia:

Gene	Symbol	Baseline A	Ischemia A	Baseline B	Ischemia B	Baseline C	Ischemia C	Fold Δ	p-value
Fc receptor, IgE, high affinity I, gamma polypeptide	Fcer1g	26927	19555	21773	21268	19015	21467	0.94	0.50
macrophage galactose N-acetyl-galactosamine specific lectin 2	Mgl2	1450	942	960	707	351	828	1.25	0.79
mannose receptor, C type 1	Mrc1	168	213	203	72	415	231	0.73	0.39
platelet-activating factor acetylhydrolase, isoform 1b	Pafah1b1	2328	1877	1570	1782	1606	1701	1.00	0.86
prosaposin	Psap	27140	22879	27001	28059	23643	25701	0.99	0.85
transforming growth factor, beta 1 triggering receptor expressed on myeloid cells 2	Tgfb1	1207	910	1138	789	134	885	2.69	0.92
arginase type II	Vti1b	1509	1564	3011	4073	3548	3429	1.12	0.75

M2 genes not expressed:***

Gene	Symbol	Baseline A	Ischemia A	Baseline B	Ischemia B	Baseline C	Ischemia C	Fold Δ	p-value
Fc receptor, IgE, low affinity II, alpha polypeptide	Fcer2a	8	-3	-6	-2	8	-5	N/A	N/A
chemokine (C-C motif) ligand 22	Ccl22	-3	10	8	14	12	8	N/A	N/A
chemokine (C-C motif) ligand 17	Ccl17	10	5	3	6	19	-1	N/A	N/A
macrophage scavenger receptor 1	Msr1	-4	-1	-1	-2	7	3	N/A	N/A
macrophage scavenger receptor 2	Msr2	11	-2	17	5	27	-2	N/A	N/A
chemokine (C-C motif) ligand 2	Ccl2	43	43	14	11	21	12	N/A	N/A
chemokine (C-C) receptor 2	Ccr2	-6	-1	5	1	9	-2	N/A	N/A
interleukin 13	Il13	18	23	28	26	30	25	N/A	N/A
interleukin 1 receptor antagonist	IL1RA	62	53	24	18	13	16	N/A	N/A
interleukin 10 (Il10)	Il10	-8	-3	-3	-4	6	-1	N/A	N/A

chemokine (C-C motif) ligand 17	Ccl17	10	5	3	6	19	-1	N/A	N/A
chemokine (C-C motif) ligand 22	Ccl22	-3	10	8	14	12	8	N/A	N/A
chemokine (C-C motif) ligand 24	Ccl24	12	16	5	10	16	3	N/A	N/A
resistin like alpha/Fizz1	Retnla	-6	-4	-1	-3	12	0	N/A	N/A
cadherin 1	Cdh1	6	6	1	6	12	10	N/A	N/A
folate receptor 2 (fetal)	Folr2	-2	-4	-4	-6	9	9	N/A	N/A
CD163 antigen	Cd163	-7	-2	2	-4	9	-2	N/A	N/A

*All genes which were expressed at levels greater than 40 relative expression units and which showed consistent induction or reduction across all three data sets are reported as "changed."

**Genes which did not show consistent induction or reduction across all three data sets are reported as "unchanged."

***Genes with a relative expression value <40 are reported as "not expressed."

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Chapter 4

Summary and Future Directions

4.1 SUMMARY

The studies outlined in this work lend insight into the mechanisms governing the regulation of angiogenesis by monocytes and also may have important implications for the design of cellular therapies for angiogenic disorders. In this work we demonstrated that monocytes of the inflammatory subset are rapidly recruited to the ischemic tissue post-hindlimb ischemia. A further proof of the importance of these cells in regulating post-ischemic angiogenesis was the accelerated revascularization observed when these cells were adoptively transferred into an ischemic recipient. We also identified several factors that were produced upon adoptive transfer—VEGF, MCP-1, and MMP—each of which is a known player in the angiogenic repair. Additionally, we identified apolipoprotein A1, a factor with a previously unknown role in angiogenesis, to be upregulated post-adoptive transfer. Interestingly, this protein must play a critical role in the mechanism by which adoptively transferred cells accelerate revascularization as ApoA1^{-/-} monocytes did not mediate this effect.

Having established that an adoptive transfer of monocytes does potently affect revascularization, we next sought to identify the ways in which the ischemic recipients' own endogenous monocytes differed functionally from the non-ischemic donor cells used for the adoptive transfer. We demonstrated that an adoptive transfer of monocytes obtained from an ischemic donor do not accelerate revascularization in the hindlimb ischemia model. Our studies revealed that post-hindlimb ischemia there

is a marked increase in systemic levels of IL-6 and G-CSF and this is associated with increased STAT3 phosphorylation in bone marrow-resident monocytes. In addition to the increased STAT3 activation observed, bone marrow resident monocytes also exhibited increased expression of M2 genes and a reduction in expression of M1 genes. In keeping with this finding, an adoptive transfer of SHIP^{-/-} monocytes which are preferentially skewed to the M2 phenotype, does not accelerate revascularization in the hindlimb ischemia model. This finding runs counter to tumor angiogenesis studies in which M2 macrophages are major contributors to angiogenesis and underscores the need to explore macrophage-regulated angiogenesis in a variety of ischemic settings. Taken together our data identify a potent pro-angiogenic subset of monocytes (CX3CR1^{lo}Gr-1⁺ inflammatory subset) and also reveal a profound defect in the ability of ischemia-conditioned monocytes to accelerate recovery from ischemia. These findings can be exploited clinically by modifying current cell-based therapies for ischemic disorders such that they contain only this pro-angiogenic monocyte population (and not simply the unfractionated mononuclear cell compartment) and also potentially to obtain these cells from a matched, non-autologous donor in order to avoid the negative impact of the ischemic-conditioning observed in our studies.

4.2 APPLICATION TO OTHER ISCHEMIC MODELS

A limitation of the studies described in this work is that only one model of ischemia and angiogenesis has been explored. The mouse model for hindlimb ischemia we utilized generates an acute ischemia that is largely resolved after

approximately three weeks. Therefore it would be useful to compare the findings we obtained using this model and compare with other models of acute ischemia such as myocardial infarction or acute ischemic kidney injury to see if our findings regarding the efficacy of a monocyte adoptive transfer hold true in these settings as well. While differences between these models will certainly exist, shared findings with regard to monocyte subsets involved and factors produced will corroborate and strengthen our conclusions from the hindlimb ischemia model.

In the majority of patients with critical limb ischemia, atherosclerosis—which is a chronic, persistent inflammatory state—is the underlying cause. In this way, our model for hindlimb ischemia does not accurately replicate this disease state. It would be useful to compare our findings from the acute ischemia model utilized in our studies to a model for chronic ischemia. For example, a model has been recently described in which the femoral artery excision surgery is performed in an ApoE^{-/-} mouse; the combination of the femoral artery excision with the persistent inflammatory state in this mouse model better replicates chronic ischemia than femoral artery excision alone (Kang, 2008). Results obtained from this model would provide insight into the ways in which the monocytic response differs between an acute versus a chronic ischemic insult.

In addition to exploring the ways in which treatment with monocytes may be used as a pro-angiogenic therapy for ischemic disorders, it would also be useful to explore ways in which our findings may regulate pathologic neovascularization such as that observed during macular degeneration. It would be useful to extend our findings to oxygen-induced retinopathy (OIR) and laser-induced choroidal

neovascularization (CNV) studies which model this disorder. Such studies could focus on which macrophage subsets accumulate during the course of neovascularization. It may also be useful to attempt to prevent neovascularization by neutralizing the pro-angiogenic factors we described in Chapter 2—for example treating these animals with the apolipoprotein A1 mimetic peptide in an effort to reduce angiogenesis.

4.3 ROLE OF TYPE I INTERFERON

In addition to revealing that several STAT3 targets were upregulated in ischemia-conditioned monocytes, microarray data revealed that several type I interferon-regulated genes were significantly downregulated (Table 3.2). The role of type I interferons in the response to ischemia has not been extensively examined, however one study reported that IFN α/β , and not IFN- γ , is specifically induced in a mouse model of acute liver ischemia (Zhai, 2008). The role that IFN α/β may play in regulating the development of bone marrow-resident monocytes is unclear. One recent set of in vitro studies has indicated that bone marrow monocytes cultured under M2-polarizing conditions produce more endogenous IFN- β and express a far greater number of type I interferon target genes than do monocytes cultured under M1-polarizing conditions (Fleetwood, 2009). If this finding hold true in vivo it may represent an important regulatory mechanism for the downstream polarization of bone marrow resident monocytes. Further studies are required in order to determine if tonic IFN α/β signaling does, in fact, occur in the bone marrow and what specific implications this regulation has on the downstream phenotype of these cells.

4.4 CLINICAL TRIAL USING MOBILIZING AGENTS TO RECRUIT MONOCYTES

Our laboratory is currently enrolling patients with critical limb ischemia for a clinical trial in which they are treated for 10 days with G-CSF, a potent mobilizing agent, or placebo. A total of 60 patients will be enrolled with 30 patients each in the treatment and placebo arms. Eligibility criteria for patient enrollment includes a diagnosis of critical limb ischemia, defined as the presence of a non-healing ulcer or rest pain, secondary to peripheral arterial disease (PAD). Patients in this study must not be candidates for a surgical revascularization procedure. Additionally patients must have an absolute neutrophil count in the normal range and may not demonstrate an active infection or malignancy. CD14⁻/CD16⁺ monocyte subsets as well as endothelial progenitor cells (EPCs) and lymphocytes will be measured by flow cytometry in peripheral blood obtained from patients prior to treatment as well as at and day 10 post-treatment. The efficacy of this treatment will be assessed clinically by measuring ulcer size, ankle-brachial index, toe pressures, as well as a pain assessment. The primary endpoint for these studies will be the rate of amputation at one year post-treatment. The results of this study will demonstrate the specificity and magnitude of recruitment of the CD14⁻CD16⁺ and CD14⁺CD16⁻ human monocyte subsets in response to treatment with G-CSF. This study will also determine if mobilization of bone marrow-derived hematopoietic cells represents an efficacious treatment for critical limb ischemia. If the results of this trial are disappointing, a modified protocol in which an alternative mobilizing agent, such as AMD-3100 may

yield better results based on the finding presented in this work that G-CSF, while it actively recruits monocytes to a site of ischemia, may also reduce their pro-angiogenic potential.

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