MACROPHAGE-VASCULAR SMOOTH MUSCLE CELL INTERACTIONS IN NEOINTIMAL HYPERPLASIA

by

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ABSTRACT

The objective of this thesis work was to understand how the dynamic cross talk that occurs between vascular smooth muscle cells (SMCs) and macrophages (Mϕ) recruited to regions of arterial injury promotes the pathogenesis of neointimal hyperplasia.

Macrophages were the predominant myeloid cell type recruited to wire-injured femoral arteries in mouse, as assessed by flow cytometry. Recruited macrophages from injured vessels exhibited a distinct expression profile relative to circulating peripheral blood mononuclear cells. This phenotype was largely recapitulated in vitro by maturing rat bone marrow cells in the presence of macrophage-colony stimulating factor (M-CSF) and 20% conditioned media from cultured rat SMC (sMϕ), compared to maturation in M-CSF alone (M0). The physical parameters of the active factor present in SMC conditioned medium were consistent with the properties of TGF-β. Recombinant TGF-β1 recapitulated the effect of SMC conditioned medium on macrophage phenotypic modulation. Macrophage maturation studies performed in the presence of a pan-TGF-β neutralizing antibody, a TGF-β receptor 1 inhibitor, or conditioned medium from TGF-β-depleted SMCs confirmed that the SMC-derived factor responsible for macrophage activation was TGF-β. Finally, we assessed the effect of SMC-mediated macrophage
activation state on SMC biology. SMCs cocultured with sMϕ exhibited increased rates of proliferation relative to SMCs cultured alone or with M0 macrophages.

We assessed whether macrophage phenotype is differentially modulated in mice with targeted deletion of the protein and lipid phosphatase PTEN in SMC (PTEN iKO). At 15 days following injury there was an increase in the number of macrophages present in PTEN iKO lesions, and a larger percentage of macrophages were Ly6c<sup>high</sup> by flow cytometry. This suggests that, at this time point, monocyte recruitment in PTEN iKO lesions is enhanced relative to recruitment in wild type lesions.

These data are significant since macrophages have been shown to play an important role in promotion of neointima formation following vascular injury, and these results begin to explain the mechanisms involved. Our data show that TGF-β produced by SMCs is critical for promoting a macrophage phenotype representative of that observed in injured vessels. These activated macrophages would be predicted to signal to the SMC in vivo, resulting in feed-forward promotion of neointima formation.

The form and content of this abstract are approved. I recommend its publication.

Approved: Raphael Nemenoff
To my husband Gabe Ostriker,

and to my parents Pat and Chris Cherry.
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LIST OF ABBREVIATIONS

AKT- Protein kinase B
Arg I – Arginase I
β-gal- β-galactosidase
BMC – Bone marrow cell
BMDC – Bone marrow-derived macrophage
BrdU – 5-Bromo-2’-Deoxyuridine
CaMKII – Calcium/calmodulin-dependent protein kinase II
CC’d - “cocultured with”
CCL5 – Chemokine (C-C motif) ligand 5; RANTES
CCR3 – Chemokine (C-C motif) receptors 3
CCR7 – Chemokine (C-C motif) receptor 7
CD206 – Mannose receptor
cGMP- Cyclic guanosine monophosphate
CM – Conditioned medium
CPI-17- PKC-dependent 17-kDa C-protein-potentiated inhibitor of protein phosphatase
CSFR1- Colony-stimulating factor receptor 1
Ctrl- Control
CypB- Cyclophilin B
DAPI- 4’,6’-diamidino-2-phenylindole
DOC – Deoxycholic acid
ECM- Extracellular matrix
FACS – Fluorescence-activated cell sorting
FAK – Focal adhesion kinase
FBS – Fetal bovine serum
FS – Forward scatter
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
GPCR – G protein-coupled receptor
IL-10- Interleukin-10
IL-12- Interleukine-12
IL-6- Interleukin-6
iNOS- Inducible nitric oxide synthase
iRFA – Injured right femoral artery
KD- Knockdown
kDa – Kilodalton
KLF4/5 – Kruppel-like factor 4/5
LDL – Low density lipoprotein
LFA- Left femoral artery
LPS – Lipopolysaccharide
Luc- Luciferase
M0 – Macrophage matured only in the presence of M-CSF
M1- Macrophage stimulated with LSP/IFN-γ
M2- Macrophage stimulated with IL-4
MADS- MCM1, Agamous, Deficiens, SRF
MAPK – Mitogen-activated protein kinase
MCP-1 - Macrophage chemoattractant protein-1; CCL2
M-CSF – Macrophage-colony stimulating factor
MHC-II - Major histocompatibility complex II
MLCK – Myosin light-chain kinase
MMP9 - Matrix metalloprotease 9
mRNA – Messenger RNA
Mφ – Macrophage
Mφ* - Macrophage preconditioned by coculture with SMC
NIH – Neointimal hyperplasia
NLR – Nod-like receptor
NO - Nitric oxide
NT – Non-targeting
oxLDL – Oxidized LDL
P65 – NF-κB p65 subunit; RelA
PBMC – Peripheral blood mononuclear cell
PBS – Phosphate buffered saline
PCI – Percutaneous coronary intervention, i.e. surgery to restore flow in a blocked artery
PCR – Polymerase chain reaction
PDGF- Platelet-derived growth factor
PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC – Protein kinase C
PKG – Protein kinase G
PLC-γ – Phospholipase C, gamma
PM - pharmacomechanical
PTEN – Phosphatase and tensin homologue
PTEN iKO – Inducible, SMC-specific PTEN knockout
PTEN KO - SMC-specific PTEN knockout
qPCR – Quantitative real-time PCR
RNA – Ribonucleic acid
RNA-seq – RNA sequencing
rRNA-Ribosomal RNA
RT-PCR – Reverse transcription PCR
SB2- SB203580; p38 kinase inhibitor
SB4-SB431542; TBRI kinase inhibitor
SDF-1α- Stromal cell-derived factor 1-α; CXCL12
SE/SEM- Standard error of the mean
Si-RNA – Small interfering RNA
SMA – Smooth muscle α-Actin
SMC- (Vascular) smooth muscle cell
SMC* - Smooth muscle cell preconditioned by coculture with Mφ
SM-MHC – Smooth muscle myosin heavy-chain
sMφ – SMC conditioned medium-matured macrophage
SRF- Serum response factor
SRM – Serum-reduced medium (0.1% FBS)
SS- Side scatter
TBR – TGF-β receptor
TBR- TGF-β receptor
TCA – Trichloroacetic acid
TCE – TGF-β control element
TGF-β- Transforming growth factor β
Th1- T helper 1
Th2 – T helper 2
TLR – Toll-like receptor
tMϕ – TGF-β-matured macrophage
TNF-α – Tumor necrosis factor-α
Vs- Versus
WCL- Whole cell lysate
Wrt – With respect to
WT – Wild type
CHAPTER I

INTRODUCTION: ETIOLOGY AND EPIDEMIOLOGY OF
ATHEROSCLEROSIS AND RESTENOSIS

Cardiovascular Disease

Atherosclerosis-associated coronary artery disease and peripheral artery disease currently put the lives of approximately 17.6 and 8 million Americans at risk, respectively\(^1,2\). Approximately 30% of patients who undergo surgical intervention to treat these occlusive diseases develop restenosis\(^3\), or the subsequent re-occlusion of the artery lumen by the formation of a neointima composed of proliferating vascular smooth muscle cells (SMCs), inflammatory cells such as macrophages, and excessive deposition of extracellular matrix (ECM)\(^4\). Vessel occlusion and inhibited blood flow, particularly in overweight and/or diabetic patients, results in significant morbidity, mortality, and strain on the healthcare system. Considering that the incidence of diabetes is predicted to approximately double in the next 40 years\(^5\), and that obese and diabetic patients represent a large portion of the individuals undergoing these surgical procedures, the impact of morbidity and mortality due to neointimal hyperplasia is likely to increase in the near future. In addition to restenosis following surgical treatment of atherosclerotic blockages, bypass grafts, organ transplants, and hemodialysis vein grafts all result in significant numbers of patients experiencing graft or transplant failure due to vessel occlusion from neointimal hyperplasia\(^6\). Current therapies for preventing restenosis, such
as drug-eluting stents, have improved, but have not eliminated the occurrence of restenosis. Furthermore, stent placement is not feasible for alleviating the restenotic response in bypass grafts, transplants, or hemodialysis grafts, and there have been reports of increased risk of late stent thrombosis following placement of drug-eluting stents. In order to develop better targeted therapies, with a decreased risk of stent-induced thrombosis, a better understanding of the pathogenesis of neointimal hyperplasia is required. In this dissertation we sought to understand the molecular cross talk that occurs between two prominent cell types present in these lesions - SMCs and macrophages - in order to gain such an understanding.

Structure of the Artery and Resident Cells

Neointimal hyperplasia is largely a condition affecting the arteries. Arteries consist of three layers, or tunicae, that serve separate roles in the tissue’s function. The structure and physiology of the artery, and it’s composite layers and cells, is reviewed in Kumar ed., 2010, and McPhee ed., 2010. The most luminal layer is the tunica intima. The intima is comprised of a single layer of endothelia cells joined by tight junctions that serve as a selectively permeable lining of the artery lumen in all vessels. In the context of normal vascular function, this endothelial layer is responsible for creating an interface between the blood and tissue that represses thrombosis, or clotting, unless required. The endothelial layer is largely impermeable to serum proteins, but allows for the diffusion of oxygen and nutrients from the circulation into the more sub-luminal cell layers, or tissues in the case of capillaries. The endothelium also plays a role in blood vessel dilation and constriction, due to SMC relaxation and contraction, and
Figure 1: Schematic of the arterial tunicae and of neointima formation. 
A) Histological section of the mouse femoral artery with the intima, media, and adventitia indicated. B) Cartoon of the normal artery (left) and the artery with a neointimal lesion present (right).
is reviewed in VanHoutte, 2009. In response to a variety of stimuli, including mechanical forces (shear stress, stretch) and vasoactive substances, vasodilation in SMC is largely achieved by upregulation of endothelial nitric oxide synthase and subsequent release of nitric oxide that permeates to, and acts on, the underlying SMCs. Conversely, endothelial cells can affect the contraction of SMCs, or induce vasoconstriction, by the release of factors such as endothelin-1 and, importantly, prostacyclin.

The aforementioned SMCs are the primary constituent, along with interspersed and varying numbers of layers of elastic laminae, of the tunica media. The smooth muscle cells of the media function to affect vasodilation/constriction and to regulate blood pressure. SMCs are a remarkable cell type in that they retain the ability to undergo profound changes in phenotype in response to a host of environmental and local stimuli. In this context, SMCs may lose their contractile phenotype and begin to exhibit a dedifferentiated, migratory/ proliferative /secretory phenotype. It is this dedifferentiated phenotype that contributes to disease progression as it pertains to this dissertation. The outer-most layer of the artery is the tunica adventitia. Until recently, the adventitia was thought to be a rather loosely organized layer of fibroblasts and connective tissue. Recent studies (reviewed in Majesky el. al, 2011) have demonstrated that the adventitia is a highly ordered tissue, consisting of leukocytes and resident progenitor populations that may contribute to vascular pathologies. In addition, the adventitial microvasculature (vaso vasorum), regulates circulating inflammatory cell trafficking between the blood and the tissue. Finally, a major function of this layer, in the healthy vessel, is to anchor the vessels to the surrounding tissue.
Vascular Smooth Muscle Cell Function and Phenotypic Modulation

The contractile and elastic characteristics of the arteries allow them to modulate changes in blood pressure due to the pulsatile blood flow delivered by the heart, and to dilate or constrict in order to regulate oxygen delivery to tissues depending on metabolic requirements (reviewed in Webb, 2003 and Pappano, 2013). Vascular smooth muscle cells are the cellular constituent of arteries that physically mediates vascular tonus. SMCs are mononucleate cells that form several concentric layers in the mediae of larger arteries, down to single cell layers in mediae of arterioles. Contraction and relaxation of vascular SMCs is elicited by either neural or humoral stimulation. Contraction of vascular smooth muscle occurs through a different mechanism than it does in cardiac or skeletal muscle. Rather than Ca\(^{++}\) binding to troponin-tropomyosin to allow myosin binding to actin, in vascular SMCs contraction occurs by Ca\(^{++}\)-calmodulin phosphorylation of myosin light-chain kinase (MLCK). MLCK then phosphorylates myosin light chain allowing for formation of myosin-actin cross bridges. Contraction is antagonized by myosin light-chain phosphatase, the activity of which is negatively regulated by rho-kinase, PKC-dependent 17-kDa C-protein-potentiated inhibitor of protein phosphatase (CPI-17), and positively regulated by endothelial cell-derived nitric oxide (NO) through soluble guanylate cyclase-cGMP-PKG. In vascular SMCs, intracellular Ca\(^{++}\) concentrations may be increased, to induce contraction, by influx from outside the cell through voltage-gated (also receptor-operated and store-operated) calcium channels that activate upon cellular depolarization, or from the sarcoplasmic reticulum by ryanodine or IP\(_3\) receptor channels (calcium-induced calcium release). Vasoactivity in SMCs may be induced by either excitation-contraction coupling (i.e.
action potentials) or pharmacomechanical (PM) coupling. Both mechanisms can result in either contraction or relaxation. Contraction-inducing PM agonists, such as norepinephrine, angiotensin-II, endothelin, and vasopressin, act through their respective GPCRs to activate $G\alpha_{q/11}$ or $G\alpha_{12/13}$ that engage downstream signaling pathways to activate MLCK and represses MLCP, respectively. Relaxation-inducing PM agonists- such as atrial natriuretic factor- repress MLCK and increase potassium channel conductance via a similar intracellular signaling mechanism as NO$^{16}$. The majority of the vasculature is innervated by the sympathetic nervous system, and this innervation typically mediates vasoconstriction by norepinephrine release at varicosities, or axonal swellings on neurons from which norepinephrine is released, to act on nearby SMCs$^{17}$. Conversely, the effect of epinephrine, produced by the adrenal medulla, is a long-range endocrine effect$^{15}$.

In addition to smooth muscle $\alpha$-actin and smooth muscle-myosin (the main mechanotransducers in the contractile SMC), several additional contractile apparatus proteins are expressed in the differentiated SMC such as SM-22$\alpha$ and calponin. (Aortic carboxypeptidase-like protein, desmin, caldesmon, vinculin, metavinculin, telokine, $\alpha$- and $\beta$-tropomyosin, and smoothelin are additional smooth muscle-specific genes (SM-genes) that have been used to assess SMC differentiation state$^{18,19}$ but their role in contraction or reliability as marker genes will not be discussed here.) SM-22$\alpha$ and calponin associate with the cytoskeletal apparatus, and their expression is repressed in neointimal lesions$^{20,21}$. The precise function that SM-22$\alpha$ serves in SMC contraction is not well understood$^{22}$. Calponin is an inhibitor of actino-myosin Mg$^{++}$ATPase activity (which decreases the kinetics of cross-bridge formation), but also serves to decrease the
rate of dissociation of the actin-myosin complex and increase force generation\textsuperscript{23}. Calponin is inactivated by phosphorylation in response to contractile stimuli by either PKC or CaMKII\textsuperscript{24}.

As progenitor cells give rise to mature SMCs, expression of SM-\(\alpha\)Actin (\(\alpha\)Actin, or SMA), SM-myosin heavy chain (SM-MHC), calponin, and SM-22\(\alpha\) is induced, transcriptionally, by serum response factor (SRF)-myocardin complex binding to CArG boxes (CC(AT)\textsubscript{6}GG motifs; also known as serum response elements) in SM-gene promoters or intronic sequences\textsuperscript{25}. SRF is a ubiquitously expressed MADS box transcription factor that binds to GArG boxes as a homodimer\textsuperscript{25}. SRF is required for initiating transcription of both growth factor-induced genes, such as \textit{c-fos}, as well as SM-genes in response to differentiating stimuli such as arginine vasopressin\textsuperscript{25, 26}. In 2004 the Olson group explained how SRF can exhibit such dichotomous activities by showing that mitogenic signals induce association of the ETS-domain transcription factor Elk-1 with SRF, allowing it to associate with immediate early gene promoters; SRF-Elk-1 association results in dissociation of the muscle-specific transcription factor myocardin from SRF, preventing transcriptional initiation at SM-gene promoters\textsuperscript{27}. Formation of multiple SRF-myocardin complexes on at least two of the three CArG boxes in SM-genes permits the TATA box-bound RNA polymerase II complex to initiate transcription\textsuperscript{28}. SRF-myocardin-dependent transcription may also require \textit{cis}-activators present at TGF-\(\beta\) control elements (TCE) and E-box elements\textsuperscript{25}. The extrinsic signals (such as mechanical forces, contractile PM agonists, collagens I and IV, reactive oxygen species, TGF-\(\beta\), and prostacyclin\textsuperscript{25, 29}), that induce SMC differentiation and SM-gene upregulation have
largely been characterized \textit{in vitro}, and with the availability of conditional knockout technologies, are presently being validated \textit{in vivo}.

The dedifferentiated vascular smooth muscle cell is the primary cellular constituent of the restenotic neointimal lesion, and is thus typically considered diseased. However, it is useful for healthy SMCs to retain the ability to dedifferentiate in order to contribute to non-pathologic arteriogenesis. Unfortunately, as with many normal cellular processes, control of SMC dedifferentiation may escape from normal negative feedback mechanisms, resulting in pathology.

SMC dedifferentiation is characterized by repression of contractile proteins, aberrant proliferation, and expression of proteins characteristic of a pro-inflammatory phenotype such as cytokines, chemokines, matrix proteoglycans, and adhesion molecules\textsuperscript{30}. Following vascular injury, several factors, in addition to the injury itself, have been shown to induce SMC dedifferentiation and cytokine secretion from SMCs\textsuperscript{31}, and remaining endothelial cells in the vessel wall\textsuperscript{32}. Damaged endothelial cells and activated platelets that bind to the exposed medial matrix secrete cytokines and chemokines such as PDGF-BB, angiotensin II (Ang II), and IL-1β\textsuperscript{4}. SM-gene repression has been suggested to be mediated by PDGF, in part, by association of the transcription factor KLF4 (Kruppel-like factor 4) from SM-gene promoters\textsuperscript{33}. SMC proliferation and migration have been suggested to be the result of engagement of several parallel, and potentially interacting, downstream pathways such as PI3K/AKT, PLC-γ, Ras/Raf/MAPK, and SRC/FAK\textsuperscript{34}. The functional redundancy of the many engaged pathways, and cross talk between pathways, has made identifying critical pathways or nodes elusive. Similar to PDGF, Ang II has been shown to drive SMC proliferation,
migration, ECM deposition, hypertrophy, and inflammatory signaling. Ang II has been shown to exert its activating effects on SMCs through angiotensin receptor 1 (AT$_1$)$^{35}$. AT$_1$ antagonism by the peptide Ang(1-7) has been shown to reduce neointima formation following denudation injury in rat carotid arteries$^{36}$, denudation and stent placement in rat abdominal aortas$^{37}$, and denudation in rabbit aortas$^{38}$. The chemotactic and mitogenic effect of Ang II on SMCs has been suggested to be mediated through EGFR transactivation and downstream AKT and ERK signaling$^{39}$. However, unlike PDGF, which induces repression of SM-genes, Ang II induces upregulation of SM-genes through upregulation of myocardin$^{40}$. The heterogeneous effects of PDGF and Ang II on SMC phenotype and function are illustrative of how multifactorial the regulation of SMC phenotype in the vessel wall can be.

**Atherosclerosis**

Primary atherosclerotic lesions (reviewed in Lusis, 2000$^{41}$ and Stocker, 2004$^{42}$) tend to initiate in regions of arterial branching or curvature where blood flow is not laminar. In these regions, the arterial endothelium exhibits non-uniform apical-basolateral polarization and sub-optimal barrier function. These regions of disturbed flow accumulate sub-pathologic fatty streaks within the first few decades of life. Endothelial heterogeneity is associated with increased deposition of lipid (low density lipoprotein: LDL) molecules that diffuse passively through disturbed endothelial cell junctions, or are deposited in the sub-endothelial space by transcytosis, and are retained due to interactions of LDL apolipoproteins with proteoglycans. Sub-endothelially deposited LDL molecules are modified by lysosomal enzymes such as cathepsin D and
lysosomal acid lipase, resulting in the formation of LDL microaggregates. These aggregates are readily taken up by SMCs and macrophages. Retained LDL molecules in the sub-endothelial space undergo oxidation in response to exposure to oxidative species produced by resident vascular cells. Virtually all cells in the vasculature generate superoxide radicals by reducing molecular oxygen with electrons derived from NADPH via NADH/NADPH oxidases. Generation of reactive oxygen and nitrogen species can occur through the activity of several other enzymes in addition to NADH/NADPH oxidases, such as xanthine oxidase, nitric oxide synthase, myeloperoxidase, and lipoxygenase. Released oxygen radicals, which are themselves primarily reductive, can then react with other species to generate strong oxidants that go on to oxidize fatty acids and cholesterol as well as proteins such as LDL apolipoproteins. Oxidation products of fatty acids, such as linoleic, hydroxyoctadecanoic acid, and hydroxyeicosatetraenoic acids, have been detected in atherosclerotic lesions, typically as cholesterol esters. Cholesterol oxidation products, or oxysterols, may be generated by non-enzymatic oxidation of cholesterol molecules or by the enzymatic modification of cholesterol by mitochondrial 27-hydroxylase. Finally, protein oxidation can result in the production of hydroperoxides of the parent protein, as well as parent proteins with oxidant molecule adducts covalently attached to them. Oxidation of LDL molecules allows for their LDL receptor-independent uptake into macrophages, leading to the development of foam cells. Foam cells are sub-endothelially localized, lipid-laden, macrophages. Monocytes that give rise to foam cells transmigrate into the arterial media in response to upregulation of selectins on endothelial cells exposed to oxidized lipids. In early atherosclerosis, accumulation of foam cells and sub-endothelial lipid results in increasing stable plaque
bulk. Over time the complexity of the lesion increases. Eventual death of foam cells and release of their lipid contents contributes to the formation of an unstable plaque with a necrotic core. Cytokines, chemokines, and growth factors secreted by plaque-infiltrating macrophages, foam cells, as well as by other adaptive and innate immune cells, contribute to the activation of resident SMC. SMC activation results in migration of SMCs from the medial layer of the vessel into the burgeoning plaque mass, as well as increased production of fibrotic extracellular matrix from SMCs. This fibrous neointimal lesion, characterized by an SMC and ECM-rich fibrous cap containing an underlying necrotic core is predisposed to erosion at the luminal surface, as well as to hemorrhage of intra-plaque neo-vessels. These events can trigger thrombosis and associated acute arterial occlusion resulting in infarction or stroke.

**Neointimal Hyperplasia**

This dissertation is primarily concerned with the pathologic state termed neointimal hyperplasia (also termed stenosis, or restenosis if recurrence of stenosis follows surgical re-vascularization) that commonly occurs, in humans, following surgical treatment of atherosclerotic blockage. (This will be discussed in further detail in the following section on epidemiology.) The neointima is a pathologic tissue comprised primarily of extensive ECM, activated medial smooth muscle cells, as well as leukocyte infiltrates (Figure 1B). This pathologic tissue expands inward from the media toward the center of the artery lumen. (Further details on this process will be presented in the following section entitled Pathogenesis of Neointimal Hyperplasia.)
Stenosis of arteries due to development of a neointimal lesion results in significant mortality and morbidity in Western populations, and increasingly in non-Western populations. These lesions, either primary stenotic lesions or restenotic lesions, can be sufficiently occlusive so as to inhibit blood flow, resulting in angina pectoris, myocardial infarction, or significant ischemic peripheral artery disease. Artery occlusion due to neointima formation is not exclusive to atherosclerosis or following angioplasty and stenting. This pathologic state can also occur at the site of vessel grafts in organ transplant, in vascular grafts such as in coronary by-pass surgery, or in the setting of vascular access dysfunction in hemodialysis patients. Vessel occlusion in these scenarios can result in transplant or graft failure, or systemic infection.

**Epidemiology**

Percutaneous revascularization procedures to alleviate primary atherosclerotic lesions include atherectomy or endarterectomy procedures, which mechanically remove the built up plaque, and angioplasty and stent deployment, which expand the vessel lumen outward to displace the plaque. These procedures, while immediately effective at restoring blood flow, are not without complications (as reviewed in Mitra, 2006, Seedial, 2013, and Pourdjabbar, 2011). The incidence of restenosis following balloon angioplasty was approximately 34% prior to the introduction of intravascular stents. Stent placement at the site of intervention serves to prevent negative remodeling of the vessel due to stretch-induced vessel recoil. The first stents available to interventionalists were bare-metal stents. Incidence of restenosis in patients receiving bare-metal stents after angioplasty was reduced by approximately 10%. However, incidence of restenosis
was still reported to be somewhere in the range of 10-60% of surgical cases. Since the mid-2000s drug-eluting stents have become more prolific. These devices, typically eluting either rapamycin or paclitaxel analogues, have further reduced the incidence of restenosis by approximately 30% and 42%, respectively, as compared to bare-metal stents\textsuperscript{44, 45}. However, these anti-proliferative agents not only reduce SMC proliferation, but also inhibit endothelial cell proliferation, which results in delayed healing. This delay in re-endothelialization of the vessel results in increased incidence of late stent thrombosis and associated mortality and/or morbidity\textsuperscript{44}. It is for this reason that a more complete understanding of the pathogenesis of neointimal hyperplasia is necessary; which will facilitate development of more targeted therapies to reduce neointimal hyperplasia while encouraging re-endothelialization of vessels denuded during re-vascularization procedures.

**Pathogenesis of Neointimal Hyperplasia**

As discussed above, the clinical methods for treating occlusion due to primary atherosclerotic lesions involve mechanical removal or displacement of the atherosclerotic plaque, which, in turn, denudes the arterial endothelium. While these procedures are required to restore flow through occluded vessels, they also trigger a cascade of events that can eventually result in the formation of a neointimal lesion (reviewed in Chaabane, 2013\textsuperscript{4}, Mitra, 2006\textsuperscript{43}, and Simon, 2012\textsuperscript{46}). Upon surgical interventions, denudation of the arterial endothelium, and the presence of stent struts, triggers platelet adhesion, even with the use of anti-platelet therapy. Platelet deposition and de-granulation results in the release of high concentrations of pro-inflammatory cytokines, chemokines, and growth
factors. These pro-inflammatory mediators influence both leukocyte recruitment as well as modulation of SMCs toward a dedifferentiated, synthetic, and proliferative - or activated - phenotype. In addition to increased secretion of pro-inflammatory ligands, SMC activation in response to mechanical injury as well as primary atherosclerotic plaque build-up, results in extracellular matrix deposition and remodeling.

Aberrant ECM deposition is a significant aspect of the pathogenesis of neointimal hyperplasia because it contributes substantially to the bulk mass of the neointimal lesion. The lack of an endothelial lining allows for serum proteins such as fibrin and fibronectin to accumulate, encouraging adhesion of circulating monocytes. Over time this provisional matrix is stabilized through deposition of collagen, hyaluronan, and proteoglycans such as versican, by SMCs. Deposition of these proteins results in monocytes becoming trapped in the developing neointima, and reduces the anti-thrombotic and elastic properties of the vessel. This collagen-rich matrix also has been implicated in inducing apoptosis of SMCs in lesions, which has been suggested to correlate, perhaps counterintuitively, with worsened neointima formation.

A major contributor to the pathogenesis of neointimal hyperplasia is sterile inflammation (i.e. inflammation in the absence of microbial infection) that can be defined as the recruitment of inflammatory cells, elaboration of pro-inflammatory factors and pro-inflammatory cross talk between resident cells and recruited leukocytes. Many critical pro-inflammatory factors secreted in response to mechanical injury or primary atherosclerosis have been characterized. Factors such as PDGF-BB, TGF-β, Ang II, platelet factor 4 (CXCL4), SDF-1α (CXCL12), RANTES (CCL5), Mip-1α (CCL3), thromboxane A2, and many others, are secreted by adhered platelets within a short
period of time following injury. These factors act to drive both smooth muscle cell activation as well as leukocyte recruitment. As the lesion develops, activated SMCs and lesional leukocytes respond to platelet-derived factors, secrete many of the same pro-inflammatory factors as well as additional factors, and engage in paracrine cross talk and autocrine signaling. Indeed SMC activation and inflammation constitute two inputs into a feed-forward loop, and as such, identifying which of the two is the initiating event is extremely difficult.

As discussed above, a key aspect of sterile inflammation (in the absence of infection) is inflammatory cell recruitment to the site of injury. Several leukocyte populations have been shown to be recruited to vascular lesions following mechanical denudation injury\(^50\). While T-cells and B-cells have been shown to be present in vascular lesions, they have not been shown to significantly contribute to lesion pathogenesis\(^51-53\). Neutrophil recruitment to lesions has been shown to be an early and acute event following injury\(^54,55\). Interestingly, it has also been reported that systemic neutrophil depletion exacerbates neointimal lesion severity following denudation injury\(^56\), suggesting that acute neutrophil recruitment is an anti-stenotic event. In contrast, several groups have published data that implicate macrophage recruitment to lesions as a pro-stenotic factor. For example, systemic neutralization of SDF-1\(\alpha\) or MCP-1 has been shown to result in decreased monocyte-derived macrophage recruitment to lesions, as well as concomitant reduction in lesion size\(^11,12\). Further studies have shown that systemic bisphosphonate liposome or anti-leukocyte common antigen antibody-mediated macrophage depletion is sufficient to reduce neointima formation in response to mechanical injury in the mouse or rat, and rabbit, respectively\(^57-59\). Additionally, studies
in humans have found that the presence of macrophages in atherectomy samples predicts, with high significance, future development of restenosis. Due to the overwhelming body of data implicating macrophages as pro-stenotic cells, we decided to focus on the role of macrophages, and their bi-directional cross talk with SMCs in driving the pathogenesis of neointimal hyperplasia. As the term ‘bi-directional’ indicates, while we have described the process of SMCs becoming activated to recruit macrophages as a linear process, we hypothesized that this actually occurs as a feed-forward loop where SMCs modulate macrophages which then signal back to SMCs to exacerbate or maintain their activation to recruit more monocytes and modulate their phenotype, and so on. We hypothesized that initial SMC activation contributes to monocyte recruitment to lesions, which mature into macrophages in the presence of SMC-derived factors. We further hypothesized that these macrophages may be phenotypically modulated, as compared to resident vascular tissue macrophages or monocytic precursors, and then subsequently signal back to the resident SMCs to either maintain or exacerbate SMC activation.

**Macrophage Function and Phenotypic Modulation**

In addition to the epithelial barrier, dendritic cells, eosinophils, neutrophils, natural killer cells, and certain plasma proteins, macrophages are a major component of the innate immune system (as reviewed in Abbas, 2014). Macrophages play several important roles in host defense as well as in normal tissue homeostasis. For example, they are responsible for phagocytizing antibody coated microbes, or opsonized or damaged cells via toll-like receptors (TLRs), NOD-like receptors (NLRs), or scavenger receptors. They also present antigen to effector cells, and secrete cytokines that modulate
other adaptive and innate immune cells, and resident tissue cells. Depending on the
tissue they reside in, macrophages may exhibit different combinations of the above
functions. For example, in the spleen macrophages phagocytize old erythrocytes, while
in the lung they phagocytize microorganisms and particulate matter.62

The origin of macrophages in normal and diseased tissues is an area of much
current research. It is a commonly held belief among immunologists who focus on
macrophages that being able to discriminate these cells by developmental origin will
allow for a better understanding of their function. Mature tissue macrophages can derive
from self-renewing yolk sac progenitors that arrive in the tissues early in development, or
they may arise through the more well appreciated process of maturation from bone
marrow-derived monocytes upon extravasation from the blood into the tissues.63 It is not
currently known whether resident vascular macrophages originate from yolk sac-derived
progenitors or from circulating monocytes. However, while both of these mechanisms
likely contribute to neointimal macrophages, it is known that upon vascular injury or
atherosclerosis in murine models, at least some percentage of the cells that go on to
become lesional macrophages are derived from bone marrow-derived monocytes.64,65

Maturation of monocytes to macrophages is mediated by M-CSF (CSF1) and/or IL-34
(both ligands for CSFR1) via activation of the transcription factor PU.1.62,66,67 Upon
vascular injury, or in primary atherosclerosis, it is known that bone marrow-derived
Ly6c\textsuperscript{high} inflammatory monocytes, as well as Ly6c\textsuperscript{low} patrolling monocytes, are recruited
to the lesion, and contribute to lesion progression.68,69 It is possible that in addition to the
heterogeneity in the neointimal macrophage population due to hematopoietic origin (bone
marrow/medullary hematopoiesis versus extramedullary hematopoiesis of resident
vascular progenitors), additional heterogeneity may be the result of whether or not lesional macrophages mature from inflammatory or patrolling monocytes.

A strong correlation between monocyte/macrophage infiltration to sites of vascular injury-induced neointima, and worsened neointimal hyperplasia, has been established by several laboratories\textsuperscript{58, 60, 70, 71}. For example, Moreno et. al. found that positive staining for macrophages in human atherectomy sections predicted, with high significance, whether or not patients would go on to develop restenosis. Danenberg et. al. and Hong et. al. showed that bisphosphonate liposome depletion of macrophages \textit{in vivo} decreased neointima formation in rat and mouse vascular injury models, respectively. Additionally, Furukawa et. al. showed that the neointima to media ratio was significantly smaller in rats treated systemically with macrophage chemoattractant protein-1 (MCP-1) neutralizing antibody, versus isotype control, in the balloon catheter carotid artery injury model. MCP-1 has been repeatedly shown to be upregulated following vascular injury and to be involved in macrophage recruitment to vascular lesions (reviewed in Charo, 2004\textsuperscript{72}). Although many studies have noted a correlation between inflammatory cell (e.g. macrophage) infiltration to sites of neointimal hyperplasia and severity of vessel occlusion, to date the specific mechanisms driving macrophage-induced exacerbation of neointima formation have remained largely undefined.

Understanding macrophage phenotypic modulation upon maturation of monocytes to macrophages in vascular lesions may lead us to understand how these cells drive the pathogenesis of neointimal hyperplasia. Tissue macrophages are exposed to a vast array of activating stimuli as they surveil normal tissue and phagocytize apoptotic cells, or as they are recruited to injured or infected tissue. There exists a corresponding
vast continuum of macrophage activation states that are tailored to allow the macrophage to function appropriately depending on the stimulus. It has become relatively accepted to assess macrophage phenotype, or polarization, by monitoring the extremes of the spectrum: M1 and M2 (characteristics of these two populations reviewed in Van Ginderachter, 2006\textsuperscript{73} and Mosser, 2008\textsuperscript{74}). These activation states were originally characterized in the setting of *Leishmania major* infection, where T lymphocytes from resistant mouse strains (C57BL/6, B10D2) were shown to express a T-helper 1 (Th1) response (generation of interferon γ (IFN-γ) or tumor necrosis factor-α (TNF-α); resulting in Th1-modulated macrophages or M1 macrophages), while T cells from susceptible mouse strains (BALB/c, DBA/2) were shown to express a T-helper 2 (Th2) response (generation of IL-4; resulting in Th2-modulated macrophages or M2 macrophages)\textsuperscript{75}. M1, also called classically activated macrophages, are described as pro-inflammatory, and are considered pro-stenotic but anti-tumorigenic. The M1 phenotype is commonly modeled *in vitro* by stimulation with IFN-γ, and/or the pathogen-associated molecular pattern molecule lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria. These M1 macrophages express markers/cytokines such as iNOS, IL-12, TNF-α, and IL-1β. Functionally, they produce cytotoxic compounds such as nitric oxide through the metabolism of L-arginine by iNOS, hydrogen peroxide, and superoxide. TNF-α and IL-1β are functionally important pro-inflammatory cytokines that add to the macrophage’s innate immune reactivity. These reactive compounds and pro-inflammatory cytokines are minimally harmful to host tissues in the short-term. However, chronic exposure to M1 products can have deleterious effects that lead to cell death and fibrosis. Transcriptional regulation of many M1 products is thought to be
largely dependent on STAT1 and NF-κB transcriptional activity downstream of toll-like receptor and/or INF-γ receptor activation\textsuperscript{76, 77}.

In contrast to M1 macrophages, M2, or alternatively activated macrophages, are associated with injury resolution, and are considered to aid in stabilization of atherosclerotic plaque but to be pro-tumorigenic. The M2 phenotype is commonly modeled \textit{in vitro} by stimulation with IL-4 or IL-13. These M2 macrophages express markers/cytokines such as arginase I (Arg I), IL-10, TGF-β, and MHC class II. Functionally, M2 macrophages are capable of antigen presentation, exhibit increased endo- and phagocytosis, reduce inflammatory signaling through production of IL-10 and TGF-β (TGF-β abrogates the pro-inflammatory effect of endotoxins, such as LPS\textsuperscript{78}), and generate urea and L-ornithine through the metabolism of L-arginine by arginase I\textsuperscript{73}. Transcriptional regulation of macrophage phenotype downstream of IL-4 has been reported to be largely dependent on STAT6 activity, while IL-13 has been reported to exert its transcriptional effects via STAT1/3/5A, and B as well as via STAT6\textsuperscript{79}.

In order to study the effector functions of macrophages of the aforementioned polarization states, several \textit{in vitro} culture models have been used (described in Murray, 2010\textsuperscript{80}). Mouse and rat macrophages have been isolated from the peritoneum by thioglycollate elicitation\textsuperscript{81}, by alveolar lavage, or matured from precursors from PBMCs or bone marrow cultured in the presence of macrophage-colony stimulating factor (M-CSF). In order to study human macrophages in culture, they may be matured from precursor cells isolated from the peripheral blood in the presence of M-CSF. As described above, M1 or M2 polarization is modeled by stimulating macrophages \textit{in vitro} with LPS and IFN-γ, or IL-4, respectively. The control condition for these experiments is
a macrophage that is not exposed to either stimulus, but cultured in only M-CSF, often
termed an M0 macrophage. Because macrophage maturation in vivo probably does not
occur only in the presence of M-CSF it is unclear how the in vitro matured M0
macrophage relates to normal tissue macrophages in vivo. It is for this reason that it is
critical to validate data generated in vitro, using artificially M1/M2 polarized
macrophages compared to un-polarized M0 macrophages, in an in vivo system.

In human primary atherosclerosis, both M1 and M2 macrophages have been
observed in lesions. To our knowledge, the phenotype of macrophages in human
restenotic lesions has not been thoroughly assessed. In animal models, macrophage
phenotype has been studied in the murine apolipoprotein E (ApoE) and low-density
lipoprotein receptor (LDLR) knockout models of atherosclerosis. In both ApoE−/− and
the LDLR−/− models, macrophages have been observed exhibiting hybrid or heterogeneous
phenotypes, but with a prevalence of M1 macrophages and a correlation between lesion
severity and proportion of M1-like cells. Apolipoprotein E has been shown to
repress a pro-inflammatory M1-like phenotype in macrophages by p38 MAP kinase-
mediated inhibition of STAT1 and NF-κB activity. In the context of mechanical
vascular injury, Iwata et. al. attempted to characterize the molecular signature of
neointimal lesion macrophages by comparing mRNA from macrophage-rich areas in
neointimal lesions, as compared to macrophage-devoid areas. This was accomplished by
transplanting C57BL/6J mice with GFP bone marrow, performing wire-induced femoral
artery injury, and then isolating GFP-rich neointimal tissue and GFP-devoid neointimal
tissue by laser capture microdissection. Real time PCR array of these microdissected
samples revealed a hybrid/heterogeneous phenotype with upregulation of M1 markers
(IL-1β, CCL5, IFN-γ, TNF-α) as well as upregulation of M2 markers (IL-10, IL-13, TGF-β)\textsuperscript{86}.

Characterization of macrophage phenotype in sterile inflammation based on the characteristics exhibited by macrophages in infectious disease may not be adequate. Logically it stands that while IFN-γ and IL-4 may well be expressed in sterile disease, and thus participate in macrophage phenotypic modulation, endotoxin is unlikely to be a major contributor to macrophage polarization in sterile inflammation. Additionally, the bi-modal M1/M2 polarization model is clearly an over simplification. The observation of a hybrid and/or heterogenous phenotype in human and animal models of atherosclerosis and restenosis suggest that there is more going on than simple M1 or M2 polarization. Indeed, several other factors present in vascular lesions have been shown to modulate macrophage phenotype. Platelet factor 4 (PF4), a major factor released during platelet degranulation, has been shown to induce a pro-stenotic “M4” macrophage phenotype characterized by inability to upregulate heme oxygenase-1 (HO-1) in response to hemoglobin-haptoglobin complexes due to loss of CD163 (a scavenger receptor for hemoglobin-haptoglobin complex) expression. This M4 macrophage also is characterized by increased expression, as compared to M-CSF matured control M0 macrophage, by increased IL-6, TNF-α, TRAIL, CCL18 and 22, mannose receptor (CD206), CD86, MHC-II, MMP7 and 12\textsuperscript{87}. Another example of an atherosclerosis-relevant but non-M1/M2 macrophage phenotype is the oxidized phospholipid-modulated macrophage, or Mox macrophage\textsuperscript{83}. Mox macrophages are characterized by upregulation of HO-1, COX-2, VEGF, IL-1β, and several other genes, as compared to M0, M1, or M2.
macrophages. Arg-1, iNOS, TNF-α, MCP-1, and IL-12 were notably not induced in Mox macrophages.

Macrophage phenotypic modulation, and cross talk with non-immune cell types, is an active area of research in several other disease areas. Characterization of macrophage phenotype in cancer models has illustrated that these cells exhibit polarization toward an M2-like or immunosuppressive phenotype which functionally allows tumors to escape cytotoxic killing and promotes angiogenesis. In a model of renal fibrosis, mesenchymal stem cells (MSCs) were found to induce an M2-like phenotype in macrophages which the authors suggest may be contributing to the reparative effect of MSCs on fibrosis. In contrast with renal fibrosis, but in concordance with cancer metastasis, M2 macrophage polarization has been reported to be associated with Th2-type inflammation and eosinophilia in allergic exacerbation of asthma.

Macrophage phenotypic maker regulation, as observed in rodent disease models, and in vitro assays, is often somewhat discordantly regulated as compared to in human samples (reviewed in Murray, 2011). Analysis of macrophage phenotypic modulation in murine models is difficult to translate to human disease due to discordance of human and mouse macrophages’ gene regulation in response to Th1 and Th2-type cytokines. Human monocyte-derived macrophages do not express iNOS in response to LPS/IFN-γ, nor do human macrophage cell lines. However, tissue samples from tuberculosis infected individuals have been shown to express iNOS. Additionally, in humans, arginase I seems to be expressed only by neutrophils and not macrophages whereas murine macrophages and neutrophils express this protein. These discrepancies may be due to the origin of...
cells isolated for analysis. For example, studies on human *in vitro* matured macrophages are typically derived from peripheral blood while mouse-derived *in vitro* matured macrophages are usually isolated from bone marrow or elicited into the peritoneum by intraperitoneal injection of thioglycollate. Alternatively, it is possible that macrophage effector functions are specialized differently in human as compared to mouse, and/or rat, and that differential protein expression in these cells reflects that difference in specialization. Several other markers typically used to differentiate M1 from M2 macrophages in the mouse do not have homologues in the human such as Fizz1, and Ym1. Significant work is currently underway in order to characterize molecular markers that are concordantly regulated in human and mouse macrophages in response to M1 and M2 stimuli. Recent proteomic analysis of human and mouse-derived macrophages suggests that mannose receptor 1 (CD206) may be reliable discriminator for identification of M2-polarized macrophages. M1-polarized macrophages from both species have not been directly compared in an un-biased manner. However, two recent studies comparing human-derived M0, M1, and M2 macrophages suggest that increased expression (at both the transcriptional and translational levels) of CCR7, CXCL10 (interferon-inducible protein 10), CXCL11 (interferon-inducible protein 9), and CCL5 may be characteristic of human M1 macrophages. In contrast to the mouse, where low IL-10 and high IL-12 expression is well characterized, these studies in human samples suggest that IL-10 and IL-12 are not markedly regulated transcriptionally, but that they are both increased translationally in response to M1 stimulation.
SMC-Macrophage Interactions in Atherosclerosis and Restenosis

Observational studies of phenotypic modulation of macrophages in various disease states is informative for hypothesis generation, but we are particularly interested in how macrophages functionally modulate other cell types in the vessel wall with which they interact. In clinical samples, the presence of macrophages in human atherectomy samples has been shown to predict future incidence of restenosis with high significance. However, existing clinical data are of limited utility in understanding how SMCs and macrophages interact and how that interaction exacerbates disease, as these data can establish correlation but not causation. Pre-clinical disease models and in vitro studies have provided more insight into these interactions.

Several studies performed in rodents have established a causative link between monocyte/macrophage recruitment to vascular lesions and progression of neointimal hyperplasia, as discussed above. In vitro studies dating back to the late 1980’s showed evidence that vascular SMCs and macrophages interact in a pro-stenotic fashion; Rennick et. al. showed that coculture of macrophages and SMCs resulted in enhanced proliferation of SMCs relative to SMCs cultured alone. More recent, mechanistic studies have provided some insight into how macrophages and SMCs cross talk with respect to macrophage recruitment to lesions (adhesion and migration), macrophage phenotypic modulation, apoptotic signaling, and SMC activation. For example, in the setting of atherosclerosis, oxidized LDL induces CCR2^high^CX3CR1^low^ monocytes to switch phenotype to CCR2^low^CX3CR1^high^, allowing for CX3CR1-dependent adhesion to SMCs. The Natarajan group has performed studies that specifically address macrophage SMC interactions such as would be expected to occur in the early stages of
restenosis triggered by endothelial denudation (i.e in the context of SMC exposed to the circulation). This group observed that angiotensin II and PDGF-BB, two molecules known to promote pathologic vascular remodeling, and diabetic stimuli (elevated glucose or pharmacologic activation of the receptor for advanced glycation end products) increased monocyte adhesion to SMCs. Further, they showed that interaction of monocytes to SMCs in direct coculture resulted in increased surface expression of CD36 by macrophages, which might be predicted to participate in TGF-β activation and subsequent paracrine effects on both macrophages and SMCs (see the section TGF-β’s Role in Atherosclerosis and Restenosis).

Correlative studies have assessed expression of molecules by SMCs that would be predicted to modulate macrophage phenotype upon monocyte recruitment. Plenz et. al. showed that SMCs in the non-diseased vessel express GM-CSF (granulocyte-macrophage colony-stimulating factor), which is increased in SMCs from lesions as well as in activated SMCs in vitro. This cytokine acts as a maturation factor for dendritic cells and as a chemoattractant and activation/proliferation factor for macrophages.

Several groups have also characterized the effect of cross talk between SMCs and macrophages with respect to modulation of apoptosis in either cell type. In addition to their findings related to the effect of SMCs on macrophage CD36 expression, the Natarajan lab has also published that physical interaction of macrophages with SMCs, via VCAM-1, can reduce the macrophage apoptotic response to serum deprivation by increasing AKT pathway activation and downstream Bcl-2 expression. From the other perspective, activated lesional macrophage-derived NO induces surface expression of Fas and TNF-α receptor 1 by SMCs, making them more susceptible to pro-apoptotic signals.
The same lesional macrophages are known to express surface Fas ligand and TNF-α\textsuperscript{103}. Further, it has been shown \textit{in vitro} that macrophages are capable of inducing Fas-mediated apoptosis of SMCs\textsuperscript{104, 105}, and SMCs undergo apoptosis when plated on Mφ-modified collagen I, versus unmodified collagen I\textsuperscript{106}.

While it is appreciated that apoptosis of SMCs and macrophages is involved in the pathogenesis of neointimal hyperplasia, SMC activation is considered the more prominent contributor. The Natarajan group published informative studies in the early 2000s assessing the interactions of SMCs and monocytes \textit{in vitro}. They are one of the few groups that have tried to assess the interactions of these two cells not in the context of super-physiologic stimulation of macrophages with LPS. Several studies in the vascular disease literature have used LPS-polarized macrophages in order to model the M1-like phenotype that has been observed to be a prevalent population in atherosclerotic lesions\textsuperscript{107-109}. For example, Chen et. al. showed that LPS-stimulation of human monocytes resulted in synergistic upregulation of pro-inflammatory cytokines from both macrophages and cocultured saphenous vein SMCs (which agrees with our data presented in Appendix A)\textsuperscript{110}.

Investigators performing such studies in the vascular literature have looked to the infectious disease literature, where the effects of LPS on macrophage phenotype is more relevant, to understand how other growth factors influence the phenotype of macrophages. The difficulty in extrapolating from the infection literature to the setting of sterile-inflammation (such as cardiovascular disease or cancer) is that activation of macrophages does not occur by the same mechanisms, and the modulatory effects of other growth factors may not be the same in the presence and absence of LPS stimulation.
For example, several studies by Carl Nathan’s lab have established that TGF-β - specifically in the presence of LPS - inhibits activation of macrophages (see section TGF-β’s Role in Atherosclerosis and Restenosis). TGF-β has thereafter been accepted to be a macrophage de-activating factor, particularly in the field of vascular biology. However, we will present data in this dissertation showing that TGF-β, in the absence of LPS, promotes an activated macrophage phenotype, and is ultimately pro-stenotic.

**TGF-β’s Role in Atherosclerosis and Restenosis**

TGF-β is a multifunctional 24 kiloDalton (kDa) dimeric peptide growth factor (reviewed in Suwanabol, 2011, Taylor, 2009, and Annes, 2003) that is part of a family of cytokines that includes bone morphogenic proteins, growth differentiation factors, activins, nodals, and anti-mullerian hormone. There are three isoforms of TGF-β (TGF-β 1, 2 and 3) which are encoded by different genes (TGFB1, TGFB2, and TGFB3, respectively), and are expressed differentially across cell or tissue types. TGF-β1 is known to be expressed in epithelial cells, smooth muscle cells, hematopoietic cells, and by fibroblasts. TGF-β2 is preferentially expressed by epithelial cells as well as neurons, and TGF-β3 is primarily expressed by mesenchymal cells. While data exists that all TGF-β isoforms are upregulated in vascular injury, studies from many groups suggest that the TGF-β1 isoform is the isoform that influences neointimal hyperplasia to the greatest extent. The TGF-β gene product is expressed as a 75kDa dimeric pro-protein that is cleaved in the Golgi by furin-type enzymes into the truncated mature 24kDa dimer plus a pro-domain latency-associated protein, or LAP, dimer. This inactive form of the cytokine is referred to as the small latent complex, or SLC. The SLC is further stabilized
and inactivated by the association with one of several isoforms of a latent TGF-β binding protein (LTBP) via di-sulfide linkages. This greater than 200kDa form of TGF-β, of the large latent complex (LLC), is secreted and can form covalent cross-linkages with extracellular matrix molecules by transglutaminases. When bound by LAP or LAP-LTBP dimers, TGF-β is sterically unable to bind to its receptors to engage signaling cascades. The evolutionary reason for why TGF-β may be constitutively produced and held in an inactive form in the ECM is not well understood. However, it is due to this primed but inactive state that assessing TGF-β activation state is essential for understanding its biology. There are several biologic and physical mechanisms for the activation of latent TGF-β. TGF-β can be activated enzymatically by liberation of the mature dimer from LAP-LTBP, such as by MMPs, plasmin, or by furins or other convertases\textsuperscript{112}. Active TGF-β can also be liberated from the LLC by a conformational change induced by LAP-binding receptors, such as the thrombospondin 1/CD36 complex\textsuperscript{114}, mannose 6-phosphate receptor, and several αV-containing integrins\textsuperscript{112}. It is unclear whether this latter mechanism of TGF-β activation requires both conformational change as well as protease activity, or if conformational change in the LLC is sufficient to liberate fully active mature TGF-β dimer. In addition to these protein-mediated mechanisms of TGF-β activation, the mature active form can also be released from latent complexes due to exposure to low pH, reactive oxygen species, as well as by freeze-thawing\textsuperscript{113}.

Active TGF-β engages signaling cascades by binding to tetrameric receptor complexes consisting of two type I receptors (TBRI) and two type II (TBRII) receptors. TGF-β receptors (TBRs) are transmembrane serine-threonine kinases. Ligand binding to
the TBRII dimer results in association of the TBRI dimer to form the tetrameric receptor complex. TBRII then phosphorylates TBRI, initiating canonical and non-canonical signaling pathways. In canonical signaling, ligand binding initiates association of a receptor Smad (R-Smad; Smad2 or 3) and Smad anchor for receptor for activation (SARA) with the activated receptor. The R-Smad is phosphorylated, allowing for co-Smad (Smad-4) association and nuclear translocation. This R-Smad/co-Smad complex is then able to bind to Smad-binding elements, TGF-β control elements, or CarG boxes in promoter regions of genes to modulate gene expression. An inhibitory aspect of canonical TGF-β signaling is the repression of R-Smad/Co-Smad nuclear localization by inhibitory Smads. These proteins may act as part of a negative feedback loop that serves to limit TBRI/II signaling by acting as a dominant negative to prevent R-Smad activation, or by inciting receptor degradation. In contrast to canonical TGF-β signaling, non-canonical signaling is not mediated through Smad proteins. In a cell type-dependent manner JNK/p38, ERK, Rho-GTPase, and AKT signaling pathways may be engaged to modulate, positively or negatively, Smad-dependent effects. These modulatory effects may also vary in a cell type-dependent manner in terms of whether they exert their effects by parallel signaling or by direct physical interaction with Smad signaling molecules.

Upregulation of TGF-β and TGF-β signaling has been observed in human primary atherosclerotic as well as restenotic lesions. Retrospective cohort studies in humans have linked altered TGF-β production or mutations in TGF-β signaling components to cardiovascular disease. Further, McCaffrey et. al. published that TBRI/II expression ratios were found to be altered in SMCs in atherosclerotic human arteries; in vitro data by Grainger et. al. suggests that a high TBRII:TBRI ratio results
in a differentiating response to TGF-β stimulation, but that a low TBRII:TBRI ratio results in a pro-synthetic response. However, the inability to perform genetic manipulations in the human have made it difficult determine the net contribution of TGF-β signaling to atherosclerotic plaque development or restenotic neointimal hyperplasia, and even more so to determine the contribution of cell type-specific TGF-β signaling on these disease processes.

Pre-clinical models of vascular disease have offered some insight into the role TGF-β may be playing in the pathogenesis of atherosclerosis and restenosis. As early as the late 1980’s animal studies demonstrated that TGF-β was upregulated transcriptionally following mechanical vascular injury\textsuperscript{124, 125}. It has more recently been shown that latent TGF-β is activated by mechanical arterial injury, and that systemic inhibition of TGF-β signaling with a TBRI kinase inhibitor (SB-505124) is sufficient to inhibit NIH\textsuperscript{126}. Additional studies in rat and/or mouse injury models have also shown reduced NIH with systemic administration of anti-TGF-β neutralizing antibody, soluble receptor, or ribozyme oligonucleotide. Correspondingly, several groups have found overexpression or infusion of TGF-β sufficient to induce exacerbated NIH\textsuperscript{111}. The role of TGF-β signaling in murine atherosclerotic models suggests a similar effect on cellular behavior, but a different result with respect to disease progression due to mechanistic differences between atherosclerosis and restenosis. At least two groups have published that neutralization of TGF-β in ApoE null mice was sufficient to reduce ECM production in the lesion leading to decreased fibrosis and more unstable plaques\textsuperscript{127, 128}. When extrapolated to restenosis, where ECM production is a major contributor to pathogenesis, these data suggest that TGF-β neutralization would be anti-stenotic.
In order to further interrogate TGF-β’s role on cell biology, many laboratories have studied the effect of this cytokine on cells in vitro. Studies in primary cultured vascular smooth muscle cells have shown consistently that TGF-β acts as an anti-proliferative/pro-differentiation factor for SMCs. TGF-β has also been shown to be a critical differentiation signal responsible for driving embryonic stem cell differentiation to an SMC lineage\textsuperscript{115,129}. Additionally, while TGF-β has been reported to induce ECM production by SMCs, suggesting promotion of a synthetic phenotype, it also induces upregulation of smooth muscle contractile apparatus proteins and repressed migration, suggesting net promotion of a contractile phenotype\textsuperscript{123}. Mechanistically, TGF-β has been shown to regulate SM-gene transcription (reviewed in Guo, 2012\textsuperscript{115}) by inducing expression of miR143 and 145, which repress expression of the transcription factor KLF4. KLF4 has been shown to repress SM-gene transcription by binding to TGF-β control elements, whereas KLF5 exerts the opposite effect on SM-genes via TCE binding. TGF-β is also known to induce nuclear translocation of, and nuclear complex formation at Smad binding elements (SBEs) by Smad3 and 4 along with CArG element-bound myocardin. This effect is specific to Smad3 and is not seen with Smad2. TGF-β’s effect on SM-gene regulation is also mediated through RhoA/ROCK-dependent regulation of serum response factor (SRF) expression, and nuclear translocation and binding to CArG boxes. RhoA has also been reported to be required for TGF-β-dependent Smad phosphorylation and promoter activation.

In contrast with TGF-β’s effect on SM-gene expression and SMC differentiation state, TGF-β’s role in macrophage phenotypic modulation has been studied in less detail. By the late 1980s, TGF-β had been shown to induce tissue granulation involving collagen
production, fibroblast proliferation, angiogenesis, and leukocyte recruitment\textsuperscript{130}. Subsequent \textit{in vitro} studies showed that TGF-β is potent as a chemoattractant for monocytic cells at femto- to low picomolar concentrations, and that TGF-β induced cytokine (IL-1, FGF, and TNF) production by monocytes\textsuperscript{131}. TGF-β (and not colony stimulating factors, interleukins, interferon γ, or endotoxin) has also been shown to induce FcγRII, or CD16, on cultured monocytes, which allows them to bind IgG multimers to effect immunophagocytosis and oxidative burst. In seeming contrast to these pro-inflammatory effects of TGF-β on cultured monocytes, it was also reported around the same time, by the Nathan lab, that TGF-β is a macrophage de-activating factor\textsuperscript{78,132,133}. This concept has persisted across many research areas where the contribution of macrophages to disease progression in the setting of primarily sterile inflammation (cancer, cardiovascular disease) is studied. However, these studies that presented TGF-β as a de-activating factor for macrophages were focused on the effect of TGF-β on macrophage phenotype in the presence of microbial (or helminth) infection \textit{in vivo}, or endotoxin \textit{in vitro}. From these data it is clear that TGF-β is capable of repressing the effects of endotoxin on macrophage activation, however our data, along with data from Wahl et. al.\textsuperscript{131} and Pang et. al\textsuperscript{134}, suggest that the role of TGF-β on macrophage phenotype in the setting of sterile inflammation is pro-inflammatory.

**Overview of this Dissertation**

The objective of this project was to define the role of SMCs in modulating the phenotype of macrophages, the role of macrophages in the dedifferentiation of SMCs,
and the cross talk between SMCs and macrophages using both \textit{in vitro} and \textit{in vivo} approaches.

The results of these studies are presented in Chapters 2-4. In Chapter II we investigate how macrophage phenotype was modulated \textit{in vivo} in response to the wire-induced femoral artery injury model of angioplasty. In Chapter III we use an \textit{in vitro} culture-based system to investigate how SMC-derived factors modulate macrophage phenotype, and to determine what factor is responsible for modulation of macrophage phenotype. Lastly, in Chapter IV we begin to examine how monocyte recruitment to neointimal lesions is influenced by the expression level of PTEN in SMCs. Chapter V of this dissertation summarizes the results of these studies and presents follow-up studies and potential translational implications of this work.
CHAPTER II

QUANTITATION OF MYELOID CELL POPULATIONS, AND CHARACTERIZATION OF MACROPHAGE PHENOTYPE IN A MURINE MODEL OF RESTENOSIS

Introduction

Neointimal hyperplasia is known to occur in the context of atherosclerosis, or following percutaneous interventions, bypass grafting, or transplant. Despite the implementation of drug-eluting stents, luminal narrowing due to restenosis remains a significant clinical problem. A more complete understanding of the pathogenesis of neointimal hyperplasia is required to develop more precisely targeted therapies that will allow for complete re-endothelialization of re-vascularized or grafted vessels and vessel repair in order to improve outcomes.

Neointimal lesions have been shown to be largely composed of activated or dedifferentiated vascular smooth muscle cells that migrate from the tunica media to the developing neointima. Activation of resident SMCs is a complex, multi-faceted process that promotes a transition to a highly proliferative, inflammatory phenotype characterized by down-regulation of SM contractile genes and increased production of multiple growth factors, cytokines, and chemokines. Many of these factors

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participate in neointima formation through direct effects on SMCs combined with recruitment of inflammatory cells that sustain lesion progression. As SMCs are critical in initiating and promoting neointima formation, targeting SMCs is a logical strategy to inhibit progression of vascular disease. However, simply focusing on blocking SMC proliferation is likely an ineffective means to prevent disease as these therapies inhibit proliferation of other cells (i.e. endothelial cells) that are required for vessel recovery. Preventing vascular inflammation is an equally important approach to limit restenosis since it also plays a central role in intimal growth. Several studies have demonstrated a critical role for recruited macrophages in the progression of neointimal hyperplasia.\(^58,\ 64,\ 71,\ 137-140\) For example, clodronate depletion of macrophages has been shown to reduce injury-induced neointima formation in both the mouse\(^57\) and rat\(^58\). However, the mechanisms underlying how macrophages specifically cross talk with SMC, and vice versa, to induce and/or perpetuate SMC activation and neointimal hyperplasia are not well understood.

Macrophages are highly plastic cells which undergo phenotypic modulation in response to signals from the microenvironment. Macrophage phenotypic modulation, or polarization, is typically described as either LPS+interferon-\(\gamma\)-mediated classical M1 activation or IL-4/IL-10-mediated alternative M2 activation.\(^73,\ 75\) Modulation of macrophage phenotype in the vasculature has largely been studied in the context of atherosclerosis, where these cells have been found to exhibit a spectrum of phenotypes.\(^68,\ 84,\ 141,\ 142\) As macrophages have been identified as the predominant inflammatory cell infiltrating injured vessels, we sought to characterize the phenotype of macrophages recruited to neointimal lesions \textit{in vivo}. In Chapter III of this dissertation, we will
describe studies defining how signals from SMCs contribute to this macrophage phenotype, and studies assessing if macrophages modulated by SMCs gain the ability to signal back to SMCs to promote or maintain SMC activation.

**Materials and Methods**

**Reagents**: Antibodies: CD68-FITC (Biolegend), SM-α-actin-Cy3 and unconjugated (Sigma), F4/80 (Cl:A3-1, Abcam), anti-rat-Alexa 488 (Life Technologies). Flow cytometry antibodies: biotinylated-F4/80 (Cl:A3-1, AbD Serotec), streptavidin-RPE, CD11b-FITC, Ly6g-PE-Cy7, SiglecF-PE, CD11c-APC-Cy7 (all BD Biosciences), MHC-II-PerCP eFluor 710 (eBiosciences).

**Femoral Artery Injury**: All animal procedures were approved by, and performed in accordance with the guidelines of the University of Colorado IACUC. Guide-wire denudation femoral artery injury was performed, as shown by us previously, on right femoral arteries of male 20-28g C57BL/6 mice (Figure 2A). Mice were anesthetized with 50μg/g sodium pentobarbital (diluted to 5mg/ml in sterile saline) administered intraperitoneally (IP). Anesthetized animals’ right inguinal regions were depilated. Approximately 100μl of lidocaine solution was delivered subcutaneously (SQ) at the incision site. After allowing local anesthesia to take effect for about a minute, a ~one centimeter incision was made to expose the superficial vasculature from the knee proximally to the abdominal wall (for illustrations of the murine vasculature see The Anatomy of the Laboratory Mouse website). The femoral artery, and the attached vein, were isolated from the surrounding tissue and femoral nerve by blunt dissection. Silk 6-0
suture was used to ligate the proximal end of the femoral artery where a branch enters in between the rectus femoris and the vastus medialis muscles. Another suture was used to stabilize the proximal end of the femoral artery (separated from the femoral vein) where a branch moves superficially toward the subcutaneous fat. An arteriotomy was created in the femoral artery between these two ligatures. A 0.38mm guide wire (Cook, C-SF-15-15) was then inserted into the femoral artery at least until the bifurcation of the abdominal aorta – where the aorta becomes the common iliac arteries and the caudal artery. The guide wire was inserted fully, and pulled back ~50% of the way, and inserted fully again three times to mechanically denude the arterial endothelium. The wire was then left in place for one minute to induce a stretch-induced injury. Upon removing the wire the two ligatures were tied tight. Blood flow was restored to the portion of the artery proximal to the artery branches just proximal to the upper ligature. The incision was then closed using 5-0 silk suture with curved cutting blade (Covidien). In order to ascertain if femoral arteries were being sufficiently denuded retro-orbital injection of 100μl of filtered Evans Blue dye was injected, in non-experimental animals, immediately following terminal anesthesia with isofluorane overdose (Figure 2B). Evans blue dye stains vessels with disrupted endothelial cell monolayers. Injured femoral arteries, contra-lateral control femoral arteries, aortas, and carotids were harvested on day 15 post-injury following euthanasia and flushing of the vasculature with heparinized PBS (80kU/L).

Flow Cytometry: Isolated injured, and contralateral control vessels, were digested to single cells by digestion at 37°C for 2hr in collagenase buffer (3.2mg/ml collagenase II,
Figure 2: Anatomy of the medial aspect of the mouse hind-limb and Evans Blue staining of guide wire-denuded femoral artery.
A) The vasculature of the mouse was perfused with trypan blue. The artery is stained blue. The vein is perfused with blood and appears red. Locations of ligatures and arteriotomy used in the femoral artery injury model are indicated. B) Immediately following injury, Evans blue dye was injected retro-orbitally. Regions of disrupted endothelial integrity, due to wire-injury, are stained blue. Shown is an isolated femoral artery from the distal ligature to the iliac bifurcation of the aorta.
0.7mg/ml elastase (Worthington), 0.2mg/ml soybean trypsin inhibitor (Sigma) in Hank’s buffered saline solution (HBSS, pH7.5). Red blood cells were lysed for 2-5 min in RBC lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA (pH 7.2)). Single cell suspensions were stained for CD11b, F4/80, Ly6g, MHC-II, Siglec-F, CD11c, and DAPI to stain for dead cells. Flow cytometry was performed on a Galios cytometer (Becton Dickenson) as demonstrated by our group previously\textsuperscript{145}. Gating strategy for flow cytometry is shown in Figure 3A. Briefly, after gating out dead cells and debris, live cells were plotted for CD11b versus Ly6g. Neutrophils appear as a distinct double positive population\textsuperscript{146}, and were gated out. Remaining traces were then plotted for CD11b versus F4/80. The remaining double positive population contains macrophages, eosinophils, and dendritic cells. The eosinophil population was gated out by plotting the CD11b+/F4/80+ population for forward scatter versus side scatter. This high forward scatter, low side scatter side population was verified to be CD11b+/SiglecF+ eosinophils\textsuperscript{145} (red traces, Figure 3A, upper right). Dendritic cells were enumerated and gated out by plotting CD11c high cells for MHC-II expression versus forward scatter, and selecting the MHC-II+ low forward scatter population.

**CD11b Magnetic Bead Positive Selection of Cell Suspensions**: Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood spun over a Histopaque 1.077g/ml (Sigma) gradient at 500xg for 30min as shown by us previously\textsuperscript{147}. Buffy coat layers were then washed with sterile PBS and spun at 300xg for 10min to separate mononuclear cells from platelets. Single cell suspensions were prepared from injured, or contralateral control, vessels as described under Flow Cytometry. Anti-
CD11b antibody-conjugated magnetic beads (Miltenyi) were used to separate CD11b positive and negative populations from PBMC and vessel suspensions as per the manufacturer's specifications. Single cell suspensions from PBMC and injured vessel CD11b positive selections were analyzed by flow cytometry for purity of macrophages as described under Flow cytometry. Total RNA was prepared from CD11b positive cell isolates using RNEasy Plus Micro columns (Qiagen).

**Real Time qPCR:** cDNA was prepared from total RNA isolates (equal total μg/reaction based on maximum allowable in the lowest concentration sample) using the iScript cDNA Synthesis kit from Bio-Rad. Real time qPCR then performed using the Power Sybr Green supermix from Applied Biosystems as shown previously\textsuperscript{147-149}. Reactions were carried out on an iCycler (Bio-Rad). Cycling parameters were: 5min at 95°C; 15sec at 95°C, 30sec at the annealing temperature optimized for each primer pair, and 30sec at 72°C for 50 cycles (60 cycles for CD11b pulldown samples). GAPDH was used as a reference gene.

**qPCR Primers:** See Table 1.

**Statistics:** Data are expressed as means ± standard error. Student’s T-tests were used to compare data sets with two samples. Data sets with more than two samples were analyzed by ANOVA followed by Tukey multiple comparisons post-tests.
## Table 1: Chapter II qPCR primers

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<th>Gene Description</th>
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<th>Reverse Sequence</th>
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<tr>
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<td></td>
</tr>
<tr>
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</tbody>
</table>
Results

Macrophages Recruited to Injured Vessels are Phenotypically Distinct from Peripheral Blood Monocyte (PBMC) Precursors. Our group and others, have shown increased recruitment of bone marrow-derived macrophages to the arterial wall following endothelial denudation-mediated arterial injury in stained tissues. Flow cytometry was used to quantitatively verify enhanced presence of macrophages in the setting of vascular injury (gating strategy: Figure 3A) at 15 days post-injury, when macrophage accumulation was previously observed to be maximal, and when neutrophil recruitment is expected to have abated. We found that in injured femoral arteries, relative to contralateral control femoral arteries or aortas from injured animals and femoral arteries or aortas from naïve animals, macrophages made up a significantly larger percentage of total cells than did neutrophils, eosinophils, or dendritic cells (Figure 3B-F). Eosinophil and neutrophil recruitment was not increased at this time point following injury. We sought to determine if these recruited macrophages are phenotypically distinct from PBMC precursor monocytes. In order to compare neointima-associated macrophages versus resident tissue macrophages, or circulating monocyte controls, CD11b+ cells were isolated from injured femoral arteries, uninjured arteries, or Histopaque-separated buffy coats by positive selection using anti-CD11b antibody-conjugated magnetic beads. Positively selected cells stained for F4/80, indicating that they were largely monocytes/macrophages, whereas non-selected cells stained positive only for smooth muscle α-actin (SM-αActin), indicating SMCs, or were negative for both F4/80 and SM-αActin, indicating other cell types (Figure 4A). CD11b positive selection resulted in
Figure 3: Analysis of myeloid populations in injured arteries by flow cytometry.
Figure 3: Analysis of myeloid populations in injured arteries by flow cytometry.

Single cell suspensions were isolated from femoral arteries following 2-wks wire-induced injury (iRFA), from contralateral control femoral arteries (LFA) and aortas from injured mice, and from aorta and femoral arteries of naïve mice. Cells were stained for CD11b, Ly6g, F4/80, SiglecF+, CD11c, MHC-II, and DAPI and analyzed by flow cytometry. A) Live cells were plotted for CD11c vs Forward Scatter (FS), CD11c<sup>hi</sup> cells were plotted for MHC-II versus FS to enumerate and gate out CD11c<sup>hi</sup>/MHC-II<sup>hi</sup>/FS<sup>low</sup> dendritic cells. Live cells, excluding dendritic cells, were plotted for CD11b versus Ly6g to quantify and exclude CD11b<sup>hi</sup>/Ly6g<sup>hi</sup> neutrophils. Eosinophils were excluded from the remaining CD11b<sup>hi</sup>/F4/80<sup>hi</sup> population, based on higher forward scatter and low side scatter or Siglec-F positivity. A, Upper right) Scatter plot showing that the FS/SS eosinophil gating strategy distinguishes CD11b+/Ly6g-/SiglecF+ eosinophils (red) from the CD11b+/Ly6g-/F4/80+ macrophages (green). B) Representative CD11b vs F4/80 scatter plots from injured versus uninjured arteries at day 15 after wire-induced injury. C) Neutrophils, D) eosinophils, E) dendritic cells and F) macrophages were quantified by flow cytometry 15 days after injury using the gating strategy described above. Each point represents a pool of arteries from 3-5 animals. n=2 to 4 independent experiments. Red gates indicate populations gated out prior to subsequent analysis step; black gates indicate populations gated on for subsequent analysis step. *P<0.05.
Figure 4: Immunofluorescent and flow cytometry characterization of in vivo cell populations.
A) Single cell suspensions were isolated from femoral arteries 2-wks after wire-induced injury. CD11b positive (CD11b+) and negative (CD11b−) cell fractions from peripheral blood mononuclear cells (PBMCs), uninjured arteries, and injured femoral arteries (iRFA) were assessed for SM-αActin (red) and F4/80 (green) expression. DAPI was used to visualize nuclei. Representative images shown. B) CD11b positive cell fractions from PBMCs and iRFA were assessed for contaminating cell populations by flow cytometry using the gating strategy described in Figure 3. Representative images/plots shown.
80% monocyte/macrophages from either PMBCs or injured femoral arteries, as determined by flow cytometry analysis of CD11b positively selected cells. Flow cytometry analysis of uninjured vessels for quantity of macrophages was not performed. Injured artery isolations contained similar percentages of eosinophils and neutrophils, and slightly more dendritic cells than did PBMC isolations (Figure 4B). Since contaminating cell populations were similar in PBMC and injured vessel isolations, comparing mRNA levels in these cell isolations was valid. Expression of a panel of genes was assessed in CD11b positive cell isolates by real time quantitative polymerase chain reaction (qPCR) (Figure 5). We focused on genes that have been shown to be upregulated in bone marrow cell derived-rich regions of neointimae, as well as on genes that have been suggested to be upregulated in inflammatory mouse, rat, and human macrophages. Several genes (IL-6, CCR3, CCR7, IL-10, IL-12b, and TNF-α) were upregulated in CD11b+ cells from injured vessels relative to CD11b+ PBMCs. In contrast, expression of IL-12a and MMP9 was repressed in CD11b+ cells from injured vessels relative to PBMC controls. In addition to this 9-gene panel, we assessed expression of inducible nitric oxide synthase (iNOS) and arginase 1 (Arg I), markers of M1 and alternatively activated M2 macrophages, respectively. INOS and Arg I expression was higher in CD11b+ cells from injured arteries relative to controls. Several of these genes were modulated similarly, compared to PBMC precursors, in both macrophages from uninjured vessels as well as in macrophages from injured vessels. The genes that fell into this category were: IL-10, IL-12a, and perhaps Arg I, although Arg I data from uninjured vessels had a very large error. In contrast, several genes were modulated differentially in macrophages from injured vessels versus macrophages from injured vessels. IL-6, TNF-α, and MMP9 expression
was decreased in macrophages from injured vessels as compared to macrophages from uninjured vessels. There was a similar trend exhibited by IL-12b. Lastly, when compared to both monocylic precursors and macrophages from uninjured vessels, macrophages from injured vessels expressed markedly higher iNOS, and there was a trend toward higher CCR3 and CCR7.

CD11b+ cells were isolated from circulating peripheral blood mononuclear cells (PBMCs), and from digested uninjured femoral arteries (Uninjured) and femoral arteries following 2-wks wire-induced injury (iRFA). Total RNA was isolated and qPCR conducted for the indicated mRNAs. Shown are mRNA copy number normalized to GAPDH. Means±SE; n=4 independent experiments. *P<0.05 vs PBMC.

<table>
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<th></th>
<th>PBMC</th>
<th>Uninjured</th>
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<tbody>
<tr>
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<tr>
<td>Arg1</td>
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Copy #/GAPDH

![Graph showing mRNA copy number normalized to GAPDH.](chart.png)
Discussion

Progression of vascular remodeling in response to vascular injury involves complex interactions between vascular cells and recruited inflammatory cells. Inflammatory cells, including circulating monocytes, are recruited to the site of injury where they undergo differentiation into macrophages. In human atherectomy samples, presence of macrophages predicts development of restenosis with high significance\textsuperscript{60}. Macrophages have been shown to contribute to pathogenesis of restenosis through secretion of growth factors, cytokines, and chemokines which induce SMC and endothelial cell proliferation, as well as contribute to SMC activation. Neointimal lesion macrophages additionally contribute to the developing neointima by secreting matrix-remodeling enzymes (eg. MMPs) that facilitate development of fibrosis\textsuperscript{4}. Macrophages exhibit remarkable phenotypic plasticity in response to signals from the local tissue microenvironment. The phenotype of macrophages in an injured vessel will depend on both the lineage commitment and environmental signals generated through interactions with other cell types in the lesion, including smooth muscle cells\textsuperscript{68}. The goal of this study was to define characteristics of macrophages in the developing neointima that are distinct from other immature/circulating monocytes and to define the role SMCs play in influencing this macrophage phenotype. Understanding the signals critical for macrophage phenotypic modulation may shed light on novel therapeutic strategies to limit SMC proliferation and ECM deposition while allowing for re-endothelialization and reduction of thrombotic events.
**Conclusions**: Using flow cytometry, we were able to quantitatively show increased recruitment/retention of monocyte/macrophages to injured vessels. We were then able to define an expression signature that distinguishes lesional macrophages from resident tissue macrophages or precursor monocytes. These macrophages manifest several features of classically activated M1-like macrophages, although there were some noted dissimilarities (e.g. decreased TNF-α, and increased arginase I expression), possibly suggesting a complex activation state, or heterogeneous population. Gene regulation in these samples could be clustered into three categories: genes that were expressed at similar levels in macrophages from injured and uninjured vessels but at different levels in PBMCs (IL-10, IL-12a, arginase I), genes that were expressed at similar levels in PBMCs and macrophages from uninjured vessels but differentially in macrophages from injured vessels (CCR3, and possibly CCL5), and genes that were expressed differentially in all three populations (IL-6, Il12b, CCR7, TNF-α, MMP9, iNOS). These different categories of gene modulation patterns may reflect that regulation of some genes in both macrophage populations, relative to precursor monocytes, may be regulated by monocyte-to-macrophage maturation while other genes may be regulated by injury-associated factors, or finally a combination of maturation and injury associated factors. Together, these data suggest that, in addition to increased recruitment upon vascular injury, monocytes recruited to vascular lesions receive injury-specific molecular signals that result in promotion of an altered activation state that is distinct from precursor monocytes and macrophages present in uninjured vessels, and exhibits characteristics of both M1 and M2 phenotypes.
Based on these data, and numerous studies presented in the atherosclerosis literature supporting the occurrence of phenotypic modulation in plaque macrophages\textsuperscript{68}, we hypothesized that factors produced by SMC were critical in promoting this injury-associated phenotype. Chapter III of this dissertation discusses \textit{in vitro} studies performed to ascertain if SMC-derived factors have the ability to modulate the phenotype of macrophages as they mature from immature precursors.

\textbf{Limitations and Future Directions:} Interpretation of the study presented in this chapter is complicated by several technical limitations. First, we decided to assess the phenotype of both the macrophages present in the uninjured vessels and the monocytes present in the peripheral blood as control populations because it has not been determined in the vascular biology field whether the cells that give rise to macrophages in restenotic lesions derive predominantly from resident mature macrophages present in normal vessels, from myeloid progenitor populations present in the arterial adventitia, or from circulating monocytes. It is most likely that more than of these scenarios occur concomitantly. Therefore the control population that would most accurately represent a control population with which to compare the macrophages present in the injured vessel might be a combination of macrophages (and/or progenitors) present in the uninjured vessel and precursor monocytes. However, determining exactly how to isolate a control population of monocyte/macrophages that represents the population present in an injured vessel, but just prior to exposure to the factors elaborated at the site of injury, is far from being elucidated.
An additional technical limitation of these studies is the fact that the cell populations that we were able to isolate by CD11b positive selection are not homogenous, as shown by flow cytometry in Figure 4B. Indeed we do not know if the macrophage population present in these isolations would be proven to be homogenous, with respect to phenotypic polarization or growth factor production, even if they appeared to be purely macrophages by flow cytometry analysis. It is possible that if we were to develop FACS methodologies to isolate pure populations of monocyte/macrophages from injured and uninjured vessels, and from peripheral blood, with respect to surface marker expression that these populations would still be heterogeneous with respect to function. We may be able to assess this heterogeneity in future studies by identifying more reliable cell surface markers to isolate subpopulations of macrophages by FACS. For example, if we were to find, using immunofluorescent staining, that a large percentage of macrophages in the injured vessel co-expressed iNOS and arginase I, but do not express CD206 (see Figure 12B), we could potentially isolate iNOS/ArgI positive macrophages, versus CD206 positive macrophages by laser capture micro-dissection and subsequent RNA-seq to truly characterize the transcriptome of, at least, these two populations. Transcriptome analysis might then yield cell surface markers that could be used to isolate macrophages of different polarization states from injured and uninjured vessels for use in ex vivo SMC cross talk studies to understand how these different subpopulations may influence SMC biology.

A final complicating factor is that, in these studies, we have only assessed macrophage phenotype at a single time point. It is possible that some of the similarity we observed between macrophages in the injured vessel compared to macrophages in the
uninjured vessel (as well as the discordance we observe between these data and the *in vitro* data presented in Chapter III), may be due to temporal effects on macrophage phenotype. Perhaps if we were to assess the phenotype of macrophages at earlier time points we may observe a phenotype in macrophages from injured vessels that is markedly distinct from macrophages in uninjured vessels with respect to more of the genes in our panel.

The studies performed in this chapter assessed macrophage phenotype in the setting of endothelial denudation injury in non-atherogenic mice. A future direction of this project would be to expand these studies to understand how monocyte recruitment and phenotypic modulation in vascular injury is influenced by hyperlipidemia (ApoE or LDLR knockout plus high fat diet). Additionally, it would be informative to assess how macrophages isolated from hyperlipidemic lesions may differ in terms of cross talk with SMCs in *ex vivo* assays.
CHAPTER III

RECAPITULATION OF NEOINTIMAL LESION-LIKE PHENOTYPE IN MACROPHAGES IN VITRO IS DEPENDENT ON SMC-DERIVED TGF-BETA²

Introduction

In Chapter II we showed that macrophages present in injured vessels express a distinct phenotype as compared to precursor monocytes. We hypothesized that this phenotype was the result of monocytes maturing into macrophages in the presence of SMC-derived factors in vivo. In this chapter we assess this hypothesis using an in vitro model of macrophage maturation from immature precursors in the presence or absence of SMC-derived factors.

As discussed above (see Chapter I: Macrophage Function and Phenotypic Modulation), macrophages are phenotypically plastic cells that exhibit altered phenotype and function in response to environmental cues. It is clear that while in vivo studies are critical for assaying the effect of disease on tissues and cells, and ultimately for validating strategies for intervening in disease pathogenesis in a pre-clinical setting, it is often essential to turn to in vitro model systems in order to identify mechanisms of pathogenesis. Several groups have assayed the effects of other cell types on macrophages in vitro in order to characterize the effects of cross talk on, and identify factors

responsible for, macrophage phenotypic modulation. For example, studies from our lab showed that in-direct Boyden chamber coculture of murine lung adenocarcinoma cells with bone marrow-derived macrophages was capable of inducing arginase I expression in macrophages, indicative of induction of an M2-like phenotype. Cai et. al. cultured macrophages in direct contact with human vascular SMCs and observed that both VCAM-1 and MCP-1-mediated upregulation of CD36, indicative of a pro-atherogenic phenotype, as well as enhanced resistance to death in response to serum deprivation.

Whether in direct or in direct coculture, the aforementioned methods assay the net result of bi-directional cross talk. Performing such studies with macrophages and SMCs is complicated by the fact that these two cell types are very plastic. Macrophages are capable of dramatic phenotypic modulation in response to paracrine or autocrine factors, and SMCs are capable of converting from a differentiated, contractile, phenotype to a dedifferentiated, non-contractile, motile, and synthetic phenotype. Because cell types will undergo phenotypic changes in coculture, these approaches may be of limited utility in assessing the effect of one cell on the other.

In contrast with coculture studies, conditioned medium studies allow for dissection of uni-directional aspects of intercellular cross talk. For example, a study originating from the Karin lab showed, using conditioned medium isolated from Lewis lung carcinoma cells, that a soluble form of the matrix proteoglycan, versican, was capable of inducing an activated (increased production of TNF-α, IL-6, and IL-1β) phenotype in in vitro matured bone marrow-derived macrophages. We decided to employ a similar strategy to assay uni-directional effects of SMC-derived factors on macrophages. In addition to focusing on the uni-directional effects of SMCs on Mφ, we
also wanted to study those effects in the context of monocyte-to-macrophage maturation. We hypothesized that we could more closely recapitulate the in vivo scenario of a recruited monocyte maturing to a lesional macrophage by stimulating immature bone marrow-derived monocyte/macrophages with SMC-derived factors prior to them attaining a mature macrophage fate; as opposed to stimulating fully mature macrophages with SMC-derived factors. Using this approach we have identified TGF-β as a factor produced by SMCs which produces an activated macrophage phenotype.

We performed our in vitro studies using rat SMCs because we have found that mouse-derived aortic SMC isolations in culture are highly heterogeneous. Rat-derived SMCs are a well-characterized system, and isolations of SMC from rat aortas are reliably homogenous. In order to perform cross talk studies using cells from one species, we studied the effects of rat SMC CM on rat BMC maturation to macrophages. Rat macrophage maturation from whole bone marrow has been characterized previously\textsuperscript{153}; in these studies, mouse L929 cell conditioned medium was used as a source of M-CSF to induce maturation of rat BMC to mature macrophages. We have found the concentration of M-CSF to be variable in L929 cell conditioned medium. We therefore purchased recombinant mouse M-CSF to mature BMCs to macrophages in a way that has been previously characterized and with an invariant concentration of M-CSF.

Materials and Methods

**Reagents:** Antibodies: CD68-FITC (Biolegend), SM-α-actin-Cy3 and unconjugated (Sigma), phospho-SMAD2 (Cell Signaling), β-actin (Sigma), SM-22α (Abcam), SM-MHC (Biomedical Technologies), Calponin and β-tubulin (Santa Cruz Biotechnologies),
BrdU (BD Biosciences), F4/80 (Clone A3-1, Abcam), anti-rabbit-AP and anti-mouse-HRP (Santa Cruz), anti-rat-Alexa 488 (Life Technologies), Universal Elite HRP Kit (Vector Labs), IRDye 800CW Donkey anti-mouse IgG and IRDye 680RD donkey anti-rabbit IgG (Li-Cor). Flow cytometry antibodies: biotinylated-F4/80 (Clone A3-1, AbD Serotec), streptavidin-PE, streptavidin-PerCP-Cy5.5, CD11b-FITC, Ly6g-PE-Cy7, SiglecF-PE, CD11c-APC-Cy7, and CCR3-Alexa Fluor 647 (all BD Biosciences), CCR7-PE (eBioscience). Recombinant proteins: human TGF-β1 (R&D Systems), mouse M-CSF (eBiosciences). Small molecule agonists/antagonists: SB203580, BAY 11-7082, LY-294002, sulindac sulfide, and PD-98059 (Calbiochem), SB431542 and pioglitazone (Cayman).

**Cell Culture and Isolation of Conditioned Medium:** Primary aortic SMCs were isolated from Sprague-Dawley rats as previously described. SMCs were used for experiments between passages 8 and 15. Rat SMCs were plated at 7*10^6 cells/150 mm dish in MEM medium supplemented with 10% fetal bovine serum (FBS) and 1x penicillin/streptomycin (20 IU/ml penicillin, 20ug/ml streptomycin). Twenty four hours after plating, cells were serum restricted in 0.1% serum medium (reduced serum medium; SRM) for 24 hours, then new SRM was refreshed and allowed to condition for a further 24 hours. This conditioned medium was cleared by centrifugation at 500xg for 10min, and stored at -80°C until use. Primary bone marrow-derived Mφ were isolated from Sprague-Dawley rats, or C57BL6/j mice, as previously described. Whole bone marrow was frozen as described, and thawed for each experiment without passaging in the presence of 20ng/ml M-CSF (eBioscience).
**Macrophage Maturation In Vitro:** Bone marrow cells (BMC) were thawed and plated at a density of $2 \times 10^6/35$mm dish in DMEM supplemented with FBS (10% for rat BMC, 20% for mouse BMC), 1x penicillin/streptomycin, and 20ng/ml M-CSF. Two days after plating, control medium (M0), 20% SMC conditioned medium (sMϕ), or 500pg/ml TGF-β (tMϕ) was added to BMCs (final concentration: FBS, 10%; M-CSF 20ng/ml). At day five of maturation, media was replaced with fresh media containing the same concentrations of SMC CM, TGF-β, FBS, and M-CSF as at day 2. Total RNA was isolated from 7-day matured macrophages for qPCR. To analyze cytokine protein levels, macrophage media was changed to SRM, collected after 48 hours, and concentrated using 3kDa cut-off Amicon Ultra centrifugal filters. To study interactions with SMCs in coculture, macrophages were trypsinized, re-plated in 6.5mm diameter transwells (0.4 μm; Corning) and added to SMC cultures.

**Immunofluorescent Staining:** Bone marrow derived macrophages were matured on glass chamber slides in the absence or presence of SMC CM, respectively, for 7 days. Cells isolated from vessels or PBMCs by positive selection by CD11b magnetic beads were plated onto glass chamber slides in 0.1% serum culture media for 48hrs. Cells were fixed in 4% PFA, permeabilized with MeOH, and incubated with anti-ED1-FITC, or anti-F4/80 and anti-smooth muscle α-actin-Cy3 primary antibodies overnight at 4°C. F4/80 antigen:antibody complexes were visualized using Alexa Fluor-488 conjugated secondary antibodies (Life Technologies). Coverslips were mounted with VectaShield medium (Vector Laboratories) containing DAPI to detect all cell nuclei and cells imaged using an
Olympus fluorescent microscope (TE200-S). Images were analyzed using Metamorph software\textsuperscript{149}.

**Real Time qPCR**: cDNA was prepared from total RNA isolates (1ug/rxn) using the iScript cDNA Synthesis kit from Bio-Rad. Real time qPCR then performed using the Power Sybr Green supermix from Applied Biosystems as shown previously\textsuperscript{147-149}. Reactions were carried out on an iCycler (Bio-Rad). Cycling parameters were: 5min at 95°C; 15sec at 95°C, 30sec at the annealing temperature optimized for each primer pair, and 30sec at 72°C for 50 cycles. GAPDH was used as a reference gene.

**qPCR Primers\textsuperscript{149:** See Table 2.

| Mouse and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Forward: cgt gga gtc tac tgg cgt ctt cac  
| Reverse: cgg aga tga tga ccc ttt tgg c |
| --- | --- |
| Mouse and Rat TNF-α | Forward: ctc ttc tgt cta ctg aac ttc ggg  
Reverse: gagagaagatgtactggagtgaggg |
| Rat CCL5 | Forward: gtc gtc ttt gtc act cga ag  
Reverse: agg atc aga atg gag aga cc |
| Rat IL-6\textsuperscript{1} | Forward: aac tcc atc tgc cct tca gga aca  
Reverse: aag gca gtc gct gtc aac aac atc |
| Rat IL-10 | Forward: gca gga ctt taa ggg tta ctt ggg  
Reverse: ctt gat ttc tgg gcc atg gtt ctc |
| Rat Il-12a | Forward: ggt gat gaa cta tct gag ctc ctc c  
Reverse: ttc cta cag gag ctc aag gtc aac |
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**Western Blotting:** Whole cell lysates were prepared using protein lysis buffer (50mM β-glycerophosphate [pH 7.2], 100μM sodium vanadate, 2mM magnesium chloride, 1mM EGTA, 0.5% Triton X-100, 1mM DTT, and protease inhibitor cocktail [1:100 Sigma, P8340]) and cleared by centrifugation. Samples were normalized by protein concentration and SDS-PAGE was performed as described previously\(^{155}\).

Electrophoresed proteins were transferred to polyvinylidene difluoride membranes, and blocked with 5% bovine serum albumin or non-fat dry milk in tris-buffered saline. Membranes were probed with primary antibodies, and subsequently with horseradish peroxidase or alkaline phosphatase-conjugated secondary antibodies. Antibody binding was visualized with either enhanced chemiluminescence substrate (Perkin Elmer) or Lumiphos (Thermo Scientific), respectively\(^{148}\).

**Enzyme-Linked Immunosorbent Assay:** ELISA for TGF-β was performed on SMC conditioned media, as per the manufacturer’s instructions (R&D Systems, DY240). Total
TGF-β was determined by acidification of samples as recommended in the manufacturer’s instructions.

**Multiplex Luminex Assay:** 48 hour macrophage SRM conditioned media samples, prepared as described above, were concentrated ~20x with 3kDa Amicon Ultra spin filters (Millipore) and then assayed for IL-6, CCL5, TNF-α, IL-10, and IL-12p70 using Milliplex Map beads (Millipore). Samples were quantified using a MagPix instrument (Luminex). Analyte concentrations obtained from Luminex assay were normalized to total protein or RNA per sample.

**Gelatin Zymography:** Whole cell lysate, or 10x concentrated low serum conditioned medium, was electrophoresed under non-reducing, non-denaturing conditions on 10% gelatin zymography gels (Novex/Invitrogen). Renaturation, development, and staining with Simply Blue Safe Stain (Invitrogen) were performed as per the manufacturer’s specifications.

**SMC Proliferation Assay:** SMC proliferation was assessed as previously described\(^\text{[137,147]}\). Briefly, SMCs were plated in 24-well plates at a density of 3*10^4 cells/well, allowed to attach and spread overnight, and media replaced with 500μl/well SRM. Seven-day matured macrophages were trypsinized, resuspended in SRM at 4*10^5 cells/ml and re-plated on 0.4μm transwell inserts (Corning) in wells containing SMCs. 20μM BrdU was added to cocultures after 24hours. At 48 hours SMC were fixed with 4%
paraformaldehyde, and immunohistochemically stained for BrdU incorporation. BrdU index was calculated as the percent of BrdU-positive nuclei.

**Femoral Artery Injury**: All animal procedures were approved by, and performed in accordance with the guidelines of the University of Colorado IACUC. Guide-wire denudation femoral artery injury was performed, as shown by us previously\textsuperscript{137}, and as described in the Materials and Methods section of Chapter II.

**Flow Cytometry**: Isolated injured, and contralateral control vessels, were digested to single cells by digestion at 37°C for 2hr in collagenase buffer (3.2mg/ml collagenase II, 0.7mg/ml elastase (Worthington), 0.2mg/ml soybean trypsin inhibitor (Sigma) in Hank’s buffered saline solution (HBSS), pH7.5). Red blood cells were lysed for 2-5 min in RBC lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA (pH 7.2)). Single cell suspensions were stained for CD11b, F4/80, Ly6g, CCR3, CCR7, and DAPI to stain for dead cells. Flow cytometry was performed on a Galios cytometer (Becton Dickenson) as demonstrated by our group previously\textsuperscript{145}, and as described in the Materials and Methods section of Chapter II.

**Statistics**: Data are expressed as means ± standard error. Student’s T-tests were used to compare data sets with two samples. Data sets with more than two samples were analyzed by ANOVA followed by Tukey multiple comparisons post-tests. Bonferroni multiple comparisons tests were used to compare select samples within a data set. Finally, Dunnett multiple comparisons tests were used to compare all samples to a single
control. In instances, within a data set with more than two samples, when variances were different between groups (as determined by T-Test) serial T-tests were performed followed by Bonferroni correction to account for multiple comparisons.

Results

Phenotypic Modulation of Macrophages by Conditioned Media from SMCs. We hypothesized that the signals driving macrophage phenotypic modulation are derived, at least in part, from medial SMCs. We therefore developed an in vitro system to determine if maturation of bone marrow-derived monocytes in the presence of factors secreted by SMCs results in phenotypic modulation resembling lesion-associated macrophages. Rat bone marrow cells (BMCs) were matured, as previously described\(^{153}\), with recombinant mouse M-CSF in the presence (sMφ) or absence (M0) of conditioned media (CM) obtained from rat aortic SMCs. Aortic vascular smooth muscle cells isolated from the rat, and cultured ex vivo, have a proliferative index of about 24% when serum-restricted in 0.1% serum medium for 48 hours as they were for our conditioned medium collection procedure. This proliferative index is in stark contrast to the SMC proliferation rate in vivo in an uninjured vessel, which has been documented by several groups as less than 0.1% per day\(^{156,157}\). We previously showed that even out to 9 days in 0.1% serum media the proliferative index of cultured aortic SMC remains elevated above a truly quiescent in vivo level\(^{158}\). Therefore, while serum-restricted to rule out confounding effects from serum-derived factors, SMCs in culture maintain a somewhat activated phenotype and model an activated SMC phenotype. Bone marrow cells were exposed to CM from SMCs at day 2 of culture and harvested at day 7 for analysis. As shown in Figure 6A,
Figure 6: Maturation of macrophages in the presence of SMC conditioned media (CM) promotes an activated phenotype similar to macrophages from in vivo vascular lesions.

A) Rat bone marrow cells (BMC) were matured with 20ng/ml macrophage-colony stimulating factor (M-CSF) in the absence (M0) or presence of 20% SMC CM (sMϕ). Representative phase-contrast micrographs of M0 and sMϕ. B) Rat bone marrow-derived cells (BMC) were matured with 20ng/ml M-CSF in the absence (M0) or presence of 20% SMC CM (Mϕ) on glass chamber slides. Cells were assessed for CD68 expression by immunofluorescence. SMC were assessed for CD68 as a negative control. C&D) Total RNA was isolated and qPCR conducted for the indicated mRNAs. Shown are mRNA copy number normalized to GAPDH. Means±SE; n=6 independent experiments. E) Conditioned media was collected from M0 and sMϕ at day 9, 48 hours after replacing media with SRM, concentrated, and assayed for IL-10, CCL5, and TNF-α protein levels by Luminex assay. F) MMP9 expression was assessed in whole cell lysates from M0 and sMϕ by gelatin zymography. Shown are means±SE; n=7 independent experiments. *P<0.05 versus M0.
exposure of BMCs to SMC-derived factors early in maturation resulted in a pronounced change in morphology. These cells were more rounded with shorter processes whereas M0 cells were more elongated with 2 or more extended processes. The expression of the macrophage marker CD68 was assessed in these cells by immunofluorescence. SMϕ express CD68 (rat ED1) at similar levels as M0 macrophages, indicating these cells retained a macrophage fate (Figure 6B). QPCR analysis of the previously described in vivo 9-gene panel in sMϕ, relative to M0 controls, closely recapitulated the signature found in macrophages isolated from injured vessels as compared to PBMC precursor monocytes (Figure 6C). Up- and down-regulation of CCL5, IL-6, IL-10, IL-12b, CCR3, CCR7, and MMP9 were concordant between this in vitro system and in vivo injury model. Luminex multiplex bead assays were used to verify modulation of cytokines at the protein level. Transcriptional regulation of IL-10, CCL5, and TNF-α correlated with protein production (Figure 6E) whereas IL-6 and IL-12p70 were undetectable by this method (not shown). Gelatin zymography was used to assess catalytically active MMP9 expression in these lysates. Expression of MMP9 was decreased in sMϕ relative to control M0 macrophages (Figure 6F). Expression of the M1/M2 markers iNOS and Arg I was assessed by qPCR in these cells. Similar to macrophages recruited to injured vessels, sMϕ expressed high levels of iNOS, but unlike lesional macrophages, low levels of Arg I relative to M0 (Figure 6D). We were unable to detect iNOS or Arg I protein by western blotting in these experiments (data not shown). These data suggest that maturation of bone marrow-derived monocytes in the presence of factor(s) derived from SMC recapitulates many of the features of neointima-associated macrophages observed in vascular lesions in vivo. This in vitro model system establishes a novel framework for
Figure 7: Physical characterization of SMC-derived factor(s) promoting macrophage phenotypic modulation.

A, left) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0), presence of 20% SMC CM (CM), or presence of heat-treated SMC CM (Boiled). Representative phase-contrast micrographs. A, right) qPCR analysis of indicated mRNAs. Shown are means±SE; n=4 independent experiments. B) Conditioned media was collected at day 7, concentrated, and assayed for IL-10, CCL5, and TNF-α protein levels by Luminex assay. Shown are means±SE; n=3 independent experiments. C, left) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0), presence of heat-treated SMC CM (Boiled), or presence of protease and heat-treated SMC CM (Boiled, Trypsinized). Representative phase-contrast micrographs. C, right) qPCR analysis of indicated mRNAs. Shown are means±SE; n=3 independent experiments. D, left) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0), presence of native or heat-treated SMC CM (CM and Boiled), or presence of native or heat-treated SMC CM fractionated by size (<100kDa, >100kDa, respectively). Representative phase-contrast micrographs. D, right) qPCR analysis of indicated mRNAs. Shown are means±SE; n=3 independent experiments. *P<0.05 versus M0; **P<0.05 versus CM.
Figure 8: Exogenous TGF-β recapitulates effects of SMC CM on macrophage activation.

A) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 500pg/ml rh-TGF-β1 (tMϕ). Representative phase-contrast micrographs of M0 and tMϕ. B) Total RNA was isolated and qPCR conducted for the indicated mRNAs. Shown are mRNA copy number normalized to GAPDH. Means±SE; n=6 independent experiments. C) Conditioned media was collected from M0 and tMϕ at day 9, 48 hours after replacing media with SRM, concentrated, and assayed for IL-10, CCL5, and TNF-α protein levels by Luminex assay. Shown are means±SE; n=3 independent experiments. D) Whole cell lysate (WCL) and concentrated conditioned medium (CM) were assayed for MMP9 expression by gelatine zymography. E) Total RNA was isolated and qPCR conducted for iNOS and arginase I (Arg I). Shown are mRNA copy number normalized to GAPDH. Means±SE; n=6 independent experiments. *P<0.05 versus M0.
studying macrophage maturation and function that is specifically relevant to injury-induced neointima formation. Further, this system establishes a novel in vitro platform for understanding intercellular cross talk between the SMCs and recruited macrophages.

**Characterization of the Factor(s) Produced by SMCs that Mediates sMϕ Induction.** To identify potential factor(s) secreted by SMCs responsible for macrophage phenotypic modulation, we employed several techniques to test physical properties of the factor(s). SMC CM was heat-treated in a boiling bath for 10min to denature heat-labile factors. The macrophage-modulating activity of SMC CM was enhanced by heat-treatment (Figure 7A). Exposure of macrophages to heat-treated CM resulted in enhanced morphologic alteration of macrophages, enhanced up- or down-regulation of genes, and increased levels of IL-10, CCL5, and TNF-\(\alpha\) protein (Figure 7B) compared to CM that had not been heated. Trypsinization of CM completely abolished the macrophage-modulating activity of the heat-treated SMC CM (Figure 7C), confirming that the factor(s) is a polypeptide. Size fractionation was performed with 100kDa cut-off centrifugal filters and revealed that the macrophage-activating factor partitions to the >100kDa fraction in the native CM (Figure 7D). To determine if heat-dependent activation of the factor(s) is due to dissociation of an inhibitor, or inhibitory complex, size exclusion studies were performed following heat-treatment. These studies showed that the macrophage-modulating activity partitions to the <100kDa fraction following heat-treatment (Figure 7D). Together these data suggest that a peptide-based factor is secreted from SMCs in a large (>100kDa), less active complex and activity is enhanced
by heat-treatment through a mechanism resulting in decreased molecular weight of the active component.

*Exposure of Bone Marrow-Derived Cells to TGF-β Leads to Induction of the sMϕ Phenotype.* Latent, complex-bound TGF-β is secreted into the extracellular space in the vasculature, and undergoes activation upon mechanical injury\(^{125}\). Latent TGF-β is also known to be activated *in vitro* by heat and acidic conditions\(^{113}\). Activation is associated with a change in molecular weight, similar to what we have observed with CM. Furthermore, blocking TGF-β signaling in *in vivo* vascular injury models has been shown to inhibit neointima formation\(^{159-165}\). Since the macrophage modulating activity in SMC conditioned medium had characteristics consistent with TGF-β, we next tested the effect of recombinant human (rh) TGF-β on macrophage phenotype. Maturation of macrophages in the presence of rh-TGF-β1 recapitulated the morphologic modulation observed with SMC CM (*Figure 8A*) and recapitulated the expression signature of the previously described 9-gene qPCR panel observed in sMϕ (*Figure 8B*). Increased IL-10 and CCL5 and decreased TNF-α protein levels were verified by Luminex assay (*Figure 8C*). MMP9 expression was validated using gelatin zymography on either whole cell lysate (WCL) or macrophage conditioned medium (CM) (*Figure 8D*). Similar to sMϕ, iNOS and Arg I expression was increased and decreased, respectively, in macrophages matured with rh-TGF-β (tMϕ) (*Figure 8E*). *Figure 9* shows a side-by-side comparison of gene expression changes in the 9-gene qPCR panel in macrophages matured with conditioned medium versus rh-TGF-β1. Gene expression across this panel of markers was modulated very similarly between these two conditions.
SMC-derived TGF-β Promotes Macrophage Modulation Observed in In Vitro

**Maturation Studies.** We sought to determine if the factor in SMC CM responsible for modulating macrophage phenotype was TGF-β. Latent TGF-β1 was detectable in SMC CM at an average concentration of 350pg/ml (Figure 10A). Bone marrow cells stimulated with SMC CM showed increased phosphorylation of SMAD2, which was detectable by 30 minutes, and remained elevated for at least 90 minutes (Figure 10B). The level of stimulation was comparable to that observed with rh-TGF-β1, indicating that canonical TGF-β signaling occurs in macrophages in response to SMC CM, although the kinetics were somewhat delayed. Delayed phosphorylation of SMAD2 suggested that macrophages activate SMC-derived latent TGF-β1. As shown in Figure 10B, activation
of SMAD2 in response to SMC CM was completely inhibited by a specific TGF-β receptor 1 (TBR1) inhibitor, SB431542 (10μM).

To confirm that TGF-β is a critical factor in SMC CM responsible for inducing macrophage phenotypic modulation, we employed several complementary strategies. Pharmacological inhibition of TGF-β signaling with SB431542 resulted in blockade of the morphologic change induced by SMC CM (Figure 11A). This inhibitor also abolished SMC CM-induced increases in gene expression of IL-6, CCL5, IL-10, IL-12a,
Figure 11: TGF-β receptor inhibition prevents macrophage activation by SMC CM.
Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 20% SMC CM (sMϕ) and in the presence (+) or absence (-) of TRB1 inhibitor SB431542. A) Representative phase-contrast micrographs. B-D) Total RNA was isolated and analyzed by qPCR for the indicated mRNAs. E) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 20% SMC CM (sMϕ), and with or without the TRB1 inhibitor (SB431542). Conditioned media was collected, concentrated, and assayed for IL-10, CCL5, and TNF-α protein levels by Luminex assay. Shown are means±SE; n=3 independent experiments. B-D) Shown are mRNA copy number normalized to GAPDH. Means±SE; n=3 independent experiments. *P<0.05 versus M0, **P<0.05 versus sMϕ without TBRI inhibitor.
IL-12b, CCR3, and CCR7, as well as reversed the decreases in TNF-α and MMP9 expression (Figure 11B-C). Finally the receptor inhibitor reversed the expression of macrophage phenotypic markers, decreasing expression of iNOS and increasing expression of Arg I (Figure 11D). Inhibition of cytokine up/down regulation by TBR1 inhibition was assessed by Luminex assay (Figure 11E). To confirm these findings, a pan-specific anti-TGF-β neutralizing antibody was used to inhibit SMC CM-mediated TGF-β signaling in macrophages during maturation. Compared to vehicle control, neutralization of TGF-β resulted in blockade of the morphologic change (Figure 12A) and abolished altered gene expression of IL-6, CCL5, IL-10, IL-12a, IL-12b, CCR3, CCR7, TNF-α, MMP9, iNOS, and Arg I (Figure 12B-D) induced by SMC CM. These effects were specific for anti-TGF-β and were not observed with antibodies against PDGF-BB or SDF-1α; the selectivity of this effect due to TGF-β neutralizing antibody was determined by assaying the samples for CCL5 and MMP9 expression only by qPCR (Figure 12E-F).

TGF-β neutralizing antibody and TBR1 inhibition studies cannot rule out that an unknown factor produced by SMC triggers vesicle fusion, and TGF-β release from macrophages (as has been described elsewhere\textsuperscript{166}), which then mediates these changes in an autocrine manner. Therefore expression of TGF-β was silenced in SMCs using transient transfection of siRNA and conditioned medium collected from control or TGF-β-silenced SMCs. TGF-β was decreased by greater than 80% in cells receiving the TGF-β-specific siRNA compared to non-targeting control siRNA-treated SMCs (Figure 13A). Compared to maturation of macrophages in the presence of SMC CM from non-targeting siRNA transfected SMC, maturation of macrophages with TGF-β-silenced SMC CM
Figure 12: TGF-β neutralization prevents macrophage activation by SMC CM.
Rat BMC were matured with 20ng/ml M-CSF in the absence (-) (M0) or presence (+) of 20% SMC CM (sMϕ), and pan-TGF-β neutralizing antibody. 

A) Representative phase-contrast micrographs. 

B-D) Total RNA was isolated and analyzed by qPCR for the indicated mRNAs. 

E&F) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 20% SMC CM (sMϕ) and vehicle (-), pan-TGF-β neutralizing antibody (anti-TGF-β), PDGF-B neutralizing antibody (anti-PDGF-B), or SDF-1α neutralizing antibody (anti-SDF-1α). Total RNA was isolated and analyzed by qPCR CCL5 and MMP9. 

C-F) Shown are mRNA copy number normalized to GAPDH. Means±SE; n=3 independent experiments. *P<0.05 versus M0; **P<0.05 versus sMϕ without neutralizing antibody.
showed a decrease in sMϕ morphologic modulation and a ~50% decrease in phenotypic modulation of CCL5 and MMP9 (Figure 13B-C). Taken together, these data suggest a critical role for SMC-derived TGF-β in the maturation of a phenotypically distinct macrophage population, which exhibits a hybrid M1/M2 activation state and may be specifically primed to contribute to SMC activation.

Figure 13: Conditioned media from TGF-β-silenced SMC CM fails to promote macrophage phenotypic modulation.
A) SMC were depleted for TGF-β1 by transient transfection with siRNAs. Conditioned media from untransfected, non-targeting siRNA-transfected (si-NT), and siRNA-mediated TGF-β1-silenced (si-TGF-β1) SMC were analyzed for TGF-β1 expression by ELISA. Shown are mean ±SE; n=3 independent experiments. B) Rat BMC were matured for 7 days with 20ng/ml M-CSF in the absence (M0), presence of non-targeting siRNA-transfected SMC CM (si-NT), or presence of TGF-β1 siRNA-transfected SMC CM (si-TGF-β1). Representative phase-contrast micrographs, and C) analysis of indicated mRNAs by qPCR. Shown are mRNA copy number normalized to GAPDH. Shown are mean ±SE; n=3 independent experiments. *P<0.05 versus M0; **P<0.05 versus si-NT.
Mouse and Rat In Vitro Matured Macrophages Exhibit Differential Responses to TGF-β. Since our initial in vivo studies were performed in a mouse model of arterial injury, we wanted to determine how mouse BMC-derived macrophages would behave when matured in the presence, versus absence, of purified TGF-β. We therefore matured cryopreserved whole mouse bone marrow using the same experimental conditions as was used with rat bone marrow, as shown in Figure 8. We found that mouse macrophages exhibited a similar morphologic modulation in response to 500 pg/ml rh-TGF-β1 as did rat macrophages at day 7 of maturation (Figure 14A). Interestingly, mouse BMCs only began to look like the rat macrophages, morphologically, after 6 to 7 days in culture. Prior to day six, the mouse BMC-derived macrophages exhibited a much more spindle-like morphology, which was similar to PBMC-derived mouse macrophages at that time point (data not shown). Assessment of qPCR phenotypic markers showed that several genes were regulated concordantly between mouse and rat tMϕs (Figure 14B: IL-6, TNF-α, iNOS, Arg I, and additionally CD206/mannose receptor). However, several genes were regulated discordantly or were not modulated by maturation in the presence of TGF-β (Figure 14B: CCL5, IL-10, IL-12α, CCR3, CCR7, and MMP9). These data suggest that mouse and rat bone marrow-derived macrophages may not represent equivalent cell populations when matured in vitro. To try to determine if transcriptional repression of CCR3 and CCR7 was detectable at the protein level we performed flow cytometry analysis of in vitro matured mouse M0 and tMϕ macrophages and compared CCR3 and 7 expression in these cells with expression in macrophages present in injured and uninjured vessels. We found that CCR3 expression in both in vivo macrophage populations was low and essentially unchanged in response to injury (Figure 14C top),
Figure 14: Comparison of gene expression changes rat versus mouse macrophages matured in the presence of rh-TGF-β1.

A) Rat and mouse bone marrow cells (BMC) were matured with 20ng/ml macrophage-colony stimulating factor (M-CSF) in the absence (M0) or presence of 500pg/ml rh-TGF-β1 (tMϕ). Representative phase-contrast micrographs of M0 and sMϕ from both species. B) Total RNA was isolated and qPCR conducted for the indicated mRNAs. Shown are fold change in mRNA copy number, normalized to GAPDH, relative to M0. Means±SE; n=4 independent experiments *P<0.05 versus M0. Rat data originally presented in Figure 6, including statistical analysis. C) Mouse bone marrow cells (BMC) were matured with 20ng/ml macrophage-colony stimulating factor (M-CSF) in the absence (M0) or presence of 500pg/ml rh-TGF-β1 (tMϕ). Single cell suspensions were isolated from femoral arteries following 2-wks wire-induced injury (iRFA), and from pool uninjured vessels (aorta, carotid, and left femoral artery). Cells were stained for CD11b, F4/80, Ly6g, CCR3, CCR7, and DAPI, and analyzed by flow cytometry.
whereas ~30% M0 mouse macrophages expressed high levels of CCR3 and 70% expressed CCR3 levels similar to in vivo macrophages. We did not perform this analysis with in vivo monocytes from peripheral blood. These data suggest that M0 in vitro mouse macrophages are a heterogeneous population and may not fully represent a homogeneous control macrophage population. With respect to CCR7 expression, we found that in both in vivo and in vitro cells, CCR7 was expressed at a low level, and injury or maturation with TGF-β was sufficient to reduce the percentage of CCR7 positive cells (Figure 14C bottom). This suggests that while we find discordant transcriptional regulation of some of our qPCR panel between the in vitro TGF-β-matured mouse macrophages and the in vivo lesional macrophages (when compared to either PBMC or uninjured vessel macrophage controls) the protein level expression - and percentage of cells expressing - some of these markers may be modulated in the same way by injury or maturation in the presence of TGF-β. We are not able to perform this kind of protein-level assessment of cell surface markers (or heterogeneity analysis in control M0 macrophages) in the in vitro matured rat cells due to unavailability of reagents specific to rat cell surface proteins.

**Phenotypic Modulation of Macrophages by SMC-Derived TGF-β is p38 MAPK-Dependent.** To define mechanisms mediating the effects of SMC-derived factors on macrophage phenotype, we assessed the contribution of downstream signaling pathways using a pharmacologic strategy. We assayed a panel of small molecule inhibitors of signaling pathways likely to be involved in altered maturation. Macrophages were exposed to BAY 11-7082, an inhibitor of NF-κB signaling; LY-294002, a PI-3K inhibitor; SB203580, a p38 MAP kinase inhibitor; PD-98059, a MEK-1/2 (MAP kinase
Figure 15: Macrophage phenotypic modulation by SMC-derived TGF-β is p38 MAPK-dependent.
Figure 15: Macrophage phenotypic modulation by SMC-derived TGF-β is p38 MAPK-dependent.

Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 20% SMC CM (sMϕ). M0 and sMϕ were treated ± DMSO (Veh) or the following inhibitors: 0.1µM BAY-7082 (BAY), 1µM LY-294002 (LY), 10µM SB203580 (SB2), 25µM PD-98059 (PD), or 25µM Sulindac Sulfide (SS) at the time of initial SMC CM addition. A) Representative phase-contrast micrographs of M0 vs sMϕ ± vehicle or drug at day 7. B &C) Total RNA was isolated and analyzed by qPCR for the mRNAs indicated. Shown are mRNA copy number normalized to GAPDH. D) MMP9 expression, in whole cell lysates, was assessed by western blot and E) by gelatin zymography. Shown are mean ±SE; n=4 independent experiments. *P<0.05 versus M0 vehicle control; **P<0.05 versus sMϕ, vehicle treated.
kinase) inhibitor; and Sulindac Sulfide, a cyclooxygenase inhibitor throughout the course of maturation with SMC CM. Drugs, or vehicle were added to Mϕ cultures immediately prior to addition of SMC conditioned medium. Inhibition of p38 activity with SB203580 was the only condition exerting an inhibitory effect on macrophage morphologic modulation by SMC CM (Figure 15A). In addition, p38 inhibition abrogated transcriptional changes induced in the previously described panel of genes (IL-6, CCL5, CCR3, CCR7, IL-10, IL-12a, IL-12b, TNF-α, MMP9, iNOS; no significant differences in Arg I) (Figure 15B-C). Rescue of repression of MMP9 by maturation in the presence of SMC CM was seen with SB203580 (Figure 15D-E). Exposure to SB203580 also blocked the morphologic and transcriptional changes induced by recombinant TGF-β on CCL5 and MMP9 (Figure 16A-B) and inhibited up- and down-regulation of iNOS and Arg I, respectively, mediated by TGF-β (Figure 16C). These data suggest that the ability of SMC-derived factors to induce phenotypic modulation of macrophages is dependent on p38 MAPK signaling. Interestingly, we did not observe altered levels of phosphorylation of p38 in response to either recombinant TGF-β or SMC conditioned medium in macrophages (Figure 17A). This suggested to us that basal p38 kinase

**Macrophages Matured in the Presence of SMC-Derived TGF-β Exhibit Cross Talk with SMCs.** To begin to define how the phenotypic modulation of macrophages by SMC-derived TGF-β influences macrophage cross talk with SMCs, we performed *in vitro* coculture studies. Following seven days of maturation, M0 cells or sMϕ were placed in Boyden chamber inserts and cocultured with 48hr serum-restricted SMCs for 48 hours. We observed an increase in cell proliferation, as assessed by SMC DNA synthesis
by BrdU incorporation assay, at 48 hours as compared to SMC alone or in coculture with M0 macrophages (Figure 18A). We examined expression of several cytokines previously shown to be upregulated in activated SMC\textsuperscript{137}. Exposure of SMC to either M0 or sMϕ macrophages induced expression of MCP-1, KC, and SDF-1α in SMC after 48 hours in coculture. However, there was no significant difference in the degree of induction between M0 and sMϕ coculture conditions (Figure 18B). We also assessed expression of the SM-contractile proteins smooth muscle myosin heavy chain (SM-MHC), smooth muscle α-actin (SMA), calponin, and SM-22α (SM22) by western blotting in SMCs after 48 hours in coculture. We did not find significant differences in SM-contractile protein expression in these SMCs in response to coculture (Figure 18C). Densitometry of blots from several independent experiments is shown in Figure 18D.

Figure 16: Macrophage activation by TGF-β is p38 MAPK-dependent. Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 500pg/ml TGF-β and with or without SB203580 (SB2). A) Representative phase-contrast micrographs of M0 vs tMϕ (TGF-β1) ± SB203580. B & C) Total RNA was isolated and analyzed by qPCR for the indicated mRNAs. Shown are mRNA copy number normalized to GAPDH. Means±SE; B: n=4; C: n=3 independent experiments. *P<0.05 versus M0; **P<0.05 versus sMϕ, vehicle treated.
Figure 17: TGF-β does not induce p38 phosphorylation above baseline, and inhibition of p38 does not prevent Smad2/3 nuclear translocation.
A) Time course analysis of Mφ stimulated with SMC CM in the presence or absence of the TBRI inhibitor SB431542, or with rh-TGF-1 was performed, by western blot, for phospho-p38 (p-p38), and total p38. β-Actin was used as a loading control. B) Mφ were stimulated for 30min with 20% SMC conditioned medium (CM) following 1hr pre-incubation in the absence of drug, or in the presence of either 10µM p38 inhibitor SB203580 (SB2) or 10µM TBRI inhibitor SB431542 (SB4). Representative images shown.
Figure 18: Coculture with sMφ promotes SMC proliferation.
A) Rat BMC were matured with 20ng/ml M-CSF in the absence or presence of 20% SMC CM. Macrophages were then re-plated in transwell inserts and cocultured with serum restricted SMC for 48 hours. A) Cocultures received BrdU for the final 24 hours of coculture. BrdU positive cells were quantified by counting BrdU immunopositive vs negative cells (BrdU Index). B) Rat BMC were matured and re-plated with serum restricted SMC for 48 hours. Total RNA was isolated from SMC and analyzed by qPCR for the indicated mRNAs. Shown are mRNA fold change in copy number, relative to SMC, normalized to GAPDH. C) Smooth muscle contractile proteins were also assessed after 48 hours in coculture, in SMC whole cell lysates. SM-αActin (SMA), SM-22α (SM22). D) Densitometry analysis of smooth muscle contractile proteins assessed by western blot and in C. “CC’d or +” = cocultured. Shown are means±SE; A&B :n=6, C&D: n=4 independent experiments. *P<0.05 vs untreated SMC.
**Efforts to Identify sMϕ-Derived Factors Responsible for Inducing SMC Proliferation.**

We were interested in identifying what factors secreted preferentially by sMϕ versus M0 macrophages might be responsible for increased SMC proliferation observed in coculture experiments. We therefore isolated conditioned medium from M0 macrophages and sMϕ as described for Luminex assay. We stimulated serum-restricted SMC with control, M0, or sMϕ conditioned medium for 10-90 minutes to assess what down-stream signaling pathways were engaged in SMC in response to these media. We assessed phosphorylation of signal transducer and activator of transcription 3 (STAT3), AKT, and ERK. Exposure of SMCs to CM from either M0 or sMϕ resulted in activation of both ERK and AKT signaling, as determined by expression of phospho-ERK and phospho-AKT, however, we did not find any consistent differences in either the magnitude or the kinetics of activation (**Figure 19A**). STAT3 activation was consistently slightly lower in response to sMϕ medium versus M0 medium (**Figure 19A**), suggesting that IL-6 is unlikely to be the sMϕ-derived factor responsible for inducing SMC proliferation. Our lab previously showed that PDGF induces AKT and ERK activation in SMCs\(^{168}\) in addition to inducing proliferation\(^ {169}\). Because we observed increased proliferation in SMCs in response to coculture with sMϕ, we assessed if sMϕs were secreting more PDGF than M0 macrophages. We assessed PDGF transcription in M0 versus sMϕ by qPCR (**Figure 19B**) and at the protein level by ELISA performed on macrophage conditioned medium collected, and concentrated, as for Luminex assay (**Figure 19C**). We found that PDGF mRNA and protein were repressed in sMϕ as compared to M0 macrophages. These data suggest that IL-6 and PDGF are not factors produced by sMϕ that are responsible for driving increased SMC proliferation in coculture in response to
Figure 19: Mϕ conditioned medium analysis and effects on SMC signaling pathway activation.

A) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 20% SMC CM (sMϕ). Conditioned media was collected from M0 and sMϕ at day 9, 48 hours after replacing media with SRM. SMCs were stimulated from SMCs were analyzed for phospho-STAT3 (pSTAT3), phospho-AKT (pAKT), and phospho-ERK (pERK), by western blot. β-Actin was used as a loading control. B) Rat BMC were matured with 20ng/ml M-CSF in the absence (M0) or presence of 20% SMC CM (CM), boiled SMC CM (Boiled), or rh-TGF-β1 (rTGF-β). Total RNA was isolated and analyzed by qPCR for PDGF-b mRNA. Shown are mRNA copy number normalized to GAPDH. C) Conditioned media was collected from M0 and sMϕ at day 9, 48 hours after replacing media with SRM, concentrated, and assayed for PDGF-b protein by ELISA. Shown are mean ±SE; n=3 independent experiments. *P<0.05 versus M0 vehicle control.
sMϕ. The factors mediating the effects of sMϕ on SMC proliferation remain to be determined.

**Discussion**

**Conclusions:** We hypothesized that factors produced by SMCs were critical in promoting the injury-associated phenotype observed in macrophages isolated from vascular lesions *in vivo* (Chapter II). Using an *in vitro* system we were able to demonstrate that changes in many genes in the signature seen in macrophages from injured vessels were recapitulated in bone marrow-derived macrophages exposed to factors from SMC (sMϕ), compared to macrophages not exposed to these factors (M0). Using a number of strategies, we identified TGF-β1 as the critical SMC-derived factor responsible for inducing this macrophage phenotype in *in vitro* macrophage maturation studies. Table 3 summarizes the changes in gene expression observed *in vivo* (macrophages from injured vessels compared to PBMCs) compared to *in vitro* studies, and in the setting of inhibition of TGF-β signaling *in vitro*.

TGF-β has been implicated as a critical mediator of the neointimal hyperplastic response following vascular denudation injury (reviewed in Suwanabol et. al, 2011). Mechanical injury results in the upregulation of TGF-β expression by medial SMC and the activation of latent matrix-bound TGF-β. The restenotic response following vascular injury is enhanced with TGF-β infusion or over-expression while the response is suppressed when TGF-β is blocked or neutralized. Additionally, downstream TGF-β signaling (Smad2/3) has been shown to be enhanced in injured vessels. However, TGF-β is known to have an anti-proliferative and pro-differentiating effect on SMC
Table 3: Comparison of gene expression changes across selected experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>In Vivo Rel. to PBMC (n=4)</th>
<th>Rat sMφ (SMC CM) Rel. to M0 (n=6)</th>
<th>Rat tMφ (rTGF-β) Rel. to M0 (n=6)</th>
<th>Rat sMφ + TBR1 Rel. to M0 + TBR1 (n=3)</th>
<th>Rat sMφ; sTGF-β CM Rel. to M0 (n=3; CCL5 and MMP9 only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL5</td>
<td>1.10±0.27</td>
<td>3.27±0.29</td>
<td>3.93±0.45</td>
<td>1.24±0.17</td>
<td>2.63±0.11</td>
</tr>
<tr>
<td>IL-6</td>
<td>42.6±6.00</td>
<td>3.85±0.39</td>
<td>5.02±1.3</td>
<td>2.08±0.82</td>
<td>NA</td>
</tr>
<tr>
<td>IL-10</td>
<td>71.9±15.1</td>
<td>2.74±0.32</td>
<td>2.76±0.38</td>
<td>1.15±0.15</td>
<td>NA</td>
</tr>
<tr>
<td>IL-12a</td>
<td>0.21±0.11</td>
<td>3.45±0.42</td>
<td>3.44±0.79</td>
<td>1.36±0.31</td>
<td>NA</td>
</tr>
<tr>
<td>IL-12b</td>
<td>90.8±23.1</td>
<td>2.55±0.21</td>
<td>2.70±0.46</td>
<td>1.26±0.20</td>
<td>NA</td>
</tr>
<tr>
<td>CCR3</td>
<td>1.38±0.31</td>
<td>3.02±0.63</td>
<td>2.70±0.59</td>
<td>1.40±0.15</td>
<td>NA</td>
</tr>
<tr>
<td>CCR7</td>
<td>12.5±4.58</td>
<td>2.14±0.51</td>
<td>1.79±0.15</td>
<td>1.82±0.02</td>
<td>NA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>12.6±2.46</td>
<td>0.39±0.07</td>
<td>0.58±0.10</td>
<td>1.06±0.09</td>
<td>NA</td>
</tr>
<tr>
<td>MMP9</td>
<td>0.48±0.10</td>
<td>0.12±0.02</td>
<td>0.14±0.05</td>
<td>0.86±0.09</td>
<td>0.27±0.06</td>
</tr>
<tr>
<td>iNOS</td>
<td>17700±6340</td>
<td>3.96±1.05</td>
<td>3.70±0.89</td>
<td>1.39±0.11</td>
<td>NA</td>
</tr>
<tr>
<td>Arg1</td>
<td>5.01±1.46</td>
<td>0.20±0.60</td>
<td>0.81±0.27</td>
<td>1.18±0.25</td>
<td>NA</td>
</tr>
</tbody>
</table>

Gene expression data from in vivo injury study (column 2; Fig. 1) compared with gene expression data from select in vitro macrophage maturation experiments. Column 3: Fig. 2, column 4: Fig. 3, column 5: Fig. 4, column 6: Supp. Fig. V. Data expressed as mean (Copy #/GAPDH relative (Rel.) to indicated condition) ± SEM. NA = not assessed.

(regardless of SMC developmental origin), by cooperating with SRF-myocardin complexes to induce smooth muscle contractile protein expression\textsuperscript{115, 170}. These data argue that the mechanism by which TGF-β promotes neointimal hyperplasia is not exclusively through a direct effect on medial SMCs, but rather through another cell population present in the developing neointima that then signals to SMCs in a pro-proliferative manner. Our data suggest that this involves phenotypic modulation of recruited monocyte/macrophages by SMC-derived TGF-β as well as bi-directional cross talk between these modulated macrophages and resident medial SMCs, indirectly
promoting SMC proliferation. A study published in 2005 by Kobayashi et al. supports this idea. This group reconstituted lethally irradiated wild type mice with Smad3 knockout or wild type bone marrow. They found that, three weeks after vascular injury (photochemical), there was a reduction in neointimal hyperplasia in the animals that received Smad3 deficient bone marrow\textsuperscript{171}.

**Limitations and Future Directions:** In the studies presented in this chapter, studying unidirectional signaling from one cell type to another proved to be a powerful approach that allowed us to identify TGF-β as a potent SMC-to-Mφ signaling paradigm. However, extrapolation of these results to the *in vivo* disease setting must be done with caution. There are several limitations of these studies that must be addressed further. In follow-up studies we would like to examine if TGF-β is a critical mediator of macrophage phenotype *in vivo*, and if so, why do we see discordant regulation of macrophage genes in the *in vivo* setting as compared to the *in vitro*. We are also interested to understand why there is discordance between the mouse and rat macrophages matured with TGF-β. While we feel that our macrophage maturation system is more physiologically relevant than stimulating mature macrophages, to assess phenotypic modulation in the context of vascular injury, it is unclear if macrophages matured from whole bone marrow *in vitro* truly represent the macrophages present in vascular lesions *in vivo*. In addition to validating that macrophage TGF-β signaling is required for phenotypic modulation in lesional macrophages, we would also like to assess its overall role in progression of neointimal hyperplasia. We would like to determine how p38 MAP kinase is involved in TGF-β-dependent macrophage phenotypic modulation. Finally, we would like to expand
our in vitro methodologies to include ways to assess: cross talk between SMCs and Mϕ that is not dependent on diffusible factors, to assess cross talk between Mϕ and fully quiescent versus fully activated SMCs, to assess cross talk between these SMCs and Mϕ in the presence of additional cell types that would be present in an in vivo lesion, such as fibroblasts and/or epithelial cells.

We presented data in Chapter II that defined a distinct phenotype in Mϕ isolated from injured vessels, as compared to precursor monocytes. In this chapter, we describe a phenotypically modulated macrophage in response to maturation in the presence of SMC-derived factors, as compared to in the absence of such factors. The expression signatures we observed in the in vivo and in vitro settings were not completely concordant. Discordant gene expression between in vitro versus in vivo experiments could potentially be explained by the presence of other cell types, including increased numbers of dendritic cells in injured vessels versus circulating mononuclear cells in in vivo studies (Figure 4B). It is unlikely that TGF-β is the only factor present in vivo that influences macrophage phenotype, and macrophage heterogeneity may also result in discordant gene modulation between the in vivo and in vitro setting. For example, resident arterial macrophages may be less sensitive to TGF-β signaling due to down-modulation of TGF-β receptors in mature macrophages whereas infiltrating monocytes would be expected to be more susceptible to TGF-β signaling due to higher TBR expression\textsuperscript{172, 173}. Presence of both immature and mature monocyte/macrophages in the lesion may account for why TGF-β’s effect on macrophages in vivo may exhibit a less pronounced effect on genes like CCR3 and CCR7, and CCL5. Additional autocrine and paracrine signaling mechanisms may be at play in the in vivo scenario that are absent in in vitro maturation.
studies. For example, it is possible that the induction of arginase I observed \textit{in vivo} may be an effect of a paracrine signaling loop between infiltrating monocyte/macrophages and resident SMCs, or other cells present in the neointima. Conversely, the enhanced gene modulation observed in \textit{in vitro} studies may be the result of differentiation of whole bone marrow into a heterogeneous population in the absence of a selective stimulus such as TGF-β. Flow cytometry analysis of \textit{in vitro}-matured mouse bone marrow-derived macrophages (Figure 14C) suggests that maturation in the presence of TGF-β may result in preferential survival or promotion of a homogenous population of macrophages.

In these studies, we also observed discordance between mouse and rat macrophages matured \textit{in vitro} when comparing M-CSF only-matured M0 macrophages and TGF-β-matured tMφ. We know that mouse M0 macrophages matured \textit{in vitro} are heterogeneous with respect to CCR3 (Figure 12C) and F4/80 (data not shown) expression. While it is possible that rat \textit{in vitro}-matured macrophages may exhibit a similar heterogeneity we are unable to assess this by flow cytometry due to lack of reagents. If control M0 macrophages matured \textit{in vitro} from rat do not express the same level of heterogeneity as in the mouse, then that could account for some of the discrepancies we observed in TGF-β-dependent gene modulation in these two species. Finally, it is possible that mouse Mφ require additional input from other SMC-derived factors in order to express the same phenotype as rat Mφ; we might observe less discordance if we assessed maturation of mouse Mφ in the presence of mouse SMC conditioned medium rather than purified TGF-β.

Our strategy of characterizing the effect of a stimulus on macrophage phenotypic modulation as an immature precursor matures into a macrophage is, to our knowledge,
novel. This method allowed us to identify SMC-derived TGF-β as a factor capable of modulating macrophage phenotype in a way that would be predicted to be pro-stenotic. It is known that as monocytes mature into tissue macrophages, their portfolio of surface receptor expression changes. It is possible, therefore, that many studies that use fully mature bone marrow-derived macrophages as a model system to assess the effect of cell type x, or factor y, on macrophage phenotypic modulation may have missed a critical window when macrophages precursors might have been sensitive to cell type x-derived factors, or factor y. Indeed, TGF-β receptors are known to be expressed on the surface of circulating monocytes, but are then repressed in mature tissue macrophages\textsuperscript{174}.

The effect of TGF-β on immature circulating monocytes, which express both type I and type II receptors, is activating\textsuperscript{172}. For example, picomolar concentrations of TGF-β have been shown to induce TNF-α and IL-6 expression in human monocytes\textsuperscript{173}. Interestingly, while we observed such an increase in TNF-α \textit{in vivo}, TNF-α was reproducibly repressed \textit{in vitro} maturation in the presence of TGF-β. It is possible that this discrepancy in observed TNF-α expression may be due to the kinetics of gene induction, and then subsequent repression by negative feedback mechanisms. In our \textit{in vitro} studies, we assessed mRNA and protein modulation of TNF-α at five and seven days, respectively, after TGF-β introduction. Whereas published studies showing that TGF-β induced TNF-α upregulation in human monocytes assessed a 3 hour post-stimulation time point\textsuperscript{175}. It is possible that the repressed TNF-α we observed \textit{in vitro} may be an effect of late-phase negative feedback. Whereas \textit{in vivo} the increased TNF-α message we observed could be the result of marked upregulation in a subset of recently recruited monocytes, or possibly in a population of recently generated daughter cells
from adventitial hematopoetic progenitors. In contrast to the activating effect on monocytes, TGF-β is largely considered anti-activating for mature macrophages. However, most studies suggesting that TGF-β is a macrophage de-activating factor have been performed in the context of thioglycollate elicitation, lipopolysaccharide, phorbol ester stimulation. These studies therefore may not reflect the effect of TGF-β on macrophage phenotype in the absence of potent macrophage activating agents.

In addition to ascertaining if our in vitro results hold across various populations of monocyte/macrophages, further studies will be required to ascertain if deficiency in TGF-β signaling results in a net exacerbation or amelioration of neointimal hyperplasia in response to arterial injury. Such studies could be performed in the setting of either systemic inhibition of TGF-β signaling, or in the setting of myeloid-specific TGF-β signaling deficiency. Systemic TGF-β inhibition has previously been shown to reduce neointima formation in both the mouse and rat. While systemic inhibition of TGF-β signaling in vivo is feasible, interpreting the results of such studies is problematic with respect to understanding macrophage-specific effects. Recent work by Wan et al. demonstrated that systemic blockade of TGF-β signaling, either using a TGF-β-specific neutralizing antibody or a pharmacological inhibitor of TBR1 reduced the recruitment of mesenchymal stem cells to the site of injury. These authors also demonstrated that blocking TGF-β signaling inhibited the increase in MCP-1 observed in response to injury, which would be expected to lead to an overall monocyte/macrophage recruitment defect. Our future studies seek to define how TGF-β promotes a phenotypic change in recruited macrophages, rather than to define how TGF-β influences monocyte recruitment or TGF-β-dependent paracrine effects of other cell types signaling to
macrophages. We would therefore pursue the latter approach and genetically ablate TGF-β signaling in myeloid cells. We are currently generating mice with targeted deletion of TBRII in myeloid lineage cells to address this question in an *in vivo* setting of vascular injury. A myeloid-specific TBRII depletion study published in Cancer Discovery suggests that this approach is feasible and supports the idea that our *in vivo* gene expression signature may be largely TGF-β-dependent. Recent findings from Pang et. al.\textsuperscript{134} showed that myeloid-specific TBRII deficiency was sufficient to inhibit tumor metastasis, and to abrogate macrophage modulation (decreased iNOS, Arg I, IL-6, IL-10, and IL-12; and increased TNF-α, and MMP9). In addition to suggesting that our *in vivo* expression signature is largely TGF-β-dependent, these data suggest that myeloid-specific inhibition of TGF-β signaling, upon macrophage recruitment to lesions, would be expected to significantly reduce neointimal hyperplasia following injury. We propose to assess macrophage phenotype and neointima formation in the same model: LysM-Cre x TBRII floxed mice. However, if myeloid specific TBRII knockout results in decreased monocyte recruitment to lesions, phenotypic analysis of lesional populations may not be possible or valid. It may, therefore, be necessary to generate a TBRII conditional knockout that is specific for mature macrophages – such as CD68-Cre(±ER\textsuperscript{T2}) x TBRII floxed - in order to avoid effects on TGF-β-dependent monocyte recruitment following injury.

Our results suggest that macrophage p38 MAP kinase signaling is critical for TGF-β-dependent phenotypic modulation. Several human trials have exhibited a benefit of p38 MAP kinase inhibition in terms of repressing disease biomarkers and/or vascular inflammation\textsuperscript{178,179}. While, to our knowledge, human clinical trials have not exhibited a
net benefit of p38 inhibition on neointimal hyperplasia or primary atherosclerotic plaque development, several studies in pre-clinical models have shown such results. Rabbit and rat vascular injury studies have shown reduction in NIH in response to systemic p38 inhibition\textsuperscript{180,181}. Our studies showed that pharmacological inhibition of p38 kinase activity blocked both the morphological change, and the changes in gene expression, induced in macrophages by SMC conditioned media or rh-TGF-β. However, we have not been able to detect increases in active (phosphorylated) p38 in response to either stimulus (Figure 17A, and data not shown). In fact, steady state levels of phospho-p38 in sMφ are not different from M0 macrophages at any of the time points examined. It is possible that a transient activation of p38 occurs during the 5 day time course required for sMφ formation which we have failed to detect. Alternatively, constitutive p38 activity in these cells may represent a necessary, but insufficient signal for sMφ formation. For instance, signaling by M-CSF may prime macrophages to respond to TGF-β signaling, with both pathways collaborating to produce the sMφ phenotype. The requirement for basal p38 activity in sMφ formation may partially explain the efficacy of p38 inhibitors in reducing endothelial denudation-induced stenosis\textsuperscript{180,181}. There exists data to suggest that SB203580 may actually be inhibiting upstream kinase activity. In studies from both Jung et. al. and de Nicola et. al.\textsuperscript{182,183}, SB203580 was able to prevent TGF-β induced p38 phosphorylation. The later study shows data to suggest that this is due to blockade of p38 autophosphorylation, however it is possible that SB203580 may be preventing upstream TAK1 kinase activity as the kinase domains of p38 and TAK1 are similar (Fabio Rossi, personal communication).
Our data indicate that sM\(\phi\) can reciprocally signal back to SMC to promote some aspects of SMC activation. SMC in coculture with sM\(\phi\) had increased rates of proliferation compared to coculture with M0, suggesting production of a sM\(\phi\)-specific soluble factor that stimulates SMC proliferation. We hypothesized that such a factor might be PDGF-BB; however, PDGF-BB expression was decreased in sM\(\phi\) relative to M0. Coculture of SMCs with macrophages also increased production of several pro-inflammatory cytokines by SMCs. However, a similar level of induction with either sM\(\phi\) or M0 macrophages was observed. Systematically examining quantitative levels of growth factors and cytokines produced by sM\(\phi\) versus M0 macrophages may explain the differential effect in SMC proliferation versus cytokine induction. Additional experiments examining the dose response of target SMCs to condition media from these two macrophage populations may reveal difference in response at lower concentrations of conditioned medium. Alternatively, M0 macrophages may acquire some features of the sM\(\phi\) phenotype over time in coculture with SMC, masking differences between M0 and sM\(\phi\) macrophages with respect to certain aspects of SMC phenotype. Further studies will be required to understand what sM\(\phi\)-specific factors are capable of inducing enhanced SMC proliferation, and if the factor(s) responsible for inducing differential rates of proliferation are the same as those inducing similar upregulation of pro-inflammatory cytokines.

Our studies are limited in that they are restricted to assessing the contribution of soluble factors to the cross talk between SMCs and M\(\phi\). We are also interested to understand how sM\(\phi\) might influence SMC biology, versus M0 macrophages, in the setting of direct coculture, where we may be able to assess direct physical interactions.
We have shown that several cell surface chemokine receptors, and the scavenger receptor CD206, are modulated in response to maturation in the presence of TGF-β; it is possible that other cell surface molecules may be regulated differentially between M0 and sMϕ in such a way that they would induce differential levels of SM-gene modulation in a direct coculture scenario. We would hypothesize that, unlike in the setting of indirect coculture where we did not observe repression of SM-genes, direct coculture with sMϕ would result in repression of SM-genes if, for example, macrophages express cell surface Notch ligands that engage downstream upregulation of HERP/HEY family members (which has been shown to result in repression of SM-genes by blocking SRF/myocardin association with CArG elements)\textsuperscript{40}. In order to perform these direct coculture assays we will need a way to re-isolate pure populations of SMCs and Mϕ after coculture. This could be performed by FACS, but will require performing these assays with mouse-derived cells as flow cytometry reagents are not readily available for analysis of rat cells. We will, therefore, have to first validate our previous findings in an all-mouse SMC/Mϕ culture system before proceeding to direct coculture studies.

In future studies, we would also like to expand our analysis of the effects of SMC-derived factors on Mϕ, to fully quiescent and fully activated SMCs. Aortic vascular smooth muscle cells isolated from the rat, and cultured ex vivo, have a proliferative index of about 24% when serum-restricted in 0.1% serum medium for 48 hours as they were for our conditioned medium collection procedure (data not shown). This proliferative index is in stark contrast to the proliferation rate of quiescent SMCs \textit{in vivo} in an uninjured vessel, which has been documented by several groups to be less than 0.1% per day\textsuperscript{157, 184}. We previously showed that even out to 9 days in 0.1% serum media the proliferative
index of cultured aortic SMCs remains significantly elevated above a truly quiescent in vivo level\textsuperscript{158}. Therefore, while serum-restricted to rule out confounding effects from serum-derived factors, SMCs in culture maintain a somewhat activated phenotype. In contrast, a fully activated (PDGF-BB-stimulated, or PTEN-depleted (see Chapter IV for an introduction to PTEN-depletion as a model of SMC activation)) SMC exhibits approximately 5-fold higher proliferation than a cultured, serum-restricted, SMC\textsuperscript{26, 137}. We have begun to assess the differences in cross talk between M\textsuperscript{ϕ} and PTEN-depleted SMCs. These data are presented in Chapter IV. In future studies we would like to further assess cross talk between maximally activated SMCs, but also with SMCs driven into a more quiescent phenotype than what is possible under low serum conditions. This may be accomplished by pre-treatment of SMCs with rapamycin; which has been established to induce SMC differentiation\textsuperscript{185}. Indeed, rapamycin (sirolimus)-coated endovascular stents are thought to reduce neointimal hyperplasia by promoting SMC differentiation\textsuperscript{4}.

Finally, in our in vitro studies, we have only assessed the cross talk that occurs between two of the several cell types present in the injured vessel. We would like to develop novel in vitro/ex vivo culture methodologies to assess the contributions of additional cell types to the phenotypic modulation of M\textsuperscript{ϕ} and SMCs. It is possible that some of discordance we observe between our in vitro and in vivo results is due to signals derived from adventitial fibroblasts or endothelial cells etc. – or additional factors secreted from SMCs in response to signals from other cells present in the lesion. Truly understanding the pathogenesis of this disease may ultimately require in vitro systems.
that allow for high throughput experimentation, while closely recapitulating the in vivo milieu.

In summary, this study is one of the first to successfully recapitulate an in vivo, neointimal lesion-like macrophage phenotype using an in vitro system. Our results reveal a novel, physiologically relevant model system for studying macrophages that accumulate in the developing neointima. Importantly, these studies suggest that SMC-derived TGF-β1 may be a critical mediator of the macrophage vascular injury-specific polarization observed in vascular disease states such as atherosclerosis and restenosis. Further, our cross talk studies begin to address the apparent paradox in that active TGF-β acts as a pro-differentiation factor for SMC, yet in the setting of vascular injury the net result of TGF-β antagonism is reduced neointima formation\textsuperscript{163,165}. Our results suggest that TGF-β signaling in maturing monocyte/macrophages results in an activated cell that subsequently secretes factors capable of further activating SMC upon vascular injury.
CHAPTER IV

EFFECT OF SMC-SPECIFIC PTEN DEPLETION ON MACROPHAGE RECRUITMENT AND PHENOTYPE IN VASCULAR INJURY

Introduction

Phosphatase and tensin homologue (PTEN) is a phospholipid phosphatase that is responsible for counteracting the effects of PI3K; it primarily dephosphorylates PIP\(_3\) to PIP\(_2\), which ultimately decreases AKT activation and cell proliferation. Microarray\(^{186}\) and immunohistochemical staining studies (Weiser-Evans, unpublished) have showed that PTEN is repressed in SMCs in human atherosclerotic lesions. Studies by Dr. Weiser-Evans’ lab demonstrated that balloon catheter-induced injury to rat carotid arteries results in phosphorylation, and thus deactivation, of PTEN selectively in SMCs (Figure 20)\(^{187}\). Further, it was found that cultured neointimal SMCs, which exhibit serum-independent proliferation, express high levels of active AKT, mTOR, and p70S6K.

This serum-independent proliferation was

Figure 20. Balloon-mediated injury results in repression of PTEN in the rat.
Rat carotid arteries were subjected to balloon catheter injury. Whole cell lysates from injured arteries were analyzed for phosphorylated and total PTEN; β-Actin = loading control. Phosphorylated, inactive PTEN increased from 7-14 post-injury (Studies done previously in the lab: Garl et. al. Circ Res. 2004.)
inhibited by PI3K or mTOR inhibition, or dominant negative AKT expression. It was also demonstrated that this serum-independent growth phenotype could be recapitulated in vitro by morpholino-mediated loss of PTEN in serum-dependent primary SMCs\textsuperscript{188}. Additional studies by Dr. Weiser-Evans’ group showed that serum-stimulated proliferation and AKT phosphorylation were significantly decreased in primary SMCs overexpressing a wild-type PTEN\textsuperscript{187}. These results were very interesting considering that genomic and somatic PTEN loss-of-function mutations or deletions are a common finding in various types of human cancers. Additionally, PTEN has been found to be deregulated in tumors due to inappropriate subcellular localization or aberrant proteasomal degradation\textsuperscript{189}. It is possible that germline or de novo somatic mutations in PTEN that result in decreased activity could be a contributing risk factor for development of restenosis after angioplasty. In order to further investigate the contribution of PTEN-deactivation to restenotic neointima formation, our lab previously generated a smooth muscle cell–specific knockout mouse, using Cre recombinase under control of the SM22-\(\alpha\) promoter\textsuperscript{147}. As was predicted,
AKT activation was increased in SMCs that were lacking PTEN. Significant vascular remodeling including arterial medial hyperplasia, and increased deposition of the chemokine SDF-1α (Figure 21), was observed in these mice. CXCR4, one of the cognate receptors for SDF-1α, was found to co-express with SMA, suggesting the potential for autocrine SDF-1α signaling in SMCs in vivo. In cultured SMCs from these mice, growth was induced by autocrine SDF-1α signaling, and conditioned media stimulated growth of control SMCs, which was attenuated by SDF-1α neutralizing antibody\(^\text{147}\).

![Figure 22. SMC-specific depletion of PTEN results in increased BMC recruitment to lesions.](image)

WT (left panels) and PTEN iKO (right panels) mice were subjected to femoral wire-induced injury. Mice were treated with control IgG or neutralizing anti-SDF-1α for 3 weeks. PTEN iKO mice exhibited increased neointima and macrophage recruitment to vascular lesions which is reversed by anti-SDF-1α antibody (Studies done previously in the lab: Nemenoff et. al. *Arterioscler Thromb Vasc Biol*. 2011)

Due to the fact that PTEN knockout driven by SM22-α-Cre caused these mice to survive only an average of 21 days, wire injury studies in these animals were not possible. A tamoxifen-inducible PTEN knockout (PTEN iKO) mouse was therefore generated by crossing PTEN\(^{\text{fl/fl}}\) mice with transgenic mice expressing Cre recombinase fused to a mutant tamoxifen-responsive ligand-binding domain of the estrogen receptor (CreERT) under control of the murine SM-MHC promoter (SM-MHC-CreERT\(^{+/\text{fl}}\).PTEN\(^{\text{fl/fl}}\))\(^{137,190,191}\). Wire-
induced injuries were performed on the right femoral arteries of these mice, as previously
described\textsuperscript{16}. Both bone marrow transplantation with WT GFP bone marrow, and
tamoxifen treatment for 5 days to induce PTEN knockout in SM-MHC-expressing SMCs
were administered prior to injury. These mice were transplanted with GFP bone marrow
in order to determine if bone marrow-derived inflammatory and/or progenitor cells were
contributing to neointima formation. It was discovered that not only did PTEN iKO
cause a marked increase in neointima formation at three weeks-post injury, but there was
also a significant increase in the number of bone marrow-derived macrophages recruited
to the lesion in the iKO animals. This response to injury, in the setting of chronic PTEN
depletion in SMCs, more closely recapitulates the severity of found in humans, as
compared to wild type C57BL/6J mice in which form less severe lesions that regress over
time\textsuperscript{64,192}. Finally, it was determined that systemic administration of SDF-1\textalpha
neutralizing antibody reversed the effects of PTEN iKO on both neointima formation and
macrophage recruitment (\textbf{Figure 22})\textsuperscript{137}. We hypothesized, based on these data, that
depletion of PTEN in SMCs results in altered cross talk between SMCs and recruited
monocyte/macrophages that additively contributes to the pathogenesis of neointimal
hyperplasia. We therefore wanted to ascertain how chronic SMC-specific PTEN
deficiency might alter macrophage recruitment or retention in lesions, as well as to
understand how PTEN deficiency in SMCs might differentially modulate macrophage
phenotype relative to un-manipulated SMCs.
Materials and Methods

Reagents: Flow cytometry antibodies: F4/80-APC (AbD Serotec), CD11b-FITC, Ly6g-PE-Cy7, Ly6c-PE, CD11c-APC-Cy7 (all BD Biosciences), and MHC-II-PerCP eFluor 710 (eBiosciences).

Femoral Artery Injury: All animal procedures were approved by, and performed in accordance with the guidelines of the University of Colorado IACUC. Guide-wire denudation femoral artery injury was performed, as shown by us previously (Figure 2A), on right femoral arteries of male 20-28g PTEN iKO (smooth muscle-specific, tamoxifen-inducible PTEN knockout) or control (creERT only) mice at least one week following 5-day tamoxifen (0.033mg/kg, IP) treatment. Guide-wire injures were performed as described in the Methods section of Chapter III. Injured femoral arteries were harvested on day 15 post-injury following euthanasia and flushing of the vasculature with heparinized PBS (80kU/L).

Flow Cytometry: Isolated injured vessels, were digested to single cells by digestion at 37°C for 2hr in collagenase buffer (3.2mg/ml collagenase II, 0.7mg/ml elastase (Worthington), 0.2mg/ml soybean trypsin inhibitor (Sigma) in Hank’s buffered saline solution (HBSS), pH7.5). Red blood cells were lysed for 2-5 min in RBC lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA (pH 7.2)). Single cell suspensions were stained for CD11b, F4/80, Ly6g, Ly6c, MHC-II, CD11c, and DAPI to stain for dead cells. Flow cytometry was performed on a Galios cytometer (Becton Dickenson) as demonstrated by our group previously, and as described above in the Methods section.
of Chapter III. Autofluorescent counting beads (Spherotech) were added to cell suspensions in n=1 experiment to quantitate absolute numbers of macrophages in injured vessels in control versus PTEN iKO mice. Macrophage quantitation was calculated as:

\[
\text{Volume analyzed} = \frac{\text{# of beads analyzed}}{\text{# of beads/50\mu l (from spec sheet)}}
\]

\[
M\phi/\text{volume} = \frac{\text{# of } M\phi \text{ analyzed}}{\text{volume analyzed}}
\]

\[
M\phi \text{ in sample} = M\phi/\text{volume} \times \text{total volume}
\]

\[
M\phi/\text{mg tissue/vessel} = M\phi \text{ in sample} \div \text{tissue wet weight} \div \# \text{ vessels pooled in sample}
\]

**Cell Culture:** Primary aortic SMCs were isolated from Sprague-Dawley rats as previously described\(^{149}\). SMCs were used for experiments between passages 8 and 15. Rat SMCs were plated at \(7 \times 10^6\) cells/150 mm dish in MEM media supplemented with 10% fetal bovine serum (FBS) and 1x penicillin/streptomycin (20 IU/ml penicillin, 20ug/ml streptomycin). PTEN deficient SMCs were generated by infecting cells with lentiviral particles carrying empty vector control or anti-PTEN shRNA constructs as previously described\(^{137}\). PTEN knockdown was confirmed by western blotting. Primary bone marrow-derived MΦ were isolated from C57BL6/j mice, as previously described\(^{137}\). Whole bone marrow was plated, directly following isolation from bones, for each experiment without passaging in the presence of 20ng/ml M-CSF (eBioscience).

**Macrophage Adhesion Assay:** Macrophage adhesion assays were performed using a modified protocol as described elsewhere\(^97\). Macrophages matured for seven days under
20ng/ml M-CSF were fluorescently labeled with 5ug/ml BCECF-AM (A.G. Scientific, Inc.) for 15min at 37°C in HBSS. Mϕ were then trypsinized for 5 minutes. Mϕ were washed 1x with HBSS, centrifuged at 300g for 5 minutes to remove trypsin. Cells were then resuspended to a concentration of ~10^6 Mϕ/ml in SRM. One milliliter of Mϕ suspension was then added per 35mm well of 24-hour serum-restricted control or PTEN-depleted SMC monolayers (5*10^5 SMCs plated per well). Culture clusters were agitated every 5 minutes over the course of the experiment. At 1, 5, 10, 20, 40, and 80 minutes after addition of Mϕ cell suspension non-adherent Mϕ were removed by aspiration, and washing gently once with HBSS. After washing, adhered cells were maintained on SMC monolayers in SRM. At the end of the time course, adhered fluorescent macrophages could be quantified by counting under a fluorescent microscope, or by lysing all cells and quantitating fluorescence on a fluorimeter. Lysis buffer used: 0.1% Triton X-100 in 0.1M Tris base.

**Results**

*SMC-Specific Depletion of PTEN Results in Increased Macrophage Content in Vascular Lesions, and a Higher Percentage of Ly6c<high> Macrophages.* We previously observed that there were more macrophages present in vascular lesions in PTEN iKO animals versus controls by Mac-3 immunofluorescent staining^{137}. This prior observation was validated quantitatively by this student using flow cytometry of control and PTEN iKO injured femoral arteries (**Figure 23A**). We found that when normalized to wet weight of tissue (to normalize for differences in degree of overall neointimal mass) and number of vessels per condition, there was approximately a 5-fold increase in
macrophages/mg tissue/vessel in PTEN iKO versus control injured femorals. We then wanted to understand if, in addition to increased macrophage recruitment/retention in vascular lesions in PTEN iKO lesions, if there might be a difference in macrophage phenotype or subset in the recruited cells. Flow cytometry analysis revealed that there was a significant increase in the ratio of Ly6c\(^{\text{high}}\) to Ly6c\(^{\text{low}}\) macrophages present in lesions at this 15 day time point (Figure 23B-C). We did not observe differences in expression in MHC-II or CD11c in the macrophages recruited to PTEN iKO lesions as compared to controls (data not shown). The increase in Ly6c\(^{\text{high}}\) macrophages present in

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**Figure 23. Mϕ burden is increased in PTEN iKO lesions and a higher percentage of Mϕ express high levels of Ly6c.**

A) Quantitation of macrophages present in SMC-specific, inducible PTEN knockout (PTEN iKO) wire-induced lesions compared to wild type control (ctrl) lesions. Total number of macrophages normalized to mg of tissue and number of vessels pooled for analysis. B) Representative histogram of Ly6c expression in macrophages isolated from control (green) and PTEN iKO (red) wire-induced lesions. C) Quantitation of the ratio of Ly6c high-expressing cells (Ly6c Hi) to Ly6c low-expressing cells (Ly6c Lo). Shown are means±SE. A) n = 1; B-C) n=3 independent experiments. *P<0.05 vs control injured vessels.
Figure 24. Mϕ adhesion to SMCs is increased in PTEN deficient SMCs.
A) Western blot showing efficient knockdown of PTEN in lentivirus-transfected SMCs. B) Micrograph of fluorescently labeled macrophages adhered to vector control (Vector) or PTEN knockdown (PTEN KD2) SMC monolayers. Time point shown = 40 minutes. C) Quantitation of macrophage adhesion to control and PTEN knockdown SMC monolayers at 1, 5, 10, 20, 40, and 80 minutes by fluorescence (right) of well lysate, or counting (left) of adhered (fluorescent) cells. Shown are means±SE. n=3 independent experiments. *P<0.05 vs control injured vessels.
lesions suggests an increased recruitment of inflammatory monocytes to iKO lesions at this time point versus in control lesions\textsuperscript{68}.

\textbf{PTEN Depletion in Primary SMCs In Vitro Results in Increased Macrophage Adhesion.} We performed experiments, published in 2011, suggesting that increased adhesion of monocyte/macrophages to PTEN depleted SMCs might be an explanation for why monocyte-derived macrophage recruitment may be enhanced in \textit{in vivo} lesions in PTEN iKO mice\textsuperscript{137}. In these studies we generated PTEN knockdown SMCs by lentiviral-mediated introduction of shRNAs. We found that several constructs were efficient in depleting SMCs of PTEN (PTEN KD SMC) as compared to vector-transfected SMCs (Figure 24A). We then performed an \textit{in vitro} adhesion time course assay to determine if (mouse) bone marrow-derived macrophages might adhere more readily to PTEN KD SMCs. We found that by measuring the fluorescence intensity of fluorescently labeled macrophages to SMC monolayers – as well as by cell counts – macrophage adhesion to PTEN KD SMCs was significantly greater at later time points assayed (Figure 24B-C).

\textbf{Discussion}

\textbf{Conclusions:} Given that PTEN is found to be repressed in SMCs in human atherosclerotic lesions, that our PTEN iKO model more closely recapitulates the human response to vascular injury versus wild type mice, and that we observed increased macrophage recruitment to PTEN iKO lesions, we therefore hypothesized that repression of PTEN in SMCs was responsible for differential monocyte/macrophage recruitment and/or phenotypic modulation in iKO lesions versus controls. The data from our flow
cytometry experiments suggest that there is an increased macrophage burden in PTEN iKO lesions, at a 15-day time point. The increase in Ly6c\textsuperscript{high} -expressing macrophages suggests that these macrophages are likely derived from circulating inflammatory (Ly6c\textsuperscript{high}) monocytes, or that there is an increase in inflammatory monocyte recruitment compared to patrolling monocyte recruitment in the setting of PTEN depletion. Our \textit{in vitro} adhesion data suggests that at least one aspect of this increased recruitment is likely due a greater capacity of PTEN KD SMCs to bind monocyte/macrophages versus control SMCs.

\textbf{Limitations and Future Directions}: Unpublished microarray data from our lab suggests that several adhesion molecules or matrix molecules (CD53, NCAM-1, versican) that would be expected to interact with cognate factors expressed on the cell surface of macrophages (\textit{\alpha}4\textit{\beta}1 integrin\textsuperscript{193,194}, NCAM-1\textsuperscript{195}, TLR2\textsuperscript{152}, respectively) are upregulated in PTEN KD SMCs. In future studies related to these findings we would like to validate that increased recruitment is indeed occurring at this time point, as opposed to local proliferation of macrophages expressing high levels of Ly6c. We could assay this by performing adoptive transfer experiments of CD45.1 PBMCs into our CD45.2 control and iKO mice one day prior to flow cytometric analysis of lesional macrophage populations. If recruitment is actively occurring in the iKO mice we would expect to see higher chimerism in lesions (relative to blood chimerism) in the iKOs versus the control mice. We would then perform RNA-seq studies on FACS-isolated SMCs from inducible, conditional YFP expressing SMCs (Rosa-26 x SM-MHC creERT) from injured and uninjured vessels and compare expression signatures of these \textit{in vivo} samples to our \textit{in
vitro PTEN KD SMCs. This analysis should identify a manageable number of molecules expressed by PTEN-depleted cells that may be responsible for the observed increase in macrophage adhesion to PTEN KD SMCs. We would then validate candidates in in vitro adhesion studies using transient transfection with siRNAs to deplete SMC-expressed adhesive molecules and macrophage-expressed cognate factors.

It is possible that in addition to increased adhesion, other mechanisms could be contributing to increased numbers of macrophages in PTEN iKO lesions. For example, we previously showed that PTEN repression in SMCs is sufficient to induce upregulation, and secretion, of factors that are chemotactic for monocyte/macrophages such as SDF-1α and MCP-1. It is possible that the increased recruitment of monocytes to lesions could simply be due to more mobilization of hematopoietic precursors from the bone marrow and/or higher local accumulation in the circulation due to an enhanced chemotactic gradient; allowing for enhanced net monocyte adhesion. We could assay monocyte content per volume in the peripheral blood by flow cytometric analysis of whole blood. If recruitment proves not to be responsible for increased macrophage content in iKO lesions we would next assess if local proliferation of resident vascular macrophages is responsible for increased macrophage burden such as has been suggested in the ApoE−/− murine model of atherosclerosis. Adoptive transfer of GFP+ PBMCs, in combination with a BrdU pulse, given one day prior to collecting vessels for immunostaining would allow us to ascertain what the relative contribution is to macrophage burden of proliferating resident macrophages (GFP-, BrdU+) versus recruited monocytes that are either proliferating or not (GFP+, BrdU+/−). If local proliferation of tissue macrophages is a major contributor to lesional macrophage burden
our RNA-seq data should again allow us to identify candidate SMC-derived growth factors that may be responsible.

As described in the Discussion section of Chapter III, we would ultimately like to move these studies forward to identify macrophage-derived factors that might be responsible for completing a feed-forward loop where SMCs modulate the phenotype of macrophages which then cross talk back to maintain SMCs in an activated state. Since our PTEN iKO model of vascular injury more closely recapitulates human disease, we are hopeful that understanding PTEN-deficient SMC cross talk with macrophages, as compared to control SMC cross talk with macrophages, will lead us to identify novel paracrine signaling mechanisms at work between these two cell types that may be leveraged to ultimately develop anti-stenotic therapies that are more efficient and specific than current strategies.
CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Summary

Arterial occlusion due to neointimal hyperplasia as seen in primary atherosclerosis, following arterial re-vascularization, and in the setting of graft or transplant arteriopathy, can result in death or significant morbidity. Available therapies for the prevention of neointimal hyperplasia have advanced significantly in the past 10-15 years. However, there remains room for improvement, particularly with respect to identifying treatment strategies that may limit vascular smooth muscle cell proliferation and ECM production while encouraging re-endothelialization of regions of disrupted endothelial cell integrity.

We felt, at the time of initiating these studies, that while the data present in the literature convincingly suggested that macrophages contribute to the pathogenesis of neointimal hyperplasia, a paucity of data was available to suggest by what mechanisms macrophages drive the neointimal response to vascular injury. We were particularly interested to understand how vascular smooth muscle cells and macrophages cross talk in the injured vessel and how that cross talk influences both the differentiation state of the SMCs as well as the polarization state of the macrophages. We hypothesized (Figure 25) that upon vascular denudation injury, monocytes are recruited to the lesion and undergo maturation and phenotypic modulation (or polarization) in response to factors secreted by
SMCs. These phenotypically modulated macrophages are then able to signal back to the resident SMCs to either exacerbate or maintain their activation state. This feed-forward signaling would be expected to ultimately result in worsened neointima formation in response to injury. As described above, many studies in the vascular literature, particularly in atherosclerosis, have seized upon the idea that macrophages in the neointima express an M1 polarization. Many published studies have thus used LPS/INF-γ stimulation of macrophages *in vitro* to model an activated atherosclerotic or restenotic macrophage. We felt that biasing our studies by assuming that LPS/IFN-γ stimulation is an accurate model of a restenotic macrophage would prevent us from understanding how
these two cell types modulate each other’s activation states in the setting of sterile inflammation. We therefore did not preferentially activate our in vitro macrophages prior to stimulating them with SMC-derived factors. We hypothesized further that in the setting of SMC-specific depletion of PTEN – a model that more closely recapitulates the human response to denudation injury, where lesion development in the C57BL/6J mouse is not as pronounced – that macrophage recruitment and/or phenotypic modulation would be increased or more skewed toward a pro-inflammatory phenotype as compared to in the wild type mouse.

In Chapter II of this dissertation we show that macrophages were found to be the predominant myeloid cell population present in injured vessels. This quantitative analysis is an improvement over previous attempts to quantify these cells in vessels, as prior studies have primarily assessed lesional macrophages by immuno-staining in tissue sections. We then assessed the phenotype of macrophages in injured vessels compared to macrophages from uninjured vessels, or to monocytes isolated from peripheral blood. We found a distinct difference in macrophage phenotype in injured vessels as compared to monocytic precursors that suggested a hybrid activation state, and/or a heterogeneous population. Additional replicates of these studies are needed to understand how expression of these genes in uninjured vessels relates to precursor monocytes and macrophages from injured vessels. However, these data suggest that there are maturation- (from monocyte to macrophage) specific patterns of gene regulation, as well as injury-specific patterns of gene regulation. Together, these data suggest that upon recruitment to vascular lesions, and/or upon expansion of the resident macrophage population in the lesion, macrophages express a modulated phenotype relative to
macrophages in uninjured vessels or to precursor monocytes. In future studies it would be informative to understand how macrophage phenotype, in the injured vessel as compared to both control populations, changes over time. It is possible that some of the similarities we observed between gene regulation in macrophages from injured and uninjured vessels is due to lesional macrophages reverting to a more basal-like phenotype by 15 days. Time course analysis may allow us to dissect out the maturation-dependent gene changes from the injury-dependent gene changes.

Based on the data presented in Chapter II, we hypothesized that recruited monocytes in the neointimal lesion undergo phenotypic modulation in response to factors derived from resident SMCs as they mature into macrophages. We therefore set out to develop an in vitro assay that would allow us to assess uni-directional effects of SMC-derived secreted factors on macrophage maturation from bone marrow-derived precursors. When we matured rat bone marrow cells in the presence of 20% conditioned medium from SMCs, we observed a marked morphologic change in the resulting macrophages. This morphologic change was accompanied by a pronounced change in transcription of the panel of genes assessed in our in vivo injury studies. The phenotype of the macrophages matured in vitro with SMC conditioned medium (sMϕ), relative to control macrophages, was very similar to the phenotype observed in macrophages from injured vessels compared to precursor monocytes as described in Chapter II. Using a variety of strategies to assess the physical characteristics of the SMC-derived factor, we determined that SMC conditioned medium contained a heat stable peptide-based component of a large (greater than 100kDa) complex that included a heat labile inhibitory factor. This complex responded to heating by becoming more potent and decreasing in
size to less than 100kDa. We also observed that the macrophage modulatory effects of this molecule were dependent on p38 kinase activity. These data were consistent with TGF-β large latent complex.

We then observed that purified TGF-β was capable of inducing the same phenotypic modulation of macrophages that we observed with SMC conditioned medium. Studies employing TGF-β neutralization, TGF-β receptor 1 inhibition, and TGF-β silencing in SMCs, illustrated that SMC-derived TGF-β was responsible for the phenotypic modulation observed in macrophages in vitro in response to maturation in the presence of SMC-derived conditioned medium. We assayed the effect of mouse macrophage maturation in the presence of TGF-β (tMϕ) to assess if TGF-β would recapitulate the phenotypic modulation we observed in our in vivo studies, in the same species. We found that while several genes were regulated similarly between tMϕ and macrophages isolated from injured vessels, there was discordance in several genes; more discordance, in fact, than between rat sMϕ/tMϕ and macrophages from injured vessels. This suggests to us that while TGF-β seems to be able to recapitulate the phenotype of macrophages in the injured vessel fairly well, perhaps in the mouse this phenotypic modulation requires cooperation between TGF-β and one or more additional factors. In order to validate the importance of macrophage-specific TGF-β signaling on macrophage phenotype and overall neointima formation, we would like to perform injury studies in myeloid-specific (lysozyme-2 promoter-driven Cre x TBRII floxed) TGF-β signaling deficient mice, versus littermate Cre null controls. We anticipate (particularly in light of the findings by Pang et. al, showing that in the setting of cancer a similar Mϕ phenotype was observed and that it was abrogated by myeloid-specific TBRRII knockout134) that if
macrophage recruitment to lesions is not significantly reduced by depleting TGF-β signaling in macrophages, then we should observe a similar abrogation in macrophage phenotype in injury relative to precursor monocytes. We also expect that myeloid-specific TBRII depletion will result in a net decrease in neointimal hyperplasia relative to controls.

We then expanded the use of this in vitro maturation model to study the effect of paracrine cross talk that occurs between SMCs and macrophages, with a focus on assessing feedback on SMCs. In vitro coculture of SMCs with sMϕ resulted in increased proliferation of SMCs which, interestingly, did not correlate with differential expression of pro-inflammatory cytokines or smooth muscle contractile protein repression as might be expected. Subsequent attempts to determine if upregulation of PDGF-b in sMϕ might be responsible for increased SMC proliferation in coculture showed that this growth factor was actually repressed in sMϕ relative to M0 macrophages. Attempts to isolate conditioned medium from sMϕ and M0 macrophages, and to subsequently identify signaling pathways induced in SMCs in response to these media did not provide consistent data to suggest what the sMϕ-derived factor might be that was responsible for inducing SMC proliferation. Additional physical characterization studies, and optimization of macrophage conditioned medium collection, will be needed to determine the mechanism of this observation. A difficulty in these studies is the extreme plasticity of macrophages. Removal of SMC CM may result in reversion to a different phenotype more similar to M0 macrophages. Thus novel strategies will need to be employed to stabilize the macrophage phenotype in coculture systems.
As discussed above, we were also interested to know how repression of PTEN in SMCs, in the setting of injury, might influence macrophage phenotypic modulation and subsequent feedback to SMCs. We were able to generate some preliminary data, to validate what had previously been observed in the lab by immunofluorescent staining of tissue sections, showing that macrophages are increased in lesions from mice depleted of PTEN specifically in SMCs versus in control mice. Further, we observed an increase in Ly6c\textsuperscript{high} monocyte/macrophages present in the lesions at 15 days following injury in PTEN iKO mice. This suggests to us that active recruitment of inflammatory monocytes to lesions in PTEN iKO mice is ongoing at this time point, while it seems to have abated in the control mice. Further studies will be required to characterize both monocyte recruitment and the phenotype of the macrophages present in PTEN iKO lesions over time as compared to macrophages in lesions from control animals. Ultimately, we would like to isolate macrophages from PTEN iKO and control lesions, and perform \textit{ex vivo} coculture studies with naïve mouse SMCs, to assess how the cross talk of PTEN iKO-conditioned macrophages might result in differential activation of SMCs.

If our \textit{in vitro} data related to TGF-β-dependent modulation of macrophages can be validated \textit{in vivo}, then these data suggest that pharmacologic blockade of TGF-β signaling or downstream (as of yet unidentified) Mφ-to-SMC signaling may lead to better targeted therapies, either alone or in combination with existing agents, to improve outcomes.
Future Directions

In the Summary section of this chapter, I have outlined the immediate follow up studies that we would like to perform to expand upon our findings and assess their relevance in an *in vivo* model of disease. Here I would like to expand upon those studies and discuss how these data may direct studies to develop more targeted therapies for preventing neointimal hyperplasia.

The studies presented in Chapter II: Quantitation of Myeloid Cell Populations, and Characterization of Macrophage Phenotype in a Murine Model of Resentosis, could be more informative if we could expand those studies to look at macrophage phenotypic modulation in the injured vessel over time. It is possible, in addition to the fact that these populations may be more heterogeneous than we can detect by our limited flow cytometry panel, that a more TGF-β-dependent phenotype might be observed earlier following injury. The discordance we see between some of the markers when comparing *in vivo* samples at 15 days and *in vitro* samples at 7 days may be due to the *in vivo* cells losing their TGF-β-dependent phenotype over time, or conversely they may not yet be maximally activated by TGF-β. In our *in vitro* studies, changes in this panel of genes were modulated maximally at 5 days after exposure to SMC CM (data not shown), as compared to 3 and 4 days. We did not assess later or earlier time points. Recruitment of monocytes to lesions begins within a short period of time upon vascular injury\textsuperscript{113}. These monocytes would begin maturation in the presence of TGF-β immediately upon recruitment if they are capable of activating latent TGF-β. (Activation of latent TGF-β was shown to be maximal following injury from 3-14 days\textsuperscript{163}.) If TGF-β-dependent macrophage phenotypic modulation is then expected to be maximal at 5 days post-injury,
we may be analyzing these cells as they are reverting back to a less activated state. Alternatively, if TGF-β-induced macrophage phenotypic modulation only accounts for a significant enough percentage of myeloid cells in the lesion starting at day 15, when macrophage content is maximal, the optimum time to assess phenotype might be closer to 20 days after injury. Time course analysis will be required, prior to assessing the dependency of lesional macrophage phenotype and neointima formation on myeloid TGF-β signaling, in order avoid generating falsely negative results.

We would also like to characterize the transcriptome of the macrophages present in lesions, both from wild type animals as well as from PTEN iKO animals, in order to define the phenotypes of these cells in an unbiased manner. Based on our panel of only 11 genes, we might conclude that macrophages from injured and uninjured vessels are not phenotypically different. We might find the same result if we were to compare the phenotype of macrophages isolated from PTEN iKO lesions versus wild type lesions. However making such assertions based on such a limited panel of markers may be unwise. Proteomic analysis may allow us to more confidently describe macrophages derived from vascular lesions as different from various control populations. Proteomic analysis may also allow us to develop better methods for isolating inflammatory populations from non-inflammatory populations, even within the injured vessel, by identifying reliable cell surface markers. We hypothesize that there is some level of heterogeneity present in monocytes or macrophages derived from vessels or peripheral blood, and a critical next step in moving forward with these studies will be to understand what the contribution of various subpopulations of these cells is to neointima development. In addition to assessing the heterogeneity of macrophage populations in
vivo, and their phenotypic modulation over time, it will also be important to assess the contribution of the other cell populations present in the injured vessel to macrophage modulation, to SMC phenotype, and to SMC-macrophage cross talk.

Due to the fact that TGF-β’s effect on SMCs is largely described as being anti-proliferative and pro-differentiating we hypothesize that inhibiting TGF-β signaling may not be a viable therapeutic strategy. Even if a TGF-β receptor inhibitor compound could be delivered locally, such as on a coated stent, inhibition of TGF-β signaling in local SMCs may result in net exacerbation of neointimal hyperplasia. Inhibition of TGF-β signaling in endothelial cells at the margins of the denuded area would be predicted to both encourage endothelial cell survival and re-endothelialization of the vessel if the TGF-β receptor 1 isoform ALK1 predominates; whereas the opposite effect would be anticipated if the ALK5 isoform predominates\textsuperscript{196}. Careful dose-response studies would have to be performed to determine what the net effect of TGF-β receptor inhibition is with respect to neointimal hyperplasia. A potentially more viable strategy for identifying a targetable mechanism might be to determine what macrophage-derived factors are responsible for feed-forward activation of resident SMCs. The transcriptome analysis described above, potentially in combination with proteomic data generated from macrophage conditioned medium characterization from \textit{in vitro} experiments, should provide insight into what macrophage-derived mediators signal to SMCs. Ideally this analysis would yield candidates that might exert opposing effects on SMCs and endothelial cells. Such a factor might promote activation of SMCs but induce apoptosis or repress proliferation of endothelial cells. Inhibition of such a factor, or its signaling,
would then result in repression of SMC proliferation and hopefully encourage endothelial cell proliferation and migration.

Normal function of the artery involves both the integrity of its complex structural organization as well as proper dynamic cross talk between its composite cells and cells in the circulation. Angioplasty and stenting attempts to maintain proper vessel macrostructure, while drugs eluted from stents modulate the behavior of the composite cells. Dual anti-platelet treatment’s goal is to modulate the thrombotic response of platelets in the circulation to the damaged artery and the stent itself. Unfortunately, the combination of these strategies has yet to completely prevent the occurrence of post-intervention restenosis. It is possible that therapies for preventing restenosis could be improved by targeting inflammation and intercellular cross talk rather than, or in addition to, targeting cellular proliferation as most current drug-eluting-stents do. The optimal strategy for preventing restenosis is likely to require a thorough understanding of cross talk between more than just two of the major cell populations present in the developing neointima. Our studies have begun to elucidate how intercellular cross talk between SMCs and macrophages in the lesion occurs. These studies may provide a framework for how we can go about understanding interactions between other cell populations in the future. Effective treatment for restenosis will entail development of combinatorial therapies that can modulate multiple facets of the complex pathogenesis of neointimal hyperplasia.
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APPENDIX A

EARLY COCULTURE STUDIES

Introduction

This appendix includes a discussion of the data I generated during the first year of work in Dr. Nemenoff’s laboratory. This data was determined to not be publishable due to the presence mycoplasma contamination in our primary vascular smooth muscle cell cultures. I have included this section because it may be instructive for future students who may work towards understanding the cross talk that occurs between innate immune cells and other cells present in diseased tissue. Additionally, there are several methods and experimental strategies that may prove useful for the lab and/or future students. Hopefully these methods will be less likely to be lost or forgotten if they are presented herein.

The introductory material for this data is the same as that presented in Chapter I and the introduction of Chapter III. In brief, multiple studies in the literature suggest that the recruitment of monocytes to regions of vascular injury, the subsequent maturation of those monocytes into macrophages, the retention of those cells in the lesion, and cross talk with resident arterial SMCs function to exacerbate SMC activation and disease progression. We initially hypothesized that soluble mediators produced by either or both of these cell types would initiate bi-directional activation of both cell types, and that this reciprocal activation would be expected to result in exacerbation of disease. We set out,
therefore, to characterize if and how the phenotype of these two cell types would be modulated if they were allowed to exchange soluble signals in \textit{in vitro} culture. Our subsequent objectives were to understand if coculture results in bi-directional activation, then which cell type is responsible for initiation. Further, we were then interested in identifying the mechanism of how the initiating cell type signals to the responding cell, and reciprocally how the responding cell signals back to the initiating cell to result in its activation.

The data presented in this section are not necessarily statistically significant, because experimentation was terminated when it was determined that these data did not address the research questions we set out to answer. I have discussed these data as though they were found to be statistically significant in order to explore what conclusions they support and what additional hypotheses derive from them.

\textbf{Methods and Materials}

\textbf{Reagents:} Antibodies: iNOS (BD Biosciences), arginase I, anti-rabbit-AP and anti-mouse-HRP and p65 (Santa Cruz Biotechnologies), IxBα, phospho-STAT3, phospho-AKT, phospho-ERK, and total ERK (Cell Signaling Technologies), Versican (V0/V1 neo) and β-actin (Sigma), cyclophilin B (Abcam). SiRNAs: non-targeting, si-cyclophilin B-Cy3, si- rat TLR2 (Thermo Scientific), si- rat p65 (Santa Cruz Biotechnologies). Promoter reporter plasmids: NF-κB-luciferase (triplet consensus NF-κB response elements upstream of a luciferase gene in a pGL3-basic vector (Promega) a generous gift from Dr. Tom McIntyre) and pSV-β-galactosidase (Promega) \textsuperscript{168}.
**Cell Culture**: Primary aortic SMCs were isolated from Sprague-Dawley rats as previously described\(^{149}\). SMCs were used for experiments between passages 8 and 15. Preparation of SMC conditioned medium: Rat SMCs were plated at 7*10^6 cells/150 mm dish in MEM media supplemented with 10% fetal bovine serum (FBS) and 1x penicillin/streptomycin (20 IU/ml penicillin, 20ug/ml streptomycin). Twenty four hours after plating, cells were serum restricted in 0.1% serum media (reduced serum media; SRM) for 24 hours, then new SRM was refreshed and allowed to condition for a further 24 hours. This conditioned medium was cleared by centrifugation at 500g for 10min, and stored at -80°C until use. Primary bone marrow-derived Mϕ were isolated from Sprague-Dawley rats, as previously described\(^{137}\). Whole bone marrow was frozen as described\(^{154}\), and thawed for each experiment without passaging in the presence of 20ng/ml M-CSF (eBioscience).

**In-Direct Coculture**: Bone marrow-derived macrophages were matured under 20ng/ml M-CSF for seven days. While Mϕ were being matured in transwell inserts, SMCs were plated at 2.5*10^5 cells per 35mm dish (6-well culture clusters). Twenty four hours after plating SMCs they were washed and media replaced with SRM for 24hrs to growth restrict the cells. At day 7 of Mϕ maturation, Mϕ-containing transwells were added to culture clusters containing serum-restricted SMCs for 24 to 48hrs. Mϕ and SMC lysates were collected at 24hrs for mRNA analysis, and 48hrs for protein analysis. Pre-conditioned media isolation: following 24hr coculture, transwells were removed to new culture dishes and fresh SRM added to pre-cocultured (pre-conditioned) Mϕs and SMCs.
After an additional 24hrs pre-conditioned Mϕ and SMC conditioned medium (Mϕ* and SMC*, respectively) was collected and prepared for future use as described.

**Real Time qPCR**: cDNA was prepared from total RNA isolates (1ug/rxn) using the iScript cDNA Synthesis kit from Bio-Rad. Real time qPCR then performed using the Power Sybr Green supermix from Applied Biosystems as shown previously147-149. Reactions were carried out on an iCycler (Bio-Rad). Cycling parameters were: 5min at 95°C; 15sec at 95°C, 30sec at the annealing temperature optimized for each primer pair, and 30sec at 72°C for 50 cycles. β-Actin was used as a reference gene.

**qPCR primers**: See Table 4.

**Table 4: Appendix A qPCR primers.**

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<tr>
<th>primer</th>
<th>Forward</th>
<th>Reverse</th>
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<td>aat gcc gtt ttc aat ggg g</td>
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<tr>
<td>TNF-α</td>
<td>ctc ttc tgt cta cta ctc ctt ggg</td>
<td>gagaagatgatctgagtgtgaggg</td>
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<tr>
<td>IL-6</td>
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<td>aag gca tgt gtc aac aac atc</td>
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<tr>
<td>IL-10</td>
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<td>ctt gat ttc tgg gcc atg gtt ctc</td>
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<tr>
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<td>Gene/Pseudogene</td>
<td>Forward</td>
<td>Reverse</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>MCP-1 (CCL2)</td>
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<td><code>tac agc ttc ttt ggg aca cct get</code></td>
</tr>
<tr>
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<td><code>att gta tag tgt tgt cag aag cc</code></td>
</tr>
<tr>
<td>SDF1 (CCL12)</td>
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<td><code>tgt tgt tgc ttt tca gcc ttg c</code></td>
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<tr>
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</tr>
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<td><code>tgc acc atc tgt cac tct gtt aac ctc</code></td>
</tr>
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**Western Blotting:** Whole cell lysates were prepared using protein lysis buffer (50mM β-glycerophosphate [pH 7.2], 100μM sodium vanadate, 2mM magnesium chloride, 1mM EGTA, 0.5% Triton X-100, 1mM DTT, and protease inhibitor cocktail [1:100 Sigma, P8340]) and cleared by centrifugation. Samples were normalized by protein concentration and SDS-PAGE was performed as described previously\textsuperscript{155}. One volume of conditioned medium was concentrated by addition of 1/6\textsuperscript{th} volume each trichloroacetic acid and deoxycholic acid, incubating over night at 4°C, and centrifuging at 10,000g for 10 minutes at 4°C. Precipitated protein pellets were resuspended in 30µl 1x Laemmli Buffer and neutralized by addition of 3µl 2mM Tris-base prior to electrophoresis. Electrophoresed proteins were transferred to polyvinylidene difluoride membranes, and blocked with 5% bovine serum albumin or non-fat dry milk in tris-buffered saline. Membranes were probed with primary antibodies, and subsequently with horseradish peroxidase or alkaline phosphatase-conjugated secondary antibodies. Antibody binding was visualized with either enhanced chemiluminescence substrate (Perkin Elmer) or Lumiphos (Thermo Scientific), respectively\textsuperscript{148}.

**Immunofluorescent Staining:** Bone marrow derived macrophages were matured on glass chamber slides for 7 days prior to stimulation with SMC CM for 0, 10, 30 or 60 minutes. Cells were fixed in 4% PFA, permeabilized with MeOH, and incubated with anti-p65 primary antibody overnight at 4°C. P65 antigen:antibody complexes were visualized using Alexa Fluor-488 conjugated secondary antibodies (Life Technologies). Coverslips were mounted with VectaShield medium (Vector Laboratories) containing
DAPI to detect all cell nuclei and cells imaged using an Olympus fluorescent microscope (TE200-S). Images were analyzed using Metamorph software[149].

**Transient Transfection of Primary Mφ with siRNA:** Was performed as described elsewhere[198], with several modifications. Frozen bone marrow cells were thawed and plated as described under Cell Culture at 2x10^6 nucleated cells per 35mm culture dish. At day 4 of *in vitro* maturation, cells were transfected with siRNA: 50nM siRNA oligonucleotide, resuspended to 20μM in siRNA buffer (Thermo Scientific), was added to 20μM OptiMEM media (Gibco). Six μL of Lipofectamine 2000 (Life Technologies) was added per 200μL of suspended siRNA. Liposomes were allowed to incubate for 20 minutes at room temperature. Day 4 Mφ were washed 2x with HBSS in order to remove penicillin/streptomycin (pen/strep). Liposomes were resuspended to a final volume of 2ml with pen/strep-free DMEM medium with 10% FBS and 20ng/ml M-CSF. Mφ were transfected with siRNA-loaded liposomes for 24hrs. After 24hrs (day 5), liposomes were removed and media was replaced with liposome-free pen/strep-free DMEM with 10% FBS and 20ng/ml M-CSF. Mφ were used for experiments 72hrs after transfection, or day 7 after initial plating.

**NF-κB Response Element Luciferase Assay:** Promoter assays were performed as described by Kaplan-Albuquerque et. al[168]. Briefly, day 6 Mφ were transfected with 4μg CMV β-galactosidase plasmid and 4μg NF-κB-consensus sequence luciferase reporter plasmid as described under Transient Transfection of Primary Mφ with siRNA, but using Lipofectamine (Life Technologies) as a transfection reagent, for 4hrs. After 4hrs
liposome media was removed and replaced with control medium or experimental SMC conditioned medium. After 24 hours, MΦs were lysed in reporter lysis buffer (Promega), and assayed for luciferase activity upon addition of luciferin substrate (Promega) using a luminometer (BMG Labtech). β-galactosidase activity was assayed as described previously\textsuperscript{199, 200}.

**SMC Proliferation Assay:** SMC proliferation was assessed as previously described\textsuperscript{137, 147} in response to stimulation with MΦ (non-targeting or si-TLR2 transfected MΦ, with or without prior coculture with SMCs) conditioned medium. Briefly, SMCs were plated in 24-well plates at a density of 3*10^4 cells/well, allowed to attach and spread overnight, and media replaced with 500μl/well SRM. After 48 hours of serum restriction SMCs were stimulated with 20% control medium, conditioned medium from naïve si-NT or si- TLR2 transfected macrophages (si-NT or si- TLR2 MΦ CM), or conditioned medium from si-NT or si- TLR2 transfected SMC pre-conditioned macrophages (si-NT or si- TLR2 MΦ* CM). 20μM BrdU was added to SMC cultures after 24 hours of CM stimulation. After 48 hours of stimulation, SMC were fixed with 4% paraformaldehyde, and immunohistochemically stained for BrdU incorporation. BrdU index was calculated as the percent of BrdU-positive nuclei.

**Real Time PCR Assay for Mycoplasma Contamination:** Previously isolated cDNA samples from primary SMC and MΦ cultures were assessed for the presence of mycoplasma by performing qPCR reactions with primers specific for mycoplasma 16s rRNA as described elsewhere\textsuperscript{201}. Briefly, 0.5μl of cDNA isolated SMCs or MΦs
following coculture experiments (from 20μ iScript reaction) were added to PCR reaction mixtures containing: 1x PCR buffer (Life Technologies), 10mM dNTP mix (Life Technologies), 50mM MgCl2, 10μg/ml BSA, 500pmol of sense primer (GPO-1), 500pmol of antisense primer (MGSO), and 1U of Taq DNA polymerase (Life Technologies). Cycling parameters: 40 cycles of 94°C for 1min, 55°C for 1min, 72°C for 2min.

Statistics: Data are expressed as means ± standard error. Student’s T-tests were used to compare data sets with two samples. Data sets with more than two samples were analyzed by ANOVA. Bonferroni multiple comparisons tests were used to compare select samples within a data set. In instances, within a data set with more than two samples, when variances were different between groups (as determined by T-Test) serial T-tests were performed followed by Bonferroni correction to account for multiple comparisons.

Results

SMC-Mϕ Coculture Results in Reciprocal Activation of Both Cells. We hypothesized that upon vascular injury, infiltrating monocytes/macrophages interact with resident vascular smooth muscle cells in the damaged artery and these cells cross talk to modulate one another’s phenotype and function. We initially set out to test this hypothesis in vitro by coculturing rat bone marrow-derived macrophages (day 7-matured) with rat SMCs. We found that when we cocultured these two cells together for 24 hours we saw robust activation of both cell types. Cocultured SMCs transcriptionally upregulated several pro-inflammatory cytokines: SDF-1α, MCP-1, Cxcl1 (KC), Cx3cl1 (fractalkine), and IL-6 (Figure 26A). Correspondingly, cocultured SMCs transcriptionally repressed the SM-
genes SM-αActin, SM-22α, and Calponin. SM-myosin heavy chain was not significantly repressed, but the error in these data was extremely large, perhaps suggesting a technical problem in the qPCR method for assessing expression of this gene. We also observed increased expression of iNOS in these SMCs, which has been shown to occur in SMCs in response to stimulation with IL-1β\(^{202}\). We also observed increase phosphorylation of the pro-proliferative kinases AKT and ERK (Figure 26B). Further, we observed increased...
expression, by both qPCR as well as by western blotting of TCA/DOC concentrated conditioned medium, of the pro-stenotic matrix proteoglycan versican (Figure 26C-D). This molecule was previously shown, by the Weiser-Evans lab, to be upregulated following vascular injury in the rat\textsuperscript{203}, and has been suggested to be a macrophage activating ligand that functions by serving as an endogenous ligand for TLR2\textsuperscript{152}.

This activation was, as stated previously, bi-directional. Following coculture with SMCs, Mφ became activated as well. Transcriptionally, Mφ cocultured with SMCs upregulated pro-inflammatory genes such as TNF-α, IL-1β, and IL-6. They also modulated expression of IL-10, IL-12a, iNOS, and Arg I in a classically activated/M1 fashion: increased iNOS, IL-10, and Arg I, with repression of IL-12a (Figure 27A). The ratio of IL-12a: IL-10, a metric used to characterize M1/M2 polarization in mouse macrophages\textsuperscript{74}, decreased significantly following coculture (Figure 27B). In the mouse an LPS/IFN-γ-induced M1 phenotype is characterized as increased expression of iNOS and IL-12a, with repression of IL-10 and Arg I. In this system M1 macrophages express a higher IL-12:IL-10 ratio compared to M0 macrophages\textsuperscript{141}. However, we observed that in rat bone marrow-derived macrophages, LPS/IFN-γ stimulation resulted in regulation of these genes as described in our coculture experiments (Figure 27D-E) rather than the pattern of regulation observed in mouse BMDMs. In addition to transcriptional changes, we observed induction of iNOS and phosphorylation of AKT and ERK in Mφ in response to SMC coculture (Figure 27C).

These data suggested that bi-directional cross talk between these two cell types, mediated by soluble factors, was capable of inducing robust pro-inflammatory activation
of both cells. We therefore wanted to determine which cell type was basally secreting a factor, or factors, that might be able to initiate this bi-directional cross talk.

**Reciprocal Activation of Mφ and SMC in Coculture is Dependent on an SMC-Derived Factor(s).** In order to identify which cell type was responsible for basally producing a
factor to initiate the intercellular cross talk observed in indirect coculture, we prepared conditioned medium from both cell types prior to coculture (Mϕ or SMC), or from the cocultured cells following separation (Mϕ* or SMC*). We stimulated new cultures of Mϕ or SMC with those conditioned media for 24-48hrs. We found that induction of SDF-1α, MCP-1 and IL-6 in SMCs was not induced by conditioned medium from Mϕ, but these genes were induced by Mϕ* CM (Figure 28A). Similarly, SM-αActin, SM-22α, and calponin expression was not affected in response to Mϕ conditioned medium but these contractile apparatus genes were repressed in response to Mϕ* conditioned medium (Figure 28B). We then compared signaling pathway engagement in SMCs in response to Mϕ and Mϕ* conditioned media (CM) stimulation. We found that STAT3 activation and IκBα degradation were markedly increased in SMCs in response to Mϕ* CM as compared to Mϕ CM. Similarly we observed slight increases in AKT and ERK activation in response to Mϕ* CM as compared to Mϕ CM (Figure 28C). These data indicate that production of SMC activating factors by Mϕ is dependent on the macrophages first having been exposed to SMCs.

We performed the reciprocal experiments on macrophages stimulated with conditioned medium from SMCs or SMC*s. We found that SMC and SMC* conditioned medium induced a similar degree of pro-inflammatory gene upregulation in Mϕ (Figure 29A). Further, SMC conditioned medium repressed the IL12:IL-10 ratio with similar efficiency as did conditioned medium from SMC* (Figure 29B). Since SMC conditioned medium was capable of inducing the classically NF-κB-regulated genes TNF-α, IL-6, and IL-1β in Mϕ, we sought to determine if modulation of these genes correlated with NF-κB activation in response to stimulation with SMC conditioned medium. We assessed IκBα
Figure 28: SMC-preconditioned macrophage (Mϕ*) CM stimulates SMC activation, while naïve Mϕ CM does not. Rat BMC were matured for 7 days under 20ng/ml M-CSF and then cultured alone (Mϕ) or cocultured in-directly with serum restricted SMC for 24 hours (Mϕ*). Cocultured cells were then separated and new low serum medium (SRM) was added to cells and conditioned medium was collected 24 hours later. SMCs were then stimulated for 24 hrs, or 5-60 minutes, with control SRM, Mϕ CM, or Mϕ* CM. A) Total RNA was isolated from SMCs and analyzed by qPCR for the indicated mRNAs. Shown are mRNA copy number normalized to β-Actin. B) SMCs were stimulated from 5-60 minutes with Mϕ CM or Mϕ* CM. Whole cell lysates were then analyzed for phospho-STAT3 (pSTAT3), phospho-AKT (pAKT), IκBα, and phospho-p65 (p-p65). β-Actin was used as a loading control. Shown are means±SE. n=3 independent experiments. *P<0.05 versus SRM; **P<0.05 versus Mϕ CM.
Figure 29: Naïve SMC CM stimulates Mφ activation; naïve SMC CM activates NF-κB in Mφ.
A) Serum restricted SMC were cultured alone (SMC) or in in-direct coculture with Mφ for 24 hours (SMC*). Cocultured cells were then separated and new low serum medium (SRM) was added to cells and conditioned medium was collected 24hours later. Mφ were then stimulated for 24hrs, or 5-40 minutes, with control SRM, SMC CM, or SMC* CM. A) Total RNA was isolated from Mφ and analyzed by qPCR for the indicated mRNAs. Shown are mRNA copy number normalized to β-Actin. B) Mφ were stimulated from 5-40 minutes with SMC CM or SMC* CM. Whole cell lysates were then analyzed for IκBα. β-Actin was used as a loading control. Shown are means±SE. n=3 independent experiments. *P<0.05 versus SRM; **P<0.05 versus SMC CM. D) Mφ matured for 7 days on glass chamber slides with 20ng/ml M-CSF were stimulated with naive SMC CM for 10min (10min). Cells were stained by in-direct immunofluorescence for p65. E) Mφ were transfected with β-galactosidase (β-gal) and NF-κB response element-luciferase plasmids and stimulated for 72hrs with SRM, SRM with 20ng/ml M-CSF, or naïve SMC CM. Shown are luciferase units normalized to β-galactosidase activity. N=1.
degradation, by western blot, in response to control medium or SMC conditioned medium. We found that SMC conditioned medium induced IkBα degradation in Mϕ at 10-40min following stimulation, whereas control medium did not (Figure 29C). We also assessed p65 nuclear localization by immunofluorescent staining and NF-κB transcriptional activity, by luciferase assay, in response to SMC conditioned medium. We found that SMC conditioned medium, as compared to control, induced p65 nuclear translocation within 10 minutes (Figure 29D), and it also increase NF-κB-dependent transcription (Figure 29E). This suggested to us that an SMC-derived factor was present in naïve SMC conditioned medium that was responsible for inducing NF-κB activation in Mϕ, which correlated with activation as assessed by pro-inflammatory cytokine upregulation. We assessed if NF-κB signaling in Mϕ was required for the phenotypic modulation observed in response to SMC conditioned medium, and if TLR2 activation was responsible for said NF-κB activation.

NF-κB Activation and Cytokine Upregulation in Response to SMC conditioned Medium is TLR2-Dependent. Kim et. al published in 2009 that soluble versican, secreted by lung carcinoma cells, was capable of inducing a Mϕ phenotype characterized by TNF-α, IL-1β, and IL-6, that was dependent on TLR2 expression in Mϕ\textsuperscript{152}. Because our SMCs produced versican at baseline (Figure 26D), and because we saw the same Mϕ phenotype in response to SMC conditioned medium that Kim et. al. did, and NF-κB activation, we set out to determine if TLR2 signaling was required for the phenotype induced by SMC conditioned medium.
Figure 30: Transient transfection of Mϕ with siRNAs.
Primary rat Mϕ were transfected with siRNAs (non-targeting (NT), Cy3-conjugated anti-Cyclophilin B (CypB), anti-p65 (sip65), or anti-TLR2 (si-TLR2)) as described in the Materials and Methods section of this chapter. 

A) Phase-contrast micrographs, with fluorescence overlay, of macrophages transfected with non-targeting or fluorescently labeled Cyclophilin B siRNA. 

B) Whole cell lysates from NT or si-CypB transfected macrophages were assessed for CypB protein by western blotting 96hrs after transfection. 

C) Whole cell lysates from NT or si-p65 transfected macrophages were assessed for p65 protein by western blotting 72hrs and 96hrs after transfection. 

D) 48hrs after transfection of macrophages with NT or TLR2 siRNA total RNA was isolated and assessed for TLR2 mRNA. Shown is mRNA copy number, normalized to β-Actin, relative to NT control. Shown are means±SE. n=2.
In order to perform these assays, we needed to establish a method to deplete Mφ selectively for TLR2. After optimizing modifications of a previously published method\textsuperscript{198}, we were able to show robust uptake of Cy-3-labeled siRNA (anti-cyclophilin B) into primary bone marrow-derived Mφ 24 hours after transfection (Figure 30A), and corresponding depletion of cyclophilin B at 96 hours after transfection (Figure 30B). Using this method we were also able to efficiently deplete p65 or TRL2 in rat Mφ (Figure 30C-D).

We then assessed the ability of SMC conditioned medium to induce NF-κB activation in Mφ in the presence or absence of TLR2 expression. We found that TLR2 depleted Mφ no longer exhibited p65 nuclear import in response to SMC conditioned medium (Figure 31A). Neither did TLR2 depleted Mφ exhibit IκBα degradation in response to SMC conditioned medium (Figure 31B). We observed a slight reduction in NF-κB transcriptional activity, by promoter-luciferase assay, in TLR2 knockdown Mφ as compared to a complete inhibition in p65 depleted Mφ (Figure 31C). We also assessed the effect of TLR2 depletion on downstream cytokine induction. We found that in response to SMC conditioned medium, TRL2 depletion blunted TNF-α, IL-6, and IL-12 upregulation. IL-1β upregulation in response to conditioned medium was not reduced (Figure 31D); upregulation of IL-1β in response to SMC conditioned medium could be due to a different factor that does not signaling through TLR2 and is therefore not effected by TLR2 depletion. Taken together, these data suggested to us that TLR2-dependent signaling was responsible for NF-κB activation and cytokine transcriptional upregulation observed in Mφ in response to SMC conditioned medium. We therefore
assessed if SMC pre-conditioned, TLR2-depleted, Mϕ conditioned medium would lose the ability to induce SMC activation.

**Ability of SMC-Preconditioned Mϕ (Mϕ*) to Activate SMCs is TLR2-Dependent.** In order to determine if TLR2-depleted Mϕ cannot be phenotypcially modulated by SMCs...
in coculture, and subsequently secrete SMC-activating factors, we cocultured non-targeting siRNA-transfected Mφ (si-NT) and TRL2-depleted Mφ (si-TLR2) with SMCs. We then removed the SMCs from the macrophages and collected conditioned medium from these pre-conditioned Mφ*. We also collected conditioned medium from si-NT and si-TLR2 macrophages that had not been cocultured with SMCs. When we stimulated SMCs with conditioned medium from naïve si-NT or si-TRL2 macrophages for 24 hours we did not see much modulation of SDF-1α, MCP-1, or SM-α Actin gene modulation. We did however, see increased SDF-1α and MCP-1, and decreased SM-α Actin expression in response to si-NT Mφ* conditioned medium. This effect was lost in response to si-TLR2 Mφ* conditioned medium; and SM-αActin expression increased (Figure 32A). We also assessed the effect of these media on SMC proliferation. We found that 48 hour stimulation with si-NT Mφ* conditioned medium resulted in a 40% increase in SMC proliferation compared to conditioned medium from si-NT macrophages that had not been exposed to SMCs in coculture, as assessed by BrdU incorporation. Whereas si-TLR2 Mφ* conditioned medium did not induce an increase in proliferation compared to si-TLR2 macrophages that had not been exposed to SMCs (Figure 32B). These data suggested that a significant component of the ability of SMC-preconditioned macrophages to cross talk back to naïve SMCs was due to TLR2-dependent activation of macrophages by an SMC-derived factor. We then set out to assess what SMC-derived factor was acting as an endogenous ligand for TLR2 capable of activating NF-κB signaling, downstream cytokine induction, and pro-stenotic SMC cross talk.
The Mϕ-Activating Factor Present in SMC Cultures is Not SMC-Derived. Due to the results published by Kim et. al\textsuperscript{152} suggesting that versican can act as an endogenous ligand of TLR2, we hypothesized that SMC-derived versican was responsible for the macrophage activation that we observed. We were beginning to develop methods to deplete versican in SMCs, when we ceased to be able to replicate SMC conditioned medium-dependent NF-κB activation in macrophages. While trying to replicate this data with isolation after isolation of primary SMCs, we searched the literature for other ideas.
about candidate molecules that might act as endogenous activators of toll-like receptors.

In conducting this literature search, it became clear that the concept of existence of endogenous ligands for TLRs is a controversial one. For example, some investigators feel that none of the studies presented in the literature sufficiently control for the presence (whether detectable by limulus amebocyte lysate assay or not due to association with LPS-binding proteins) of LPS in culture reagents\(^{204, 205}\). Other investigators feel comfortable accepting the evidence that both damage-associated molecular patterns and other endogenous TLR ligands do exist and are critical regulators of innate immunity\(^{206}\).
We hypothesized that the simplest explanation is likely the correct one. We therefore sought to determine if the SMC-derived factor that was capable of inducing such robust activation in our macrophages could be a contaminant.

Because we were no longer able to generate conditioned medium that activated NF-κB in Mϕ we felt that it was unlikely that any potential contaminant was present in live cultures, but that it had be present in earlier cultures. Cell wall components, such as would be present in cultures contaminated with Gram-negative bacteria, such as LPS, would activate NF-κB in a TLR4 rather than TLR2-dependent manner. However, mycoplasma lipoproteins are known activators of TLR2 signaling\textsuperscript{204}. We therefore employed a previously published method for assessing the presence of mycoplasmal 16s rRNA in cDNAs prepared from cocultured SMCs and macrophages\textsuperscript{201}. Using this method we discovered that, like no template control, SMCs whose conditioned medium was not capable of inducing NF-κB activation in Mϕ (Un-contaminated SMC) and pre-coculture macrophages (Suspect Mϕ) did not amplify positive for mycoplasma contamination. However, SMCs from the coculture experiments described in Figure 26A-D did amplify positive for mycoplasmal contamination. Additionally, macrophages from the same experiments, while not contaminated initially, became contaminated in response to coculture with contaminated SMCs (Mϕ after CC+ SMC) (Figure 33). This indicated to us that the ability of SMCs, in these early experiments, to induce robust macrophage activation was the result of mycoplasma-derived pathogen-associated molecular patterns activating TLR2 on the cultured macrophages. This discovery, unfortunately meant that the data described in this appendix was unsuitable for
publication as it did not address our research objective which was to understand the molecular cross talk the occurs between macrophages and SMCs.

Discussion

The data presented here, in Figures 26-33, were initially quite promising. Coculture, or conditioned media stimulation, of macrophages with SMC resulted in a classically activated/M1 phenotype that, as described in Chapter I, would be predicted to be pro-stenotic in the setting of vascular injury. We also observed that upon pre-conditioning with SMCs, macrophages gained the ability to induce a pro-inflammatory, dedifferentiated phenotype, and increased proliferation, in cross talk studies with SMCs. We determined that the effect of the SMC-derived factor(s) on macrophage phenotypic modulation, and subsequent cross talk with SMCs, was dependent on macrophage TLR2. All that remained, we hoped at the time, was to validate that soluble versican was the SMC-derived factor influencing macrophage phenotype, and to validate that IL-6 was the Mϕ*-derived factor responsible for Mϕ-to SMC cross talk via STAT3 signaling.

Unfortunately, the discovery that the factor responsible for modulating macrophage phenotype was mycoplamsa, or mycoplasma-derived, meant that we had not made any progress after all in addressing the hypothesis that SMCs and macrophages cross talk to modulate one another’s phenotype in the pathogenesis of neointimal hyperplasia. Many groups, due to observations from the atherosclerosis literature that lesional macrophages may express an M1-like phenotype, have used LPS/IFN-γ stimulation to model lesional macrophages. We feel that using what amounts to a model of bacterial infection-induced macrophage phenotype is not relevant to studying disease
processes that develop in a largely sterile setting. We feel that modeling macrophage phenotype with LPS is of particularly little utility if we want to understand the – mostly likely fairly subtle - effects of a smooth muscle cell on macrophage phenotype.

We feel that the data presented in the main chapters of this dissertation, in the absence of potent macrophage polarizing agents, may be more physiologically relevant to sterile inflammation-driven disease than pre-polarizing macrophages with LPS (advertent or inadvertently) prior to performing cross talk assays. We hope that any future students who take on similar projects in the lab can learn from this information to preemptively identify any such confounders in their reagents or cells. This data illustrates how attaining statistical significance does not necessarily prove physiologic relevance of an *in vitro* assay.
APPENDIX B

CCTSI TL1 PRE-DOCTORAL PROGRAM CLINICAL EXPERIENCE

In the second year of my graduate training I was accepted into the Colorado Clinical and Translational Sciences Institute (CCTSI) TL1 pre-doctoral training program. This training program entailed a seminar series designed to inform the pre-doctoral trainees about the logistics and ethical considerations of designing clinical trials, as well as about considerations relevant to working with patients from cultural backgrounds different from ourselves. The program also involved monthly meetings where trainees had the opportunity to learn about, and discuss, current and cutting-edge clinical-translational research. As part of the certificate program, I completed electives in immunology and epidemiology. The program also included “summer reading,” and book club-like discussions, of non-fiction or fictional works relevant to clinical-translational science. The books read during my participation in the program included *The Immortal Life of Henrietta Lacks*\(^{207}\), *Who Goes First?: The Story of Self-Experimentation in Medicine*\(^{208}\), and *Intuition*\(^{209}\). I also participated in the national TL1 meeting, held in St. Louis, MO, in 2011. I presented a poster at this meeting that described the then-current progress on my thesis research project. During my first year as a pre-doctoral fellow in the program, I received funding to participate in a clinical experience in addition to my thesis research.

My clinical experience for the TL1 program focused on the link between endothelial dysfunction and cardiovascular disease, and what patients with diabetes, a
condition where endothelial dysfunction is common, go through in order to manage their disease. I spent several clinical sessions shadowing my clinical mentor Dr. Jane Reusch, at the Veteran’s Affairs Hospital in Denver. Over the course of these clinics I observed Dr. Reusch discuss with her patients how best to regulate their blood sugar, in order to minimize damage to the peripheral vasculature and nerves.

The vast majority of patients I observed in these clinics presented with type 1 diabetes (reviewed in Melmed, 2011[^10]). This form of diabetes is characterized by autoimmune destruction of pancreatic beta cells that normal produce insulin, the hormone responsible for inducing uptake of glucose from the blood by skeletal muscle and adipose tissue. These patients respond to insulin, but as they cannot produce it, they must regulate their blood sugar through administration of exogenous insulin. If not sufficiently regulated, elevated blood glucose results in an inability of the vascular endothelium to generate nitric oxide. This, in turn, results in negative remodeling of the peripheral vasculature due to prolonged SMC contraction, and decreased blood flow. Decreased blood flow can result in damage to peripheral nerves leading to symptoms such as gastroparesis, or decreased movement of food through the digestive tract and associated discomfort. Decreased blood flow can also result in an angiogenic response in the retina, or proliferative retinopathy, which can impair visual acuity. Amputation of under-perfused extremities is another complication typically associated with diabetes, due to infection of wounds that go unnoticed because of damage to peripheral nerves.

In order to sufficiently regulate blood sugar, the patients I observed were required to take extensive notes on diet, exercise, and insulin administration. Keeping these notes allowed them, and Dr. Reusch, to identify when and how they were under or over-dosing
their insulin. These visits really impressed upon me how incredibly difficult and time consuming it is for these patients to accurately calculate the required dose of insulin relative to what they are consuming and how active they are. I would have thought, before participating in this clinical experience, that due to consequences as serious as amputation and blindness, that most patients would be extremely diligent about their treatment. It became clear to me, however, that managing diabetes while meeting other everyday responsibilities is nearly impossible for most people.

In addition to the many type I diabetics that I observed in Dr. Reusch’s clinic, I also observed a few type 2 diabetic patients. In type 2 diabetes (reviewed in Melmed, 2011) the pancreatic beta cells often produce a lower than normal amount of insulin, and the skeletal muscle and adipose tissue are resistant to the action of insulin. While the type 1 diabetic patients were often very rather thin, the type 2 patients were frequently significantly overweight. Obesity is a prominent risk factor for type 2 diabetes, in addition to consumption of large amounts of sugar, often from soft drinks. One of the major therapies prescribed to these patients, by Dr. Reusch, was physical activity. Patient compliance with respect to this treatment was consistently poor.

It was this observation that lead Dr. Reusch, and her colleagues Dr. Irene Schauer and Dr. Judith Regensteiner, to design a study to try to understand if elevated free fatty acids in the blood was sufficient to induce cardiac dysfunction and impaired exercise capacity in healthy subjects. Elevation of free fatty acid in the blood was used as a stimulus because it is known to induce insulin resistance. Patients with type 2 diabetes exhibit decreased oxygen consumption and oxygen uptake when they begin exercise. The principle investigators were interested to determine if free fatty acid infusion
would repress the ability of endothelial cells to induce vasodilation, making diabetic patients more uncomfortable during physical activity than healthy controls, thus possibly helping to explain why these patients have such difficulty complying with prescribed physical activity. Endothelial function was assessed non-invasively by brachial artery diameter (BAD) measurement\textsuperscript{215,216}. Briefly, in this assay, endothelium-dependent flow-mediated vasodilation is assessed by high-resolution ultrasonography of the brachial artery upon release of a pneumatic tourniquet. Vessel diameter is measured using FMD Studio software (Quipu) 1 minute, and 10 minutes after tourniquet deflation. Flow-mediated dilation is calculated as the ratio of a baseline reading to the 1min post-dilation reading. I participated in several meetings between Dr. Schauer and ultrasound technicians at the Clinical Translational Research Center at the Anschutz Medical Campus. The object of these meetings was primarily to troubleshoot transfer of the ultrasound records from the ultrasound machine to a computer equipped with the FMD Studio software. The rest of my time spent on this project involved actual analysis of flow-mediated dilation data. Unfortunately, I was not able to spend enough time working with these data, while my clinical experience was funded, to make any conclusions about the effect of lipid infusion on endothelial function. To my knowledge these studies are ongoing. Observing this study, and gaining exposure to the process of developing a clinical research protocol, gave me an appreciation for the technically challenging and ethical aspects of performing research in the clinical setting.

The experience I gained by participating in the CCTSI TL1 pre-doctoral training program has given me a much better understanding of what is involved in translating basic pre-clinical research into a clinical setting. I have learned a great deal about how
necessary it is to construct appropriate collaborations in order to perform meaningful clinical studies. I also gained an appreciation for how very different working with a patient population is from working in pre-clinical models; and the epidemiology course that I took as a requirement for this program showed me the importance of accounting for confounders and attrition when working with patient data. I would highly recommend this experience to anyone who is interested in learning from clinical studies where basic science can make the largest contribution to understanding the pathogenesis of disease, and in translating basic discoveries into clinical practice.