

REGULATION OF VASCULAR CELLS - TLR LIGAND AND GP130  
CYTOKINES

THE REGULATION OF VASCULAR WALL CELLS BY A TLR LIGAND  
AND GP130 CYTOKINES

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## **ABSTRACT**

Atherosclerosis is a disease affecting the blood vessels that is inflammatory in nature, and plays an important role in cardiovascular disease (CVD), one of the leading causes of morbidity and mortality worldwide. Oncostatin M (OSM), a member of the IL-6/gp130 cytokine family, has been implicated in atherosclerosis both in mouse models and in humans. OSM synergizes with other stimuli in various systems to regulate cells. Infectious pathogens as well as danger associated host molecules stimulate members of the innate immune system, including Toll-like Receptors (TLRs), to respond in a pro-inflammatory manner to cause cell activation and cytokine release. Experiments were performed to determine whether OSM and LPS (a TLR-4 ligand) synergize in regulation of vascular wall cells *in vitro*.

Upon stimulation of Aortic Adventitial Fibroblasts from mice (MAAFs) and humans (HAoAFs) as well as Human Aortic Smooth Muscle Cells (HAoSMCs) with LPS in combination with OSM, it was determined that there was a synergistic increase in IL-6 and VEGF levels in the cell supernatants as measured by ELISA compared to either treatment alone. MAAF were also able to synergistically express KC upon stimulation with LPS and OSM, while in HAoAFs and HAoSMCs, LPS induced IL-8 levels were suppressed by OSM. These effects were unique to OSM among gp130 cytokine members, as treatment of these cells with LPS in combination with LIF, IL-6, IL-31, or IL-11 had no marked effects compared to LPS alone. Furthermore, MCP-1 steady state mRNA

levels were elevated 6 hours post stimulation with LPS and OSM compared to either treatment alone in HAoAFs and HAoSMCs.

While OSM did not appear to modulate TLR-4 expression, OSM treatment resulted in an increased phosphorylation signal in STAT-1,-3, and -5, as well as Akt in MAAF and HAoAFs. In addition, combined LPS and OSM stimulation resulted in an increased phosphorylation signal of the MAPK p38 compared to either treatment alone. Furthermore, a neutralizing antibody to the OSMr- $\beta$  was able to inhibit HAoAF IL-6 responses to PBMC conditioned medium. Together, these findings indicate that OSM and LPS can synergize *in vitro* to induce the expression of inflammatory factors in vascular wall cells, emphasizing the potential role of OSM, TLR-4 ligands, and adventitial fibroblasts in vascular inflammation.

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**LIST OF ABBREVIATIONS**

A2AR: Adenosine A2A receptor	Elk-1: E twenty-six (ETS)-like transcription factor 1
ADAM: A disintegrin and metalloprotease	ENA-78: Epithelial neutrophil activating peptide
Ang II: Angiotensin II	ERK: Extracellular signal-regulated kinase
AP-1: Activator protein-1	FBS: Fetal bovine serum
ApoE: Apolipoprotein E	Gab1: Grb2-associating binder 1
bFGF: Basic fibroblastic growth factor	GM-CSF: Granulocyte-macrophage colony stimulating factor
CBP: CREB binding protein	GPL: gp130-like chain
CD: Cluster of differentiation	Grb2: Growth Factor Receptor-Bound 2
CLC: Cardiotrophin-like cytokine	GRO: Growth related cytokine
CM: Conditioned medium	HAoAF: Human aortic adventitial fibroblast
CNTF: Ciliary neurotrophic factor	HAoSMC: Human aortic SMC
COX-2: Cyclooxygenase-2	HDAC: Histone deacetylase
CREB: cAMP response element-binding	HIF: Hypoxia inducible factor-1
CT-1: Cardiotrophin-1	HRE: Hypoxia response element
CVD: Cardiovascular disease	HRP: Horse-radish peroxidase
DC: Dendritic cell	HSP60: Heat shock protein
DNA: Deoxyribonucleic acid	ICAM: Intra-cellular adhesion molecule
E2F: E2 factor	IFN: Interferon
eIF-4E: Eukaryotic translation initiation factor 4E	Ig: Immunoglobulin
ELISA: Enzyme Linked Immunosorbent Assay	

IKK: IκB kinase	MD-2: Myeloid differentiation protein-2
IκB: Inhibitory κB	MIP: Macrophage Inflammatory Protein
IL: Interleukin	MMP: Matrix metalloproteinase
IRAK: Interleukin-1-receptor-associated kinase	mmLDL: Minimally modified LDL
IRF: Interferon regulatory factor 3	MNK: MAPK-interacting kinases
JAK: Janus kinase	mRNA: Messenger RNA
JH: JAK homology	MSK: Mitogen- and stress-activated kinase
JNK: c-Jun N-terminal kinase	MyD88: Myeloid differentiation primary response gene 88
KC: Keratinocyte derived cytokine	NcoA/SRC1: Steroid receptor coactivator 1
kDa: Kilo Dalton	NF-κB: Nuclear factor κB
LBP: Lipopolysaccharide binding protein	NK: Natural killer
LDL: Low density lipoprotein	OSM: Oncostatin M
LIF: Leukemia inhibitory factor	PAMP: Pathogen associated molecular patterns
LPS: Lipopolysaccharide	PBMC: Peripheral blood mononuclear cell
MAAF: Mouse aortic adventitial fibroblast	PDGF: Platelet derived growth factor
MAPK: Mitogen Activated Protein Kinase	PI3K: phosphatidylinositol 3'-kinase
MAPKK: MAPK Kinase	Poly I:C: polyriboinosinic:polyribocytidylic acid
MAPKKK: MAPKK Kinase	PRR: Pattern recognition receptors
MCP-1: Monocyte chemotactic protein-1	PTB: Phospho-tyrosine-binding
M-CSF: Monocyte colony stimulating factor	RAAF: Rat Aortic Adventitial Fibroblast
MEF: Myocyte enhancer factor	
MEK: MAPK/ERK kinase	

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RSK: Ribosomal S6 kinases

SH2: Src Homology 2

Shc: Src homology and collagen

SHP-2: SH2 domain-containing protein tyrosine phosphatase-2

SMC: Smooth Muscle Cell

STAT: Signal transducers and activators of transcription

TAB: TAK-binding protein

TAK: Transforming growth factor- $\beta$ -activated kinase

TF: Tissue factor

TIR: Toll/Interleukin-1 receptor

TLR: Toll-like receptors

TNF: Tumor Necrosis Factor

TRAF3: TNF receptor-associated factor 3

TRIF: TIR-domain-containing adapter-inducing interferon- $\beta$

Tyk-2: Tyrosine kinase-2

VCAM: Vascular cell adhesion molecule

VEGF: Vascular endothelial growth factor

## **CHAPTER 1 – INTRODUCTION**

Atherosclerosis is a disease affecting the blood vessels that is inflammatory in nature [1,15,46], and involves both the acquired and the innate immune systems [2]. Atherosclerosis can begin in childhood [3], but typically does not present clinically until mid to late adulthood. It manifests as fatty, calcified, necrotic deposits that line the inside of arteries. Atherosclerosis is a major cause of cardiovascular diseases (CVD) [15,16], which include strokes and heart attacks [4]. CVD is one of the leading causes of morbidity [5] and mortality [6] worldwide. It is estimated that in 2008 the combined direct and indirect costs of CVD in the United States alone was 298 billion dollars, higher than any other disease [4], while in Canada the cost was 21 billion dollars [7]. In 2008, CVD was responsible for 811 940 deaths in the United States [4], and 69,500 deaths in Canada, accounting for 29 % of all deaths [7]. Furthermore, it is estimated that one half of Americans over the age of 45 have calcification (from atherosclerosis) in their coronary arteries [4]. Due to the prevalence of the disease, the burden on the healthcare system, and as a result of the mortality associated with this disease, the processes involved in the progression of atherosclerosis merit investigation. The current understanding of this disease will be discussed.

### **Arterial Anatomy**

An artery is composed of three layers, each separated by a thin layer of elastic lamina. Adjacent to the lumen of the artery lays the first arterial layer: the intima. The tunica intima is composed of a monolayer of endothelial cells, and is

in direct contact with the blood [8]. Endothelial cells have multiple physiological roles including acting as a protective barrier against insults to the arterial wall, releasing nitric oxide (a potent vasodilator) [9], releasing endothelin-1 [10] (a potent vasoconstrictor), and regulating extravasation of cells [10].

The tunica media, separated from the intima by the internal elastic lamina, contains smooth muscle cells (SMCs) that participate in vasoconstriction and vasodilation. The SMCs are organized in a lamellar structure, and interwoven between the cells is elastin-rich extracellular matrix [8]. In normal, healthy arteries, there is little change in the amount of extracellular matrix production or SMC proliferation in the tunica media [8].

Progressing further away from the lumen, the external elastic lamina separates the tunica media and the tunica adventitia. Mainly fibroblasts, as well as mast cells, collagen, and adipocytes reside in the adventitial layer [8,11]. Nerve endings and the vasa vasorum penetrate the adventitia [8], providing the artery with its own stimulation and blood supply respectively. Bone marrow derived cells such as dendritic cells and macrophages can also be found in all layers of the blood vessel wall, and patrol for cellular debris and noxious stimuli [12,13,14].

### **Atherosclerosis**

Atherosclerosis is the inflammatory process whereby fatty plaques develop in large or medium sized arteries [15,16]. Traditional risk factors include tobacco use [17,18], total cholesterol [19], hypertension [20], obesity [21],

physical inactivity [21], and diabetes [21]. The classical view of the atherosclerotic mechanism will be briefly outlined.

Endothelial damage is often initiated at sites of non-laminar and turbulent blood flow, especially at bifurcations in the blood vessel [22]. Endothelial damage results in altered endothelial function. Other causes of endothelial damage and/or dysfunction may include: free radicals caused by smoking [23,24], oxidized low density lipoproteins (LDL) [25], hypertension [26], and infectious microorganisms [27,8,28]. The adhesiveness of the endothelium is augmented as a result of activation of endothelial cells, caused by a variety of stimuli. Leukocytes, in particular monocytes [29], T cells [30], and neutrophils [271], are able to adhere due to an increase in expression of leukocyte adhesion molecules such as VCAM, ICAM, and P and E selectins on the endothelium at lesion sites [31,32].

Once adhered to the endothelium, chemokines such as MCP-1, and IL-8 attract leukocytes into the intima by binding to their receptors [33,34,35]. The cytokine M-CSF (Monocyte Colony Stimulating Factor) is known to play a role in the transformation of monocytes into macrophages once inside the intima [36]. M-CSF and GM-CSF also promote macrophage survival and proliferation [37] within the lesion. Vascular endothelial growth factor (VEGF) is released by macrophages and induces monocyte migration, the pro-coagulant molecule tissue factor (TF) release [38], and increases vascular permeability [39], thus increasing the ease of access for additional monocytes to enter the plaque. Macrophages within the plaque upregulate the number of scavenger receptors that they express



on their surface [40], with one stimulus for this upregulation being M-CSF [41]. Scavenger receptors on macrophages recognize and internalize oxidized LDL, causing the macrophages to develop into foam cells [42] (so named due to their foamy appearance under the microscope).

Eventually, certain cells within the plaque including some macrophages, SMCs, and neutrophils, undergo cell death, resulting in a lipid filled, necrotic, calcified core [43,44,45]. This inflammatory process stimulates proliferation of SMCs, which migrate and surround the lesion [41,15]. SMCs produce collagen, which forms a fibrous cap overlying the plaque, beneath which resides the necrotic and fatty tissue [15,46]. Should this fibrous cap be degraded (by factors such as MMPs released by activated macrophages) or abraded, then the prothrombotic elements within the plaque can come into contact with blood, causing aggregation of platelets and thrombus formation.

T cells may also have a role, infiltrating the intima by adhering to vascular adhesion molecules, and undergoing diapedesis upon recognizing chemoattractants [16]. T cells are present in atherosclerotic lesions during all of the stages of atherogenesis [15,41]. They may participate in plaque development and progression by releasing cytokines and stimulating macrophages to release MMPs, cytokines, and tissue factor [16].

### **Innate Immunity and TLR-4**

Pattern Recognition Receptors (PRRs) are important members of the innate immune system that recognize pathogen associated molecular patterns

(PAMPs) as well as alarmins (endogenous molecules that signal cell damage) [47]. Toll-like receptors (TLRs) are a group of PRRs that bind conserved bacterial, viral, fungal, and parasite-derived molecular patterns, as well as some endogenous molecules [48,49]. TLRs are type I transmembrane proteins, whose name was derived from the *Drosophila* protein with which the mammalian form shares sequence similarities. The extracellular portions of the receptors contain leucine rich repeats, while the intracellular components share homology with the IL-1 receptor [48]. TLRs are essential receptors of the innate immune system which recognize and defend against early infection [49].

In humans, 10 TLRs have been characterized [48], each binding specific ligands. The expression of these molecules appears to be relatively low as Visintin *et al.* measured only a few thousand TLRs being expressed on monocytes, and a few hundred being expressed on immature dendritic cells (DCs) [50]. Numerous cell types have been shown to express TLRs however, including airway and intestinal epithelial cells, adipocytes, cardiac myocytes, B cells, mast cells, NK cells, regulatory T cells, neutrophils, basophils and endothelial cells [48,49].

The TLR-4 signalling pathway is well characterized, and this receptor has been shown to bind various ligands. One TLR ligand is lipopolysaccharide (LPS) [51], an exterior cell membrane component of gram negative bacteria [47]. Two additional molecules are necessary for a sensitive response to LPS [48]. Before TLR-4 binds LPS, lipopolysaccharide binding protein (LBP), a lipid transferase, can bind LPS and catalyzes its transfer to CD14 [51]. CD14 is found both bound

to cell membranes of myeloid lineage cells and in its soluble form in circulation [52]. After LPS is transferred to CD14, it is then transported to the TLR-4/MD-2 receptor complex [53]. MD-2 was shown by Shimazu *et al.* [54] to be physically associated with the extracellular domain of TLR-4, and necessary for TLR-4 signalling to occur. Once LPS is bound to the TLR-4/MD-2 receptor complex, intracellular signalling pathways are activated that result in cytokine, IFN, and chemokine production. Upon binding LPS, TLR-4 molecules aggregate on the cell surface [55,56]. This allows for the interaction of the intracellular 'Toll/Interleukin-1 receptor' (TIR) domains, recruiting adaptor molecules to the receptor [55].

There are two signalling pathways downstream of TLR-4, each involving different adaptor molecules containing TIR domains [57]. The first is the MyD88 dependent pathway, and the second is the TRIF dependent pathway. There are several comprehensive reviews on intracellular TLR-4 signaling (See Brikos *et al.* [58], Newton *et al.* [59]). Briefly, upon activation, MyD88 interacts with the death domain of IRAK-4, a serine/threonine kinase [60]. This results in the recruitment of ubiquitin protein ligases, which in turn recruit the kinase TAK-1 [62]. TAK-1 initiates two pathways, the first by activating the IKK complex. The IKK complex phosphorylates I $\kappa$ B- $\alpha$ , mediating its degradation, thereby exposing NF- $\kappa$ B's nuclear localisation sequence [61]. The second pathway initiated by TAK-1 is the MAPK pathway. TAK1 has been shown to phosphorylate members of the

MAPKK family, which then phosphorylate the MAPKs ERK 1/2, JNK, and p38 [62].

The TRIF dependent pathway activates NF- $\kappa$ B in a delayed manner compared to the MyD88 pathway [63]. A key signaling molecule downstream of TRIF is TNF receptor-associated factor 3 (TRAF3), which upon binding TRIF can cause the activation of Interferon regulatory factor 3 (IRF3) [64]. The TRIF signaling pathway is necessary for TLR-4 induced IFN production [64]. TLR4 activation results in increased expression of numerous molecules implicated in inflammation, including IL-12, IL-6, MCP-1, IL-8 and TNF- $\alpha$  [59,232].

### **TLR-4 and Atherosclerosis**

TLR-4 has been implicated in atherogenesis through its production of pro-inflammatory cytokines, chemokines, and interferons. Xu *et al.* [65] performed a study demonstrating by immunohistochemistry that aortic tissue from ApoE<sup>-/-</sup> mice, but not control C57BL/6J mice, displayed TLR-4. This TLR-4 was expressed by plaque macrophages. Furthermore, on human atherosclerotic aortic tissue samples, the same phenomenon of TLR-4 expression by macrophages was observed [65]. In addition, TLR-4 mRNA expression by monocyte derived macrophages was increased *in vitro* in response to oxidized LDL [65]. As atherosclerotic plaques are laden with oxidized LDL [46], increased expression of TLR-4 provides a receptive environment for initiating and propagating an inflammatory response in the presence of an appropriate ligand. There is ample evidence that bacteria and viruses are found in atherosclerotic tissue

[27,8,66,28,28, 67, 68, 69,70], as are oxidized lipids [15], and thus could act as ligands to TLRs.

In another study, minimally modified LDL (mm-LDL) was shown to bind to CD14, resulting in TLR-4 mediated macrophage spreading and actin polymerization [71]. Macrophage spreading is known to result in increased oxidized LDL uptake by macrophages [71] and thus this study also connected TLR-4 function with a known part of the atherosclerotic process. Similarly, Walton *et al.* [72] demonstrated that IL-8 production (a chemokine involved in attracting neutrophils [73], and potentially monocytes [265]) was increased when mm-LDL was recognized by TLR-4 on aortic endothelial cells. Increased expression of both TLR-4 and TLR-2 was also seen on cultured human aortic endothelial cells in response to treatment with *P. Gingivalis* [74], a bacterium which has been implicated in atherosclerosis.

Besides being upregulated on macrophages and endothelial cells, TLR-4 is also expressed by vascular smooth muscle cells [75]. Yang *et al.* [75] completed a study involving TLR-4 receptors on vascular SMCs. They first demonstrated that human aortic SMCs produce mRNA for CD14, MD-2, and TLR-4, and that the TLR-4 mRNA levels were comparable to those produced by human monocytes. They then demonstrated that when human aortic SMCs were treated with LPS *in vitro*, there was significant IL-6 and MCP-1 production, and this production was further increased when soluble CD14 was included in the treatment [75]. Furthermore, treatment of murine aortic SMCs with LPS and

CD14 *in vitro* caused phosphorylation of ERK1/2, activation of NF- $\kappa$ B, production of MCP-1, and increased production of TLR-2 mRNA [75]. The authors concluded that vascular SMCs may participate in the inflammatory process involved in atherosclerosis by releasing chemokines and cytokines that are regulated by TLR-4 [75].

There are also several direct links between TLR-4 signalling and the development of atherosclerosis. Studies have demonstrated that certain human subjects, whose response to LPS is blunted as a result of a TLR-4 polymorphism where aspartic acid at position 299 is replaced by a glycine, present with decreased atherosclerosis [76,77]. Animal models have also been used to determine the effect of TLR-4 on atherosclerosis. Michelsen *et al.* [78] used hyperlipidemic, atherosclerotic prone ApoE knockout mice that were also deficient in either TLR-4 or MyD88 to monitor the effects of TLR-4 signalling on atherosclerosis *in vivo*. They discovered that lesion size was markedly decreased in both the TLR-4 and MyD88 null mice, in spite of elevated cholesterol levels [78]. Circulating levels of MCP-1 in the serum of MyD88 and TLR-4 null mice, and IL-12 detected in the serum of MyD88 null mice were significantly reduced compared to the control ApoE KO mice [78].

A similar study also demonstrated that atherosclerosis was decreased in ApoE and MyD88 null mice compared to ApoE null mice alone [79]. The authors demonstrated using a DNA microarray that the levels of leukocyte attracting chemokines macrophage chemotactic protein-1 (MCP-1), MCP-2, MCP-3, Gro-

1/KC, macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ), and MIP-1 $\gamma$  were much lower in MyD88 knockout mice than control mice [79]. These two studies indicate that TLR-4 and MyD88 signalling have a direct, pro-inflammatory role in the progression of atherosclerosis in mouse models.

### **Fibroblasts**

As the evidence above suggests, there is a growing body of knowledge that links innate immunity (specifically TLR-4) and atherosclerosis. The presence of TLR-4 on endothelial cells [72], macrophages [71], and vascular SMCs [75] has been well characterized. Fibroblasts that are located in the vascular adventitia are vascular wall cells that have historically been ignored, but are beginning to be recognized for their potential role in vascular inflammation [80].

There is evidence to suggest that fibroblasts in general are mediators in inflammatory disease progression. Fibroblast activation occurs in numerous chronic inflammatory conditions such as venous ulcers, granulomas, hypertrophic scarring, and fibrosis of the lungs and kidneys [81]. This activation results in chemo-attraction of leukocytes and an inflammatory environment [81]. One study correlated adventitial cellular infiltration (a sign of inflammation) with the degree of atherosclerosis in the artery [82]. A study by Scott *et al.* [83] was carried out on a porcine model to investigate lesion formation in coronary arteries after injury caused by balloon angioplasty. Three days after vascular injury, adventitial cells showed significant proliferation, while intimal and medial cells were dormant. The authors concluded that on day seven after injury, the cells that had

accumulated in the neointima were adventitial cells [83]. Furthermore, the vascular lesion formation was putatively enhanced by the PDGF (a chemoattractant and mitogen for SMCs and fibroblasts) that was released from the fibroblasts in the adventitia [83].

Adventitial fibroblasts and medial SMCs have also been shown to produce reactive oxygen species (ROS) in rabbit aortas [84]. This implicates fibroblasts in atherosclerosis as ROS play two well known roles in atherosclerosis. First, ROS can oxidize LDL [85], which can result in its uptake by macrophages [42], and second, ROS can damage endothelial cells [25], which can result in leukocyte adhesion molecule expression [32]. Chemokine and cytokine production has also been demonstrated in adventitial fibroblasts, further suggesting their role in the inflammation occurring in vascular plaques. Tieu *et al.* [86] demonstrated by immunofluorescence that IL-6 and MCP-1 were present in the aortic adventitia in close proximity to fibroblasts upon subcutaneous infusion of mice with Angiotensin II. This infusion resulted in adventitial growth, macrophage recruitment, and aortic dissections, all of which were diminished in IL-6 null or CCR2 (the receptor for MCP-1) null mice [86].

*In vitro*, human aortic adventitial fibroblasts were co-cultured with either monocytes from a human leukemia monocytic cell line, or with primary human blood monocytes. In both cases, monocytes differentiated into macrophages, and IL-6 production was increased compared to when fibroblasts and monocytes were cultured separately [86]. MCP-1 and MMP-9 production were also increased in



these co-cultures, as were CD14 and CD11b expression by macrophages [86]. In two separate studies it was confirmed that in ApoE knockout mice, mMCP-1 mRNA was expressed in developing lesions by mesenchymal adventitial cells prior to mMCP-1 mRNA expression in the intima [87,88]. Expression of mMCP-1 was increased in both the adventitia and the intima as the lesion became more severe [88]. Furthermore, early proliferation of adventitial fibroblasts and collagen synthesis by these cells was observed [88]. These studies support the role of fibroblasts in the early stages of atherosclerosis [87,88].

Of particular importance to this thesis, Vink *et al.* [89] discovered that human adventitial fibroblasts express TLR-4, and that stimulation of these fibroblasts *in vitro* with LPS induced cytokine mRNA expression and NF- $\kappa$ B activation. Therefore, although traditionally neglected in the study of atherosclerosis, there is evidence to suggest that adventitial fibroblasts can perform a role in inflammation and the development of CVD.

### **Cytokines and Chemokines Implicated in Atherosclerosis**

The advent of the ApoE  $-/-$  and LDLr  $-/-$  mice have provided valuable tools for studying atherosclerosis in mouse models *in vivo*. When these mice are fed a high fat and/or cholesterol diet, they are unable to efficiently clear circulating cholesterol. This results in the development of significant atherosclerotic plaques that resemble human plaques [90,91]. Crossing these mice with other strains of either transgenic or knockout (KO) mice has led to the discovery of numerous genes that play a role in atherosclerosis in these models.

MCP-1, which is a monocyte chemoattractant [277], was knocked out of mice that were then crossed with LDLr  $-/-$  mice. These double mutants presented with 83% less lipid accumulation in aortic plaques compared to LDLr  $-/-$  mice that were wild-type for the MCP-1 alleles [92]. TNF- $\alpha$  KO mice that were crossed with ApoE KO mice were found have 50% reduced lesion size compared to ApoE KO mice alone [93]. In addition, upon administering soluble TNF receptor to ApoE KO mice, there was found to be a 75% reduction in relative lesion size [93], implicating TNF- $\alpha$  in disease progression.

IL-1 is another pro-inflammatory cytokine that has been shown to induce cytokine and chemokine production, and increase the expression of leukocyte adhesion molecules on endothelial cells [94]. To determine the role of IL-1 in a model of atherosclerosis, ApoE KO mice were fed a high cholesterol diet, and the treatment group was administered the soluble IL-1-receptor. These mice presented with significantly smaller atherosclerotic lesions compared to control animals [95]. Similar studies have been performed with numerous other factors, including M-CSF [96], VEGF [97], and IFN- $\gamma$  [98]. These studies suggest that there are numerous factors that may be involved in atherosclerotic plaque progression. Although there are not currently OSM KO strains of mice available, OSM is another cytokine that has been implicated in the pathogenesis of atherosclerosis.

### **OSM**

Oncostatin M (OSM) is a 28 kDa cytokine that is released primarily by macrophages and T lymphocytes [99,100]. Other cells of the immune system

including eosinophils [101], mast cells [102], dendritic cells [103], and neutrophils [104] are also capable of OSM production. The human gene encoding OSM is located on chromosome 22q12 [105], while the mouse OSM gene is located on chromosome 11 [106]. The protein consists of four main helices (termed A through D); two pairs of helices that are anti-parallel in nature [107].

Oncostatin M is a member of the gp130 family of cytokines that also includes leukemia inhibitory factor (LIF), IL-6, IL-11, IL-27, IL-31, cardiotrophin-1 (CT-1), cardiotrophin-2 (CT-2), ciliary neurotrophic factor (CNTF), and cardiotrophin-like cytokine (CLC) [108,109]. Gp130 cytokine family members engage receptor complexes that include the gp130 signal transduction sub-unit with the exception of IL-31 which uses the gp130-like (GPL) chain. The mechanism by which OSM initiates signaling first involves its site 2 epitope, which contains 4 essential residues located on the A and C helices of OSM [107] binding to the cell surface gp130 ( $\alpha$ ) chain with low affinity [111]. The OSM:gp130 heterodimer then associates in human cells with either the LIFr $\beta$  [110] or the OSMr $\beta$  [111]. The phenylalanine and lysine amino acid pair located at the N-terminal region of helix D of OSM interacts with either the LIFr $\beta$  or the OSMr $\beta$ , thus forming either an OSM:gp130:LIFr $\beta$  or an OSM:gp130:OSMr $\beta$  complex [107]. Although in humans OSM can bind to the OSMR $\beta$  and LIFR $\beta$ , in mice OSM can only bind the OSMR $\beta$  [108].

Associated with the intracellular domains of the individual receptor sub-units are Janus kinase (JAK) molecules [121]. Four JAKs have been identified:

Tyrosine kinase (Tyk 2), and JAKs 1-3. JAKs 1, 2 and Tyk2 are ubiquitously expressed, while JAK 3 is present primarily in hematopoietic lineages [121]. JAKs are composed of 7 JAK homology (JH) regions, where JH1 is the canonical tyrosine kinase domain located at the carboxy terminus, while the amino terminus (JH4-7) has been shown to be necessary for interacting with the intracellular domains of cell surface receptors [112]. JAKs associate with the membrane proximal box 1 and box 2 that are found on the gp130, the LIFr $\beta$ , and the OSMr $\beta$  subunits [112]. Box 1 is an eight amino-acid proline rich motif, and box 2 is a hydrophobic followed by positively charged amino acid cluster [113]. JAKs become activated (tyrosine phosphorylated [114,108,115]) upon receptor dimerization through trans or auto-phosphorylation [116,117]. Receptor dimerization occurs as a result of the receptor binding the OSM ligand. Upon activation, JAKs phosphorylate tyrosine residues on the cytoplasmic portion of the receptor, which act as docking sites for molecules that contain Src Homology 2 (SH2) domains [108,118,119]. OSM has been shown to activate STATs 1,3,5 [147], and 6 [120].

### **Signal Transducers and Activators of Transcription**

Signal transducers and activators of transcription (STAT) molecules are a family of transcription factors that contain an SH2 domain at their C-termini and are known to act downstream of JAKs. Members of this family include STAT-1 ( $\alpha$ , and  $\beta$ , which occur as a result of differential splicing), STAT-2 [121], STAT-3, STAT-4, STAT-5a, STAT-5b, and STAT-6 [113]. STAT molecules are

ubiquitously expressed [113] with the exception of STAT-4, which is found primarily in the testis and in myeloid cells [122]. One of the functions of the SH2 domains on STATs is to mediate binding to phosphorylated tyrosine residues of the intracellular receptor motifs [118,123]. The association of a STAT molecule with the receptor allows JAK molecules to phosphorylate tyrosine residues on the STAT [124]. The STAT then dissociates from the receptor and homo- or heterodimerize with another phosphorylated STAT molecule. The SH2 domains on the STATs mediate the dimerization through recognition of the phosphorylated tyrosine motifs [125]. This dimerization is essential for DNA binding to occur [125].

The DNA binding domain of STATs is localized to amino acids 400 to 500 of the approximately 750-amino-acid-long proteins [126], and this sequence is highly conserved among the STATs [113]. Studies have demonstrated that STATs bind palindromic sequences of DNA with different numbers of base pair spacers. STAT 1 preferentially binds the sequence (5'-TTCN3GAA-3') with 3 base pair spacers [127], STAT 3 preferentially binds the sequence (5'-TTN4AA-3') with 4 base pair spacers [128], STAT-6 preferentially binds the sequence (5'-TTCN4GAA-3') with 4 base pair spacers [127], while the other STATs in general bind sequences with 5 base pair spacers (5'-TTN5AA-3') [128]. In addition STATs have been shown to interact with several non-STAT transcription factors including p48 [129], CREB [130], and c-Jun [131] which may increase the number of genes that STATs can transcribe [129]. Studies have also demonstrated

that STAT molecules 1 [132], 3 [133], and 5 [132] can be phosphorylated on serine residues. Serine phosphorylation can alter nuclear localisation and increase stability of DNA binding [132,133].

### **MAPK and PI3K Signaling**

OSM binding either of its receptors can also activate mitogen activated protein kinase (MAPK) signalling [134]. All eukaryotic cells are capable of MAPK signaling, and given this fact, it is not surprising that these kinases regulate various processes including metabolism, motility, survival, apoptosis, differentiation, and gene expression [135]. The best studied MAPKs include ERK 1/2, JNK (1, 2, and 3), and p38 ( $\alpha$ , $\beta$ , $\gamma$ , and  $\delta$  isoforms). Although activators of these kinases are numerous, generally ERK 1 and 2 are thought to be activated by growth signals and phorbol esters, while p38 and JNK are recruited in response to stressful stimuli such as radiation and cytokines [135]. The MAPKs are activated via phosphorylation of both a threonine and tyrosine residue situated in the activation loop of kinase subdomain VIII [135].

Following phosphorylation of tyrosine residues on the intracellular component of the receptor complex, the adaptor proteins 'SH2 domain-containing protein tyrosine phosphatase-2' (SHP-2) and 'Src homology and collagen' (Shc) proteins become activated. SHP-2 associates with phospho-tyrosine 759, the second tyrosine from the cell membrane of 6 tyrosine residues present on the gp130 chain, but does not bind to the OSMr  $\beta$  [136]. Shc was shown not to bind gp130, but to bind the OSMr  $\beta$  on Tyr 861 via its phospho-tyrosine-binding (PTB)

domain [136]. The PTB domain of Shc binds both phosphorylated tyrosine residues and acidic phospholipids [137].

Once SHP-2 and Shc become phosphorylated, this can lead the activation of Ras, a small GTP binding protein [138]. Ras then activates one of the MAPKKK molecules. For ERK 1/2 signaling, Ras activates Raf at the cell membrane [139]. Raf, a serine/threonine MapKKK [139] then phosphorylates the dual functional serine/threonine and tyrosine MAPKKs MEK1 and MEK 2 [140], which then cause threonine and tyrosine phosphorylation of ERK 1/2 [141]. ERK has been shown to activate numerous molecules including membrane proteins (ex. Syc), scaffolding proteins, nuclear substrates (ex. Elk-1, *egr-1*, STAT3, c-Myc, MEF2, and c-Fos) [135,142,151], and various members of the 'MAPK-activated protein kinases' (MK) family including ribosomal S6 kinases (RSKs), mitogen- and stress-activated kinases (MSKs), and the MAPK-interacting kinases (MNKs) [135].

C-Jun N-terminal kinase (JNK) is another MAPK that is activated by OSM. JNKs 1, 2, and 3 can yield 46 and 54 kDa polypeptides based on differential splicing at the C-terminus, although currently the difference in action between the two splice variants is unknown [143]. There are numerous MAPKKK that have been shown to activate both p38 and JNK, although whether any of these are activated by gp130 cytokines is currently unclear. Examples include MEKK 1-4, ASK1, TAK1, TAO1/2, and MLK2 [135]. The MAPKKs that have been shown to dually phosphorylate JNK are MKKs 4 and 7, with MKK 4

phosphorylating the tyrosine residue, while MKK 7 phosphorylates the threonine residue [144].

Activation of the third MAPK p38 generally occurs as a result of cell stressors, and most stimuli that activate JNK also activate p38 [135]. p38 is dually threonine and tyrosine phosphorylated by MEK 3 and 6 [145]. Cells that have been shown to activate p38 in response to OSM include vascular smooth muscle cells [146], mouse lung fibroblasts [147], and human cardiac myocytes and fibroblasts [148]. JNK and p38 activation results in transcription of *c-fos* genes, which then hetero-dimerize with Jun proteins to form an AP-1 transcription factor [149], which regulates numerous processes including cellular differentiation, proliferation, survival, and death [150].

SHP-2 also plays a role in phosphatidylinositol 3'-kinase (PI3K) signaling. As mentioned previously, SHP-2 binds to Tyrosine 759 on gp130 and becomes phosphorylated. This provides a binding site for Grb2-associating binder 1 (Gab1) and Gab 2, which are 110 and 100 kDa adaptor/scaffold proteins respectively [151,152]. Gab 1 and 2 interact with the p85 subunit of PI3K [151]. PI3K induces the PIP2 to become PIP3 via the addition of a phosphate group, resulting in PDK1 localizing to the plasma membrane and activating Akt [153]. In addition, studies have shown that the complex of SHP-2:Gab1:PI3K can enhance ERK activation [152], indicating that the MAPK and PI3K pathways are not completely independent.



### **OSM and Inflammation**

OSM was originally discovered in the supernatants of histiocytic lymphoma cells stimulated with the phorbol ester PMA [154]. Since then, several different stimuli have been shown to elicit OSM expression. Murine OSM mRNA is induced in response to STAT-5 activation by IL-2, IL-3, and Erythropoietin [155]. In humans, GM-CSF stimulation caused OSM mRNA and protein production in neutrophils via activation of STAT-5 [104,156]. Viral infections such as HIV have been shown to induce OSM production in humans [157,158], and OSM was expressed by macrophages upon stimulation with the complement component C5a [159]. Interestingly, LPS induced transcription and release of OSM protein in human neutrophils [104] and in human monocytes *in vitro* [103,160]. In addition, Suda *et al.* [103] performed a study which demonstrated that human peripheral blood monocyte derived dendritic cells, when stimulated with LPS produced a significant amount of OSM mRNA and protein.

The effects of OSM within the body are numerous and diverse. Depending on the context of its release, this cytokine can have various biological functions including roles in growth and development, tumour suppression, hematopoiesis, and inflammation [108]. This report focuses primarily on the inflammatory characteristics of OSM.

Like other members of the IL-6 family, Oncostatin M caused the release of acute phase proteins from hepatic cells [161], and septic shock patients had a ten fold increase in OSM production [162], implicating this cytokine in

inflammatory responses. Increased OSM expression has been implicated in inflammatory diseases such as rheumatoid arthritis, as its detection has been correlated with white blood cell infiltration into the synovium [163], and has also been shown to alter the expression of MMPs and TIMPs in various cell types [164,165].

In terms of its effects on vascular cells, OSM was shown to stimulate rabbit vascular SMC proliferation [166]. Wijelath *et al.* [167] demonstrated that treating bovine aortic endothelial cells with OSM resulted in basic fibroblastic growth factor (bFGF) production, as well as endothelial cell proliferation and migration. This suggests that angiogenesis after vascular injury could be mediated by OSM [167]. OSM also induced expression of leukocyte adhesion molecules such as P- and E-selectin, ICAM-1, and VCAM-1 on human umbilical vein endothelial cells [168]. In the same study, treating monolayered endothelial cells with OSM caused increased adhesion of polymorphonuclear cells and a ten fold increase in migration across the monolayer by these cells *in vitro* [168]. OSM stimulation did not result in IL-8 expression, but did result in the expression of other CXC chemokines: epithelial neutrophil activating peptide-78 (ENA-78), and growth related cytokine (GRO)  $\alpha$  and  $\beta$  [168]. Interestingly, GRO  $\beta$ , the human homolog of murine MIP-1 $\alpha$  [169], is involved in vascular inflammation [170], and appears to be downstream of MyD88 signalling as well [79]. Brown *et al.* [171] demonstrated that OSM induced IL-6 mRNA and protein expression in human endothelial cells, and several other cell types have been shown to produce

IL-6 in response to OSM stimulation [172,173]. In addition, OSM synergised with TNF- $\alpha$  to augment IL-6 production in human endothelial cells still further [171]. Bernard *et al.* [174] treated aortic smooth muscle cells (SMCs) with OSM and demonstrated a concentration dependent increase of IL-6 production with increasing OSM stimulation.

Observations in the Richards' laboratory indicated that by immunohistochemistry, OSM was present in the vascular intima, media, and adventitia of atherosclerotic lesions in ApoE(-/-) mice (unpublished data). Furthermore, human plaque macrophages and SMCs were shown to express OSM by immunohistochemistry [175]. Thus the presence of OSM in atherosclerotic plaques and its ability to induce inflammation suggest that it may participate in the atherosclerotic process, however its role merits further investigation, especially in the context of other inflammatory stimuli.

### **Interleukin-6**

Interleukin-6 (IL-6) is a member of the gp130 family, and like OSM, is a pleiotropic cytokine. It exerts effects on the nervous system, the endocrine system, the immune system, and also has roles in inflammation, hematopoiesis, and bone metabolism [151]. In humans the *IL-6* gene is located on chromosome 7p21 [176], while in mice it is located on chromosome 5 [151]. A number of potential promoter sequences have been identified upstream of the *IL-6* gene, including a c-fos serum-responsive element homolog, a nuclear factor for IL-6 expression (NF-IL6) binding site, a cyclic AMP-responsive element, an NF-kB

binding site, and an AP-1 binding site [151]. The protein structure is conserved among gp130 family members, and consists of four  $\alpha$ -helices arranged in pairs that are anti-parallel [177]. In addition, the IL-6 protein can be modified post-transcriptionally via serine phosphorylation [178], and it has two potential sites for N-linked glycosylation [179].

The classical IL-6 signalling mechanism is similar to that which occurs in OSM signalling. However, unlike OSM which first binds the gp130 chain, IL-6 binds to the IL-6-receptor $\alpha$  (IL-6r), which then heterodimerizes with a gp130 subunit [180,181]. Two of these IL-6/IL-6r/gp130 complexes associate, forming a functional hexameric IL-6 signalling complex [180]. The JAKs that associate with the cytoplasmic component of the gp130 subunits trans-phosphorylate one another, and the tyrosine docking sites on the gp130 subunits [151,42]. These sites are bound by molecules that contain SH2 domains [108,118,119]. In response to IL-6, STATs 1, 3 and 5 [182,183] bind to the docking sites, become phosphorylated by Jaks, and migrate to the nucleus where they initiate transcription (see 'Signal Transducers and Activators of Transcription' above). MAPK signaling is also activated by IL-6, and is mediated by SHP-2 binding to phospho-tyrosine 759 on the gp130 chain (see 'MAPK and PI3K Signaling' above).

The IL-6r is also found as a soluble protein (sIL-6r). The sIL-6r can be generated in one of two ways; it can be differentially spliced, resulting in a form of the IL-6r that lacks the trans-membrane and cytoplasmic domains [184], or the

IL-6r can undergo proteolytic cleavage by ‘a disintegrin and metalloprotease’ (ADAM) 10 and ADAM 17 [185]. Due to the fact that gp130 is a ubiquitously expressed protein, IL-6 can bind to the sIL-6r and activate cells that do not express membrane bound IL-6r in a mechanism termed ‘trans-signaling’ [186].

IL-6 has numerous physiological functions, one of which is participating in inflammatory responses. There is evidence to suggest that IL-6 plays a role in vascular inflammation. Angiotensin II (Ang II) induced vascular injury, inflammation and is believed to play a role in atherosclerosis [187]. Ang II induced IL-6 through activation of the NF $\kappa$ B transcription factor [188]. Data from Tieu *et al.* [86] demonstrated that IL-6 null mice showed significantly reduced rates of aortic dissections upon subcutaneous injection of Ang II compared with wild type mice. The localization of IL-6 production in the vascular wall of wild-type mice was greatest within the adventitial layer [86]. Furthermore, only wild-type and not IL-6 deficient murine macrophages were positive for phospho-STAT-3, indicating the role of IL-6 in macrophage activation in these models of vascular inflammation [86,42]. Upon co-culturing human aortic adventitial fibroblasts with peripheral blood monocytes, IL-6, MCP-1, and MMP-9 levels were detected [189]. In addition, these same peripheral blood monocytes were induced to differentiate into macrophages during the co-culture [86]. The authors concluded that the Ang II was mediating vascular inflammation and aortic dissections through the effects of IL-6 [86].

In a separate study, both ApoE deficient and wild type C57Bl/6 mice on high fat diets that were injected with IL-6 had a 1.9-5.1 fold increase in atherosclerotic lesion size over mice injected with saline solution [190]. Similarly, circulating IL-6 levels were found to correlate significantly with calcified aortic plaques in individuals with diabetes [191]. Importantly, IL-6 was discovered in atherosclerotic coronary artery samples [192]. IL-6 can also increase leukocyte adhesion molecules expression and chemokine production (specifically MCP-1 and IL-8) by human endothelial cells [193,194]. In cases of infection, IL-6 appears to play a role in up-regulating the procoagulant prothrombin molecules F1 and 2 (by-products in thrombin generation (indicating the presence of thrombin)) [195]. In summary, there is evidence to suggest that IL-6 can play a role in vascular inflammation.

### **Aims and Hypotheses**

**Overall Goal:** To determine whether gp130 cytokines can exacerbate TLR-activated inflammation in atherosclerosis.

**Hypothesis 1:** TLR-4 ligands regulate vascular wall cells and synergize with OSM in stimulating cells *in vitro*.

**AIM 1:** To assess cytokine and chemokine expression *in vitro* by *mouse* aortic adventitial fibroblasts (MAAFs) in response to TLR-4 ligand or OSM, alone and in combination.

**AIM 2:** To determine if *human* aortic adventitial fibroblasts (HAoAFs) are regulated by TLR ligands and synergize with OSM *in vitro* to produce pro-inflammatory cytokine and chemokine mRNA and protein.

**AIM 3)** To determine whether *human* aortic smooth muscle cells (HAoSMCs) are regulated by TLR ligands and synergize with OSM *in vitro* to produce pro-inflammatory cytokine and chemokine mRNA and protein.

**Hypothesis 2:** The mechanism of TLR-ligand/OSM synergy involves dependent up-regulation of TLRs or OSM receptors.

**AIM 4)** To determine whether the production of pro-inflammatory cytokines is associated with an increased expression of TLRs by OSM, or OSMr by TLR-ligands.

**AIM 5)** To determine whether the OSM-induced or the TLR-4-induced signalling pathways are amplified upon co-stimulation.

**Hypothesis 3:** OSM can modulate vascular wall cell activity in the presence of other physiologically relevant pro-inflammatory cytokines.

**AIM 6)** To determine whether the application of neutralizing antibodies to OSM or one of its receptors can modulate HAoAF cell activity upon stimulation with peripheral blood mononuclear cell (PBMC) conditioned medium.

## **CHAPTER 2 – MATERIALS & METHODS**

### **Cell Culture**

C57Bl/6 mouse aortic adventitial fibroblasts (MAAFs) were generated previously in the laboratory, and rat aortic adventitial fibroblasts were received as a generous gift from Dr. Hui Di Wang (Brock University). MAAFs were cultured in Dulbecco's Modified Eagle Medium, containing 10% heat inactivated Fetal Bovine Serum (FBS), 1% Penicillin Streptomycin, 0.1% L-Glutamine, and 0.1% Fungizone (all from Life Technologies, Carlsbad, CA) at 37°C in 5% CO<sub>2</sub> conditions. Experiments were performed on cells between passages 5-12, and expanded 1:5 in T150 culture flasks (Becton Dickinson (BD) and Company, Franklin Lakes, NJ).

Human Aortic Adventitial Fibroblasts (HAoAFs) and Human Aortic Smooth Muscle Cells (HAoSMCs) were purchased from Lonza Group Ltd. (Basel, Switzerland), and were cultured in Stromal Cell Growth Medium or Smooth Muscle Growth Medium-2 (Lonza Group Ltd.) respectively according to the manufacturer's instructions in 5% FBS, at 37°C in 5% CO<sub>2</sub> conditions . Experiments were performed on cells between passages 3-8, and expanded 1:3 in T150 culture flasks (BD and Company). HAoAFs were primary cells that were isolated from a 19 year old female patient, and HAoSMCs were primary cells that were isolated from a 36 year old male patient. Both patients passed away from a cause other than vascular disease.



## **ELISAs**

For assessment to responses to stimuli, cells were seeded at 10 000 cells/cm<sup>2</sup> into 96 well plates. The cells were serum deprived in media containing 2% FBS for three hours prior to stimulation, and stimulated with media containing 2% FBS for 18 hours. Following this time point, the supernatants were removed and stored in the freezer (-20°C) for future use. Soluble CD14, and all TLR ligands used to stimulate cells were generous gifts from Dr. A. Ashkar (McMaster University). All gp130 cytokines used to stimulate cells were purchased from R&D Systems (Minneapolis, MN), and were recombinant proteins derived from *E. coli*.

ELISAs were performed using DuoSet kits purchased from R&D Systems and include mouse IL-6, MCP-1, KC, and VEGF, and human IL-6, IL-8, and VEGF kits. ELISAs were performed according to the manufacturer's instructions. The degree of colour change was measured using the Bio-Tek EL 800 (BioTek, Winooski, VT) plate reader at an optical density of 450 nm.

## **Soluble CD14 treatments**

HAoAFs were seeded at 10 000 cells/cm<sup>2</sup> into 96 well tissue culture plates (BD and Company). The cells were stimulated with increasing doses of LPS alone or with LPS in combination with 100 ng/mL of soluble CD14. Separately, HAoAFs were stimulated with increasing doses of soluble CD14 in combination with 1 ng/mL of LPS. To allow for association, soluble CD14 was incubated with the LPS for 1 hour at 37 °C prior to administration to cells. Cells were stimulated

for 18 hours, at which point the supernatants were collected, and assayed for IL-6 via ELISA.

### **Cell lysates & Immunoblots**

Cells were seeded at 10 000 cells/cm<sup>2</sup> into 6 well tissue culture plates (BD and Company). The cells were serum deprived in media containing 2% FBS for three hours prior to stimulation, and stimulated with media containing 2% FBS. Cells were stimulated for either 20 minutes or four hours. Following these time points, the supernatants were removed and stored in the freezer (-20°C) for future use (ELISAs). Cells were washed twice with cold 1x PBS. Whole cell lysates were generated using Radioimmunoprecipitation (RIPA) lysis buffer containing 1% Igepal CA-630, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS) in PBS, and inhibitors 1mM Sodium Orthovanadate, 0.1 mg/mL Phenylmethylsulfonylflouride (PMSF), 5 µg/mL Aprotinin, and 1 mM dithiothreitol (DTT) . Cell lysates were scraped off the bottom of the well using cell scrapers, and were then collected and incubated on ice for one hour. Cell lysates were then sheared using a 3 mL syringe and a 21 gauge needle, centrifuged at 12 000 rpm at 4°C for 10 minutes, and then stored at -80°C for future use.

A Bradford Assay was performed to determine the protein concentration in each sample, using BSA protein (Sigma Aldrich Corp, St. Louis, MO) as the standard. Bio-Rad Protein Assay Dye Reagent Concentrate (Bio Rad, Hercules, CA) was added to make 20% volume of each sample, and the optical density was

measured at a wavelength of 595 nm, with a reference of 0 nm as measured by a Safire plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Based on values determined by the Bradford Assay, an equal amount of protein (15µg) from each sample was loaded onto either an 8 or 10% SDS-PAGE gel for electrophoresis using a standard protocol [196]. Proteins were transferred to a nitrocellulose membrane (Pall Corp., Port Washington, NY) by electrical transfer using a BioRad Mini-Protean II equipment (Bio Rad) using a standard protocol [197]. Membranes with transferred proteins were blocked using 1x Tris Buffer Saline (TBS) containing 0.15% Tween 20 (Sigma Aldrich Corp.) and 5% skim milk powder (Nestle Carnation, Vevey, Switzerland) for 1 hour at room temperature. The membranes were then probed while rocking overnight at 4°C using antibodies diluted to between 1:1000 and 1:3000 in either 5% BSA or 5% skim milk powder in 1x (TBS) containing 0.15% Tween 20. Antibodies for pSTAT-1, total STAT-1, pSTAT-3 (Tyr 705 and Ser 727), total STAT-3, pSTAT-5, p-p38, Total p38, p-SAPK/JNK, p-NFκB p65, total NFκB p65, pAkt, and Total Akt were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for p-ERK 1/2, Total ERK 1/2, Total JNK, Total STAT-5, and Total Actin were purchased from Santa Cruz Biotechnology Inc.

The following day, membranes were washed three times for 8 min each with 1x TBS containing 0.15% Tween 20. They were then incubated at room temperature for one hour with a secondary antibody diluted to 1:2500 that was conjugated to horseradish peroxidase (either mouse anti-goat IgG-HRP, Goat anti-

rabbit IgG-HRP, or goat anti-mouse IgG-HRP, from Santa Cruz Biotechnology Inc.) Membranes were then washed two times for 8 minutes each at room temperature with 1x TBS containing 0.15% Tween 20, and two times for 8 minutes each at room temperature with 1x TBS. The membranes were then incubated with Pierce ECL 2 Western Blotting Kit (ThermoFisher Scientific, Waltham, MA) reagents according to manufacturer's instructions for 5 minutes, taken to the dark room, and exposed to CL-X Posure Film (ThermoFisher Scientific, Waltham, MA).

#### **RNA purification and analysis by real-time quantitative PCR (Taqman)**

Cells were seeded at 10 000 cells/cm<sup>2</sup> into T75 flasks. The cells were serum deprived in media containing 2% FBS for three hours prior to stimulation, and stimulated with media containing 2% FBS. Following these time points, the supernatants were removed and stored in the freezer (-20°C) for future use (ELISAs). Pure Link RNA mini kit (Life Technologies, Carlsbad, CA) was used according to the manufacturer's instructions to isolate and purify the RNA. RNA was dissolved in 50 µL of nuclease free water, and stored at -80°C for future use.

RNA concentration was determined by the NanoVue Spectrophotometer (GE Healthcare, Little Chalfont, UK). Genomic DNA was removed using the Ambion DNA-free kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. RNA was then reverse transcribed into cDNA using SuperScript II RT (Life Technologies), according to the manufacturer's instructions, and stored at -20°C until future use.

Expression of IL-6, IL-8, VEGF, MCP-1, TLR-4, and OSMr- $\beta$  mRNA, as well as the house-keeping gene  $\beta$ -Actin mRNA was measured using pre-developed assay reagents purchased from Ambion (Life Technologies). TaqMan Universal Master Mix (Life Technologies), a mixture containing AmpliTaq Gold DNA polymerase, AmpErase uracil-N-glycosylase (UNG), dNTPs, a passive reference dye, and optimized buffer components was used as the source of the PCR reagents. 25 ng of cDNA was used for each Taqman reaction, and each sample was assayed in triplicate. The MicroAmp Optical 96 well reaction plates (purchased from Life Technologies) were then placed in a 7900HT Fast Real-Time PCR System (Life Technologies), which was operated by Sequence Detection System Software version 2.4 (Life Technologies). mRNA expression levels for each sample were normalized to B-Actin levels in that sample and expressed relative to control (media alone) values.

### **Flow Cytometry**

MAAF cells were seeded at 10 000 cells/cm<sup>2</sup> into 6 well plates. The cells were serum deprived in media containing 2% FBS for three hours prior to stimulation, and stimulated with media containing 2% FBS. The cells were stimulated for 2, 6, or 24 hours, and then were lifted off of the culture dish by incubating cells for 8 min. with 1 mM EDTA, and then pipetting them off of the plate. The cells were stained with either an isotype control rabbit IgG antibody or a rabbit anti-TLR-4 antibody (Santa Cruz Biotechnology Inc.) diluted 1:300 for 30 min at 4°C. The primary antibodies were washed off using FACS buffer (PBS

containing 0.3% BSA) and the cells were incubated with a FITC labelled goat anti-rabbit IgG antibody (Santa Cruz Biotechnology Inc.) for 30 minutes in the dark at 4°C. Data acquisition was performed using the BD LSR II flow cytometer (Becton Dickinson and Company), and data analysis was performed using FlowJo software (TreeStar Inc., Ashland, OR).

### **Neutralizing Antibody Experiments**

Blood was taken from a healthy volunteer, and the peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque method outlined by Fuss et al. [198] Briefly, the blood was placed in a 50 mL Falcon tube and mixed with an equal volume of room temperature PBS. The mixture was then centrifuged for 15 min at 200 x g at room temperature, and the supernatant suspension containing the platelet fraction was discarded. PBS was added to re-suspend the pellet, and the Ficoll-Hypaque solution was dispensed underneath the leukocyte/red blood cell/PBS mixture (3 mL Ficoll-Hypaque per 10 mL blood/PBS mixture). The mixture was then centrifuged at 900 x g for 20 minutes at room temperature with no brake, and the mononuclear lymphocyte layer, which is the layer above the Ficoll-Hypaque, was then collected. The cells were then washed with PBS, plated, and stimulated with 100 ng/mL of LPS. The supernatants were collected after 24 hours and were analyzed by Luminex (Millipore Corporation, Billerica, MA) for cytokine levels.

These LPS stimulated PBMC supernatants (conditioned medium (CM)) were then used to stimulate HAoAFs. The HAoAFs were stimulated with various

dilutions of CM (from 1:200, to 1:12 800) for 6 hours, and the HAoAF supernatants were collected and stored at -20°C for future use. An IL-6 ELISA was performed on these supernatants as described above.

For the neutralizing antibody experiment, HAoAFs were treated with a 1:800 dilution of CM alone, with CM and a mouse IgG antibody (Sigma Aldrich), with CM and a mouse anti-OSM antibody (R&D Systems), or with CM and a mouse anti-OSMr- $\beta$  antibody (Amgen Inc., Thousand Oaks, CA). The mouse IgG and mouse anti-OSM antibodies were incubated with the supernatants for 2 hours at 37 °C prior to treatment of the HAoAFs. The other dose of mouse IgG, and the mouse anti-OSMr- $\beta$  antibody were incubated with the HAoAFs one hour prior to treatment of the HAoAFs with CM. After 6 hours, the HAoAF supernatants were collected, and were analyzed for IL-6 protein by ELISA.

### **Statistical Analysis**

All statistical tests were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). A p value less than 0.05 was considered statistically significant. Two-Way Analysis of Variance (ANOVA) was used to determine significance between LPS alone compared to LPS combined with one of the gp130 cytokines for the ELISA data. One-Way ANOVA was used to determine significance for the mRNA data and the treatments of LPS or OSM alone data obtained via ELISA. In all cases, Bonferroni's post-test was applied. Where indicated, the effects of individual

treatments alone were summed and this predicted additive effect was compared to the observed combined effects.



### **CHAPTER 3**

#### **RESULTS PART 1**

##### ***Synergistic IL-6 induction by MAAFS in response to LPS and OSM***

To determine whether LPS and/or members of the gp130 cytokine family can regulate vascular wall cells *in vitro*, adventitial fibroblasts and smooth muscle cells from rodent and human aortic tissues have been analyzed. Experiments were undertaken using Mouse Aortic Adventitial Fibroblast (MAAF) cells that were cultured and then treated with increasing concentrations of OSM. The subsequent elevation of IL-6, a pro-inflammatory cytokine, was measured in the supernatants 18 hours post-stimulation by ELISA. Only at the highest concentration of OSM (10 ng/mL) was there detectable IL-6 elevated in the MAAF cell supernatants (Fig. 1a). MAAF cultures were then treated with increasing doses of LPS alone or in combination with a constant dose of OSM. The cells that were treated with LPS alone did not induce detectable IL-6 levels. However, there was a dose dependent IL-6 response when 0.5, and 5 ng/mL of OSM was added to the increasing concentrations of LPS (Fig. 1b).

Other members of the gp130 cytokine family were tested in combination with LPS to determine whether the observed effect was unique to OSM. When LIF or CT-1 was combined with LPS to treat the MAAF cells, there was a dose dependent response of IL-6 production (Fig. 1c,d). However the levels of IL-6 that were detected were lower than the LPS combined with OSM treatments, and were only detectable at higher concentrations of LPS stimulation. There was a slight, but statistically significant increase in IL-6 in the supernatants when IL-31

was combined with LPS (Fig. 1e), and only at the highest concentration of both LPS and IL-11 was there detectable IL-6 measured (116 pg/mL) (Fig. 1f). Therefore, OSM displayed the greatest synergy with LPS with regards to elevating IL-6 protein, while the other gp130 cytokines tested caused more moderate increases in IL-6 in the MAAF supernatants.

### ***Synergistic KC induction by MAAFS in response to LPS and OSM***

Keratinocyte derived cytokine (KC) is a neutrophil chemoattractant that was discovered to play a role in a model of atherosclerosis [199], and Lafontant *et al.* have shown that mouse fibroblasts produce KC upon stimulation with OSM [200]. The chemokine KC is not a direct homologue of human IL-8, however it, along with MIP-2, are thought to be functional homologues of this protein [201]. Thus KC expression by MAAFs was examined *in vitro*. It was observed that the MAAF supernatants contained elevated KC when treated with 1 and 10 ng/mL OSM in a dose dependent manner after 18 hours of stimulation (Fig. 2a). Similarly, when treated with 1, 10, and 100 ng/mL of LPS, the MAAFs demonstrated a dose dependent KC response. When the two treatments were combined, the KC response was more than additive (Fig. 2b) ( $p < 0.01$  at 0.1, 1, 10, and 100 ng/mL LPS in combination with either 0.5 or 5 ng/mL OSM compared to LPS at that concentration alone). In comparison with OSM, the opposite trend resulted with either CT-1 or LIF co-stimulation, as they appeared to negatively impact KC levels when combined with LPS (Fig. 2c, d). IL-6, IL-11, nor IL-31 detectably impacted the KC levels compared with LPS alone (Fig. 2e, 2f, and 2g

respectively). Therefore, of the gp-130 cytokines that were studied in these assays, OSM was uniquely able to act synergistically with LPS to cause increased levels of detectable KC in MAAF supernatants.

### ***Synergistic VEGF induction by MAAFS in response to LPS and OSM***

As published in a recent paper by Demyanets *et al.* [146], vascular endothelial growth factor (VEGF) is induced by OSM in vascular SMCs. VEGF, which is a major inducer of angiogenesis, may contribute to atherosclerotic plaque destabilization and rupture, as newly formed vessels are prone to rupturing [175]. VEGF induction was thus examined *in vitro* in response to LPS and gp130 cytokines.

Upon stimulation of MAAF cells with increasing doses of OSM, there was no VEGF detected in the cell supernatants by ELISA (data not shown). Similarly, when the cells were stimulated with increasing doses of LPS, there was no detectable VEGF in the supernatants (Fig. 3), as the limit of detection of the ELISA is 15 pg/mL, and all values were below this level. Upon stimulation with a combination of LPS and 5 ng/mL OSM, there was statistically significant elevation detected ( $p < 0.0001$ ) at 0.1, 1, 10 and 100 ng/mL LPS. Supernatants from cells treated with LPS in combination with LIF, IL-6, IL-31, or IL-11 that were assayed for mVEGF contained levels that were below the limit of detection of the ELISA (data not shown). VEGF protein levels in the supernatants of MAAF cells stimulated with LPS and OSM were not as high as were detected in

the IL-6 and KC assays, however as was seen in those two assays, VEGF levels were synergistically elevated compared to LPS or OSM treatments in isolation.

### ***TLR-3 ligand does not induce IL-6 in MAAF's***

After observing synergistic responses by MAAF cells to the TLR-4 ligand LPS in combination with OSM, it was of interest to determine whether a similar effect was observed upon activation of another TLR, TLR-3. TLR-3 has traditionally been considered to be located intracellularly, however a study has shown that on human fibroblasts and epithelial cells, it can also be located on the cell surface [202]. TLR-3 recognizes double stranded viral RNA, as well as the synthetic analog polyriboinosinic:polyribocytidylic acid (poly I:C) [203]. The TLR-3 ligand poly I:C was used to stimulate MAAFs alone or in combination with 0.5 ng/mL OSM. Poly I:C stimulation alone, or in combination with OSM resulted in no detectable IL-6 measured by ELISA after 18 hours of stimulation (Fig. 4).

### ***Synergistic IL-6 induction by RAAFS in response to LPS and OSM***

To determine whether the results seen in mouse cells were reproducible in Rat Aortic Adventitial Fibroblasts (RAAFs), RAAF's were cultured *in vitro* and the cells were stimulated with increasing concentrations of LPS with or without mOSM. TLR-4 ligand administration in the absence of mOSM did not induce IL-6 elevation in RAAF cell supernatants, even at concentrations of 100 ng/mL (Fig. 5b). Increasing doses of mOSM alone similarly did not cause detectable induction of IL-6 (Fig. 5a). However, upon stimulation with LPS and 0.5 ng/mL mOSM,

RAAF cell supernatant IL-6 levels became detectable at 0.1 ng/mL of LPS and reached a peak production of 910 pg/mL when stimulated with 100 ng/mL of LPS (Fig. 5b)( $p < 0.0001$  compared to LPS alone at 100ng/mL).

RAAF cells were also challenged with the synthetic TLR-3 ligand poly I:C. Poly I:C elicited no detectable IL-6 response from RAAF cells alone, or in combination with 0.5 ng/mL of mOSM (Fig. 5d). These results in the RAAFs are consistent with the MAAF response to poly I:C (Fig. 4), as neither cell type demonstrated an IL-6 response to TLR-3 ligand challenge.

### ***Synergistic IL-6 induction by HAoAFs in response to LPS and OSM***

After observing similar trends in both mouse and rat vascular fibroblasts with regards to IL-6 production, it was of interest to determine whether human aortic adventitial fibroblasts (HAoAFs) were regulated by a TLR-4 ligand and gp130 cytokines *in vitro* to produce pro-inflammatory cytokine and chemokine mRNA and protein. Primary HAoAFs were purchased from Lonza Bio. These cells were isolated from a 19 year old female patient that passed away from a cause other than vascular disease.

Eighteen hours post treatment, elevated human IL-6 was detected in supernatants of HAoAFs that were stimulated with 1 and 10 ng/mL of OSM (Fig. 6a). IL-6 was detected in a dose dependent manner upon stimulation with 1, 10, and 100 ng/mL of LPS (Fig. 6b and 6c). When increasing doses of LPS were combined with a constant dose of 0.1, 0.5 (Fig. 6b), or 5 ng/mL OSM (Fig. 6c), there were augmented IL-6 levels in the HAoAF supernatants compared to either

treatment alone. The IL-6 levels measured when 0.1 or 0.5 ng/mL OSM (Fig. 6b) were used to treat the cells alone were added to the IL-6 levels measured when each concentration of LPS alone was used to treat the cells, and these values (estimated additive effect) were compared statistically to the observed combined effect of either LPS plus 0.1 or 0.5 ng/mL OSM respectively. The observed IL-6 levels when LPS was administered at 10 and 100 ng/mL in combination with 0.1 or 0.5 ng/mL OSM were significantly higher ( $p < 0.0001$ ) than the estimated additive levels. The observed combined effect was significantly higher at 0.1 ng/mL ( $p < 0.01$ ), and 1, 10, and 100 ng/mL ( $p < 0.0001$ ) LPS and 5 ng/mL OSM compared to the estimated additive effect.

To compare these results with other gp130 cytokine activity, HAOAFs were stimulated with LPS in combination with 5 ng/mL of LIF, IL-31, or IL-11. Stimulation with LIF alone resulted in no significant IL-6 elevation compared to control (Fig. 6d). When LIF was combined with LPS, there was no difference in IL-6 measured compared to LPS alone (Fig. 6d). Unaccompanied, neither IL-31 nor IL-11 treatments resulted in detectable IL-6 protein in the supernatants (Fig. 6e and 6f). When IL-31 or IL-11 was combined with increasing doses of LPS, IL-6 was detected in higher concentrations than when the cells were treated with LPS alone (Fig 6e and 6f). While the maximum concentration reached with 5ng/mL IL-31 and LPS treatment was ~17000 pg/mL IL-6, and the IL-11 and LPS treatment was ~15000 pg/mL IL-6, the maximum concentration reached with the OSM + LPS treatment was ~37000 pg/mL IL-6. Thus, of the gp130 cytokines

examined for IL-6 expression, OSM was able to induce the most potent response when combined with the TLR-4 ligand LPS.

### ***OSM acts to inhibit LPS mediated IL-8 induction in HAoAFs***

Since IL-8 may be implicated in atherosclerosis [263,264], the *in vitro* IL-8 response of HAoAF cells to LPS and gp130 cytokines was determined. These cells were stimulated for 18 hours, and the supernatants were analyzed for IL-8. Increasing doses of OSM alone did not result in detectable IL-8 in the supernatants of HAoAF cells (Fig. 7a). LPS alone was able to significantly induce IL-8 that was detectable in the supernatants in a dose dependent manner (Fig. 7b). Interestingly, when combined with 0.5 or 5 ng/mL OSM, there was a suppression of IL-8 that was detected in the supernatants (Fig. 7b). When LPS was combined with LIF, IL-6, IL-31 or IL-11 there was no marked difference in IL-8 levels induced by the HAoAFs compared to LPS alone (Fig. 7c,d,e, and f respectively).

### ***Enhanced VEGF in HAoAF supernatants in response to LPS and OSM***

As shown in Figure 2b, MAAF cells responded to LPS and OSM by synergistically inducing VEGF elevation. To determine the effects of LPS and the gp130 cytokines in the human fibroblasts, HAoAFs were stimulated for 18 hours and their supernatants were analyzed by ELISA for VEGF. OSM alone at 1 and 10 ng/mL was able to induce significant ( $p < 0.0001$ ) VEGF levels in the supernatants compared to control (Fig. 8a). LPS alone at 10 and 100 ng/mL was able to induce VEGF expression ( $p < 0.05$ ) compared to control. When LPS and OSM were combined, there were enhanced levels of VEGF that were detected in

the supernatants (Fig. 8 b). When the estimated additive levels obtained by combining the effects observed after treating the cells with LPS alone or OSM alone were compared with the observed effects of the combined treatment, there was statistically significant synergy seen at the dose of 10 ng/mL LPS and 5 ng/mL OSM ( $p < 0.05$ ). None of the other gp130 cytokines tested (LIF, IL-6, IL-31, IL-11) in isolation induced detectable VEGF expression by the HAoAFs (Fig. 8 c, d, e, f), and only at the highest dose of LPS was IL-6 capable of causing augmented VEGF expression in the combination compared to LPS alone ( $p < 0.05$ ) (Fig. 8d).

### **RNA Analysis**

In order to determine the levels of steady state mRNA expression as well as to test some novel end-points that were not measured by ELISA, HAoAF RNA expression was analyzed by quantitative RT-PCR.  $\beta$ -Actin was used as the reference marker, and all values were normalized to the results obtained for the 'media' treatment (given a value of 1). In addition, the supernatants from both the 2 and 6 hour treated cells were collected and analyzed for IL-6 using an ELISA (Fig. 13)

### ***IL-6 Expression***

When analyzed by ELISA, both the 2 hour and 6 hour supernatants of cells stimulated with LPS and OSM in combination showed synergistically elevated IL-6 protein levels (Fig. 10 a and b respectively), compared to either



treatment alone, which coincides with the previous ELISA data (Fig. 6 b and c), and confirms that the cells were responding in a similar manner to the treatments. The RNA data indicates that at 2 hours post stimulation, there was an 18 fold induction of IL-6 RNA levels in LPS stimulated cells compared to media alone. In addition, 5 ng/mL of OSM alone was able to induce a 4 fold increase in IL-6 RNA, while the lower dose of OSM had no effect compared to control (Fig. 12a). When LPS was combined with 0.5 ng/mL OSM, there was no change in IL-6 RNA expression compared to LPS alone, while when combined with 5 ng/mL OSM, there was only a 9 fold induction of IL-6 RNA. This seems to be contrary to the ELISA data performed on the supernatants from these samples.

At the 6 hour time point, the ELISA and RNA data appeared to be more congruent. LPS alone was able to induce a 21 fold increase in IL-6 RNA expression compared to control, while the combined treatments resulted in 38 and 29 fold inductions of IL-6 RNA when LPS was combined with the lower and higher dose of OSM respectively compared to control (Fig. 9e). The observed RNA expression levels of LPS combined with 0.5 ng/mL OSM was higher than the estimated additive effect of LPS and 0.5 ng/mL OSM based on their induction of IL-6 RNA alone, while there was not a significant difference between the theoretical and observed IL-6 RNA levels with the LPS and 5 ng/mL OSM treatment .

### ***IL-8 Expression***

IL-8 protein expression in HAoAF cell supernatants was analyzed by ELISA which indicated that OSM resulted in suppression of LPS induced IL-8 (Fig. 7b). At both 2 (Fig. 9b) and 6 hours (Fig. 9f), LPS markedly increased the expression of IL-8 RNA levels (72 and 66 fold increases respectively compared to media). When OSM alone was applied to the cells, there was no difference in terms of RNA expression levels compared to control. There was a dose dependent inhibition of IL-8 RNA expression levels by the HAoAF cells at both time points when OSM and LPS were combined. The RNA data at 2 and 6 hours corroborates the protein expression data observed 18 hours post stimulation.

### ***VEGF Expression***

Demyanets *et al.* [146] demonstrated that Vascular Endothelial Growth Factor (VEGF) is induced by OSM in vascular SMCs. To the best of our knowledge, the same has not been demonstrated in vascular adventitial fibroblasts. The RNA data here suggests that by 2 hours, HAoAFs expressed a 3 fold increase in VEGF mRNA levels when stimulated with 5 ng/mL OSM (Fig. 9c). By 6 hours, there was a 3, and 5 fold increase in VEGF mRNA expression levels when cells were stimulated with 0.5 or 5 ng/mL OSM respectively, compared to media alone (Fig. 9g). The RNA and protein (ELISA) data seem to indicate that a potent stimulator of angiogenesis can be induced in the adventitial fibroblasts if OSM is present. At neither time point was there synergistic expression of VEGF RNA when LPS was combined with OSM despite there being elevated levels of protein when analyzed via ELISA at 18 hours (Fig. 8b).

### ***MCP-1 Expression***

As described in the introduction, the role of MCP-1 in atherosclerosis has been well characterized, and it is known to be central in monocyte recruitment to the plaque [277,278]. It was tested whether MCP-1 mRNA expression levels were being up-regulated in the HAoAFs. A 2 hour stimulation of HAoAFs with LPS resulted in a 15 fold increase in MCP-1 RNA levels, and with 0.5 or 5 ng/mL OSM resulted in a 3 and 10 fold increase respectively compared to control levels (Fig. 9d). The combined treatments at 2 hours were not additive. At 6 hours however, the combined treatment resulted in a marked increase in MCP-1 mRNA levels above LPS or OSM expression alone, with the combined treatments reaching 66 (LPS + 0.5 ng/mL OSM) and 68 (LPS + 5 ng/mL OSM) fold the expression of CCL2 mRNA levels compared to media alone (Fig. 9 h).

### ***Synergistic IL-6 induction by HAoSMCs in response to LPS and OSM***

It is currently accepted that during development of atherosclerotic lesions, vascular smooth muscle cells migrate into the vascular intima, proliferate, and are involved in forming part of the fibrous cap that protrudes into the lumen of the artery [15]. It was thus of interest to assess whether these cells can be regulated by OSM, and/or the TLR-4 ligand LPS. Primary Human Aortic Smooth Muscle Cells (HAoSMCs) were purchased from Lonza Bio. These cells were isolated from a 36 year old male patient that passed away from a cause other than vascular disease. In order to detect the inflammatory proteins secreted by these cells, HAoSMCs

were cultured *in vitro*, were stimulated for 18 hours, and then the supernatants were analyzed via ELISA.

When HAoSMCs were stimulated with OSM, there was a dose dependent response of IL-6 detected in the supernatants at OSM concentrations of 0.1 ( $p < 0.01$  vs. control), 1, and 10 ng/mL ( $p < 0.0001$  vs. control) (Fig. 11a). Upon stimulation with LPS only, a dose dependent IL-6 response was measured that was observable at 1 ng/mL of LPS and was maximal at 10 ng/mL LPS ( $p < 0.0001$  vs. control) (Fig. 11 b and c). When a constant dose of 5 ng/mL OSM was included in the treatment of cells being stimulated with LPS, there was a synergistic IL-6 response (Fig. 11c) For instance, OSM alone at 5 ng/mL resulted in detection of ~9 900 pg/mL of IL-6, LPS at 1 ng/mL resulted in detection of ~7 000 pg/mL of IL-6, while the 5 ng/mL OSM combined with 1 ng/mL LPS resulted in ~23 000 pg/mL IL-6 that was detected, significantly greater than the ~17 000 pg/mL that might have been predicted had the responses been additive ( $p < 0.01$ ). In addition, this synergistic response with LPS was also observed using low doses of OSM (50 and 100 pg/mL) (Fig. 11b). HAoSMCs were also stimulated with LIF or IL-31 in conjunction with increasing doses of LPS (Fig. 11 d and e), however IL-6 levels that were detected in the supernatants did not vary from the levels that were detected in cells stimulated with LPS alone. Upon stimulation with the combination of LPS and IL-11 (Fig. 11f) there appeared to be a suppression of IL-6 production that was detectable in the supernatants. Thus, of the gp130 cytokines assayed, OSM was unique in its ability to induce IL-6 that

was detectable in these cells alone, and in its ability to synergize with LPS in induction of IL-6 levels.

### ***OSM Suppresses LPS induced IL-8 induction in HAoSMCs***

OSM synergized with LPS in the MAAFs to induce expression of the mouse functional homolog of IL-8 (KC) (Fig. 2b), while OSM suppressed HAoAF IL-8 expression in combination with LPS compared to LPS alone (Fig. 7b). IL-8 levels were also analyzed in the HAoSMC supernatants by ELISA after 18 hours of stimulation. LPS independently caused a dose dependent increase in HAoSMC IL-8 response (Fig. 12b), while treatment with OSM alone resulted in no IL-8 detection (Fig. 12a). Similar to the human fibroblast cells, instead of augmentation, there was a dose dependent suppression of IL-8 levels when OSM was combined with LPS (Fig. 12b). There was no significant effect on IL-8 expression when LIF or IL-6 was combined with LPS compared to LPS alone (Fig. 12 c, d). Upon stimulation of HAoSMC with the highest dose (100 ng/mL) of LPS and either dose of IL-31, there was a significant increase in IL-8 expression ( $p < 0.01$ ) (Fig. 12 e). There is a trend towards suppression of IL-8 upon stimulation of HAoSMC with IL-11 in combination with LPS (Fig. 12 f).

### ***Synergistic VEGF induction by HAoSMCs in response to LPS and OSM***

In both MAAFs and HAoAFs, the combined treatment of LPS with OSM resulted in synergistic VEGF protein expression. To test whether the same phenomenon occurred in vascular wall smooth muscle cells, HAoSMCs were cultured, and treated for 18 hours, at which point the supernatants were collected

for analysis by ELISA for VEGF. As shown previously [146], OSM induced VEGF in HAoSMC supernatants (Fig. 13a). LPS treatment alone did not induce VEGF to a significant degree in these cells (Fig. 13b). The combined treatment of increasing doses of LPS and a constant dose of either 0.5 or 5 ng/mL of OSM resulted in dose dependent synergistic VEGF detection (Fig. 13b). When a constant dose of 0.5 or 5 ng/mL of LIF, IL-6, IL-31, or IL-11 combined with LPS were assessed, there was no alteration in VEGF detection levels in the supernatants (Fig. 13c,d,e, and f respectively). Thus, among the gp130 cytokines tested, only OSM was able to synergize with LPS, and induce VEGF production in the HAoSMCs.

### **RNA Analysis**

In order to assess the mRNA steady levels and their correlation to protein levels that were measured by ELISA, mRNA was isolated from the HAoSMCs. *In vitro*, HAoSMCs were cultured, and then treated for either 6 or 18 hours with different concentrations of LPS and/or OSM as indicated in Figure 14. All values were expressed relative to  $\beta$ -Actin, and were normalized to control.

### ***IL-6 Expression***

At both 6 and 18 hours post stimulation, 10 ng/mL LPS, 0.5 ng/mL OSM, and 5 ng/mL OSM treated HAoSMCs expressed increased levels of IL-6 steady state mRNA compared to control treated cells (Fig. 14 a, e). When LPS was combined with 0.5 or 5 ng/mL OSM 6 hours post treatment there was a 17.6 and a

34.8 fold induction of IL-6 mRNA respectively compared to control treated cells (Fig. 14a). When LPS was combined with 0.5 or 5 ng/mL OSM 18 hours post treatment there was an 18 and a 27 fold induction of IL-6 mRNA respectively compared to control treated cells (Fig. 14e). At both time points the estimated additive effect on IL-6 RNA expression (obtained by summing the effects of LPS alone and OSM alone) was markedly lower than the observed combined effect at both the low and the high doses of OSM.

### ***IL-8 Expression***

The expression of IL-8 RNA in the HAoSMCs corroborates the protein data obtained by ELISA both in terms of the effects of LPS alone, and the effects observed after LPS is combined with OSM. At both 6 and 18 hours (Fig. 14 b and f) LPS induced significant increases in IL-8 mRNA expression compared to control levels. At both time points when 5 ng/mL OSM was combined with LPS to treat the cells, there was a marked decrease in IL-8 RNA expression compared to control levels.

### ***VEGF Expression***

VEGF ELISA performed on supernatants from HAoSMCs determined that LPS was unable to induce significant VEGF that was detectable (Fig. 13b). mRNA steady state levels of VEGF were similarly not enhanced above control levels at 6 and 18 hours (Fig. 14 c, g). The low and high doses of OSM treatment at 6 hours resulted in a 2.5 and a 2.8 fold induction respectively of VEGF mRNA compared to control (Fig. 14 c), and at 18 hours resulted in a 2.4 and 9.4 fold

induction respectively of VEGF mRNA compared to control (Fig. 14 g). At the 18 hour time point both the low and high dose of OSM when combined with LPS resulted in increased VEGF mRNA expression compared to either treatment alone (Fig. 14 g), while at the 6 hour time point only the lower dose of OSM when combined with LPS resulted in increased VEGF steady state mRNA expression compared to either treatment alone (Fig. 14 c).

### ***MCP-1 Expression***

Upon measurement of steady state mRNA levels in HAoAFs at 6 hours there were augmented levels of MCP-1 in the cells treated with LPS combined with OSM compared to control levels (Fig. 9 h). MCP-1 transcript expression was also measured in HAoSMCs both at 6 and 18 hours post stimulation (Fig. 14 d, h). At 6 hours post stimulation, LPS alone induced a 5 fold induction of MCP-1 RNA compared to control, while the low and high dose of OSM resulted in a 4 and 8.6 fold increase respectively compared to control levels (Fig. 14 d). The combined LPS and OSM treatment resulted in MCP-1 transcript levels 11.5 and 17.4 fold higher than control levels, and higher than the predicted additive combined effect. At 18 hours post stimulation, the treatments of LPS or OSM alone resulted in increased MCP-1 mRNA expression compared to control, while only the low dose of OSM when combined with LPS resulted in an augmentation of MCP-1 transcript expression compared to the treatments alone (Fig. 14 h).



## **RESULTS PART 2**

After determining in MAAF, HAoAF, and HAoSMC cell cultures that the combined treatment of LPS with OSM was able to induce synergistic effects in certain endpoints, potential mechanisms involved in mediating these effects were of interest. OSM has been shown in other systems to synergize with molecules by increasing the expression of that molecule's receptor. For example in mouse lung fibroblasts OSM is able to synergize with IL-4 and IL-13 to produce eotaxin by markedly elevating the IL-4R $\alpha$  and the IL-13R $\alpha$ 1 [204]. Thus initial experiments were performed to determine whether increased levels of a TLR-4 associated receptor component could reproduce effects seen with the combination treatment of LPS and OSM.

### ***HAoAF IL-6 Response to LPS and/or soluble CD14***

CD14 is found in both membrane bound and soluble forms and can associate with the TLR-4 receptor [205]. Soluble CD14 has been demonstrated to enhance LPS signaling [206,207]. Thus experiments were undertaken to determine if soluble CD14 enhanced LPS signalling *in vitro* in HAoAFs. The fibroblasts were stimulated with LPS alone or with LPS in combination with 100 ng/mL of soluble CD14 for 18 hours, at which point the supernatants were assayed for IL-6 by ELISA. LPS challenge alone resulted in a dose dependent augmentation of IL-6 as observed previously (Fig 6b and c), while the addition of soluble CD14 to LPS treatment did not have a marked effect on IL-6 expression compared to LPS alone (Fig. 15a). HAoAFs were also challenged with a constant

dose (1 ng/mL) of LPS in combination with increasing doses of soluble CD14 (Fig. 15e). The increasing concentrations of soluble CD14 did not cause detectable increases in IL-6 levels. Soluble CD14 did not appear to be a rate limiting step in the production of IL-6 in these cells.

#### ***TLR-4 Expression is Not Altered by OSM Treatment***

Flow cytometry was used to test whether OSM altered TLR-4 expression levels on MAAF. MAAFs were stimulated for 2, 6, or 24 hours, and then stained with either a TLR-4 antibody or an isotype control antibody, and the mean fluorescence of the cells was measured. Of note, the difference in fluorescence between the isotype control group and the treatment groups is quite small (Fig. 16 a), indicating either a high amount of non-specific binding, a lack of specificity of the antibody for TLR-4, or a low amount of TLR-4 expression on the cells. At 2 and 6 hours post treatment, there was no increase in TLR-4 expression on the cells treated with OSM compared to control cells. At 24 hours post-stimulation, there appeared to be a slight increase in the expression of TLR-4 on the surface of the MAAFs compared to media alone, however it did not reach statistical significance.

Since the TLR-4 staining by flow cytometry on the MAAFs was not robust, examining mRNA levels was used as an alternative approach for the human cells. HAOAFs were cultured *in vitro* and then treated for either 2 or 6 hours with different concentrations of LPS and/or OSM as indicated in Figure 16 b) and c). At 2 hours post treatment there was a slight decrease in TLR-4 mRNA

levels in all samples that were treated with OSM (Fig. 16 a). At this same time point LPS alone did not alter TLR-4 RNA levels compared to control (Fig. 16b). When HAoAFs were stimulated for 6 hours with LPS alone, there was a 2.3 fold increase in TLR-4 mRNA expression relative to media alone (Fig. 16 c). When HAoAFs were stimulated for 6 hours with OSM alone or in combination with LPS, TLR-4 transcripts were expressed at equivalent levels to when the cells received media alone (Fig. 16c). Therefore in HAoAFs, TLR-4 steady state mRNA is not augmented when the cells are stimulated for 2 or 6 hours with OSM.

Analysis of TLR-4 mRNA expression was also performed on the HAoSMCs at one time point that was the same as the HAoAFs (6 hrs post stimulation, Fig. 16 d), and at a later time point (18 hrs post stimulation, Fig. 16 e) as it was possible that TLR-4 transcript levels were increasing later than 6 hours. There was a 1.8 fold increase in TLR-4 mRNA levels in LPS treated cells compared to control at the 6 hour time point (Fig. 16 d), while at 18 hours there was no change in LPS treated cell TLR-4 mRNA levels compared to control (Fig. 16 e). At neither time point did OSM alone effect TLR-4 mRNA levels (Fig. 16 d and e), and at the 18 hour time point, 5 ng/mL OSM combined with LPS seemed to cause a 2 fold decrease in TLR-4 receptor levels. At the time points that were assessed, HAoSMCs did not increase TLR-4 steady state mRNA levels compared to levels measured in control treated cells.

### ***HAoAF OSMr- $\beta$ Expression Levels***

The results for experiments on either the TLR-4 itself or the associated receptor component CD14 indicated that it was unlikely that their up-regulation was responsible for the effects observed with the combined stimulation of the vascular wall cells with LPS and OSM. Another mechanism possibly mediating these effects could be that LPS is increasing the expression of one of the OSM receptor components, thus increasing the ability of the cells to respond to OSM. Human monocytes have been shown to increase the expression of OSMr- $\beta$  in response to LPS treatment [208].

To test this hypothesis, HAoAFs were stimulated for 2 or 6 hours with LPS, OSM, or LPS and OSM, and the OSMr- $\beta$  RNA levels were analyzed using RT-qPCR (Fig. 17 a and b). The results indicated that LPS induced a 1.8 fold increase in OSMr- $\beta$  transcript levels at 2 hours (Fig. 17 a) compared to cells treated with media alone. There was no increase in OSMr- $\beta$  RNA levels when HAoAFs were stimulated with 0.5 ng/mL OSM for 2 hours, while the combined treatment of LPS and 0.5 ng/mL OSM resulted in a 1.9 fold increase in OSMr- $\beta$  RNA levels compared to control treated cells (Fig. 17a). However, when 5 ng/mL OSM was combined with LPS, there was no increase in OSMr- $\beta$  mRNA levels compared to control treated cells at 2 hours (Fig. 17a).

OSMr- $\beta$  transcript levels increased 1.7 and 3 fold compared to control when treated with 0.5 or 5 ng/mL OSM respectively at 6 hours (Fig. 17b). While LPS alone was able to induce a 2.2 fold increase in OSMr- $\beta$  transcript levels

compared to control levels at 6 hours, this effect was not additive with the effect of OSM alone in the combined LPS and OSM treatments (Fig. 17b).

### ***HAoSMC OSMr- $\beta$ Expression Levels***

The levels of OSMr- $\beta$  mRNA expression were also measured in the HAoSMCs for the purpose of detecting changes in these levels post stimulation (Fig. 17 c and d). 6 hours following treatment LPS caused a 1.5 fold induction of the OSMr- $\beta$  transcript levels compared to control levels (Fig. 17 c). The cells treated with LPS + 5 ng/mL OSM had a 2 fold higher induction of OSMr- $\beta$  mRNA compared to 5 ng/mL OSM alone (3.5 vs. 1.7), while there was no difference in mRNA levels with the combined treatment of 0.5 ng/mL OSM and LPS compared with 0.5 ng/mL OSM alone (Fig. 17 c).

18 hours post stimulation the effect of LPS alone on OSMr- $\beta$  mRNA levels disappeared (Fig. 17 d). Despite this, OSMr- $\beta$  mRNA levels were elevated following treatment with LPS and 0.5 ng/mL OSM compared to 0.5 ng/mL OSM alone (3 fold vs. 1.7 fold). Once again this effect was only seen at one dose of the combined treatment, as there was no augmentation in RNA levels when 5 ng/mL OSM is compared to the combined LPS and 5 ng/mL OSM treated samples. After measuring the mRNA expression levels in HAoAFs and HAoSMCs, there did appear to be a slight increase in OSMr- $\beta$  levels following LPS treatment in three out of the four time points tested.

### **Immunoblots**

Another possible mechanism by which synergy could occur is amplification of signaling pathways within the cell upon co-stimulation. In order to elucidate the signaling pathways that were being activated, known downstream effectors of either LPS and/or OSM signalling were analyzed by Western blotting for phosphorylation. MAAF cells were cultured and then stimulated for 20 minutes (Fig. 18) or 4 hours (Fig. 19) with media (control), LPS alone, two different doses of OSM alone, or LPS in combination with the different doses of OSM.

#### ***MAAFs – STAT Phosphorylation***

Following stimulation for 20 minutes, STAT-1 and STAT-5 appeared to be phosphorylated by OSM alone at 5 ng/mL, or in combination with LPS, however there was no observable increase in the combined treatment compared to OSM alone (Fig. 18). STAT-3 has two different phosphorylation sites. When phosphorylated at Tyr 705, STAT-3 dimerizes, translocates to the nucleus, and binds to DNA [209]. Activation of the MAPK and mTOR pathways seems to induce phosphorylation at Ser 727, and maximizes transcription [210]. MAAF cells that were treated with OSM alone or in combination with LPS, but not with LPS alone, demonstrated a marked increase in the STAT-3 phosphorylation signal at both Tyr 705, and Ser 727 that was present at both 20 minutes and 4 hours (Fig. 18 and 19). This signal was not augmented when LPS was added.

***MAAFs – Akt Phosphorylation***

OSM has also been shown to induce phosphorylation of Akt Ser 473 in mouse lung fibroblasts [211]. When MAAF cell lysates were probed for phosphorylated Akt Ser 473 following stimulation with OSM alone for 20 minutes, there was dose dependent phosphorylation signal detected. The combination of OSM and LPS resulted in no change in signal intensity compared to OSM alone (Fig. 18). There was no detectable regulation of this pathway by any treatment by 4 hours (Fig. 19).

***MAAFs – MAPK phosphorylation***

Both LPS and OSM have been shown to activate MAP Kinase signaling pathways [62,134], and thus the phosphorylation signal of p38, ERK, and JNK were assessed. MAAF treated with LPS in combination with OSM resulted in a small but detectable increase in the p38 phosphorylation signal observed at Threonine 180/Tyrosine 182 compared to either LPS or OSM treatments alone (Fig. 18). The p38 phosphorylation signal also appeared to increase as the dose of OSM that was administered increased.

Antibodies to detect the phosphorylation of ERK 1/2 were also used to probe the MAAF lysates. There was not a consistent increase in the detected ERK 1/2 phosphorylation signal following the combined LPS and OSM stimulation compared to either treatment alone after 20 minutes or 4 hours.

In examining the phosphorylated JNK signal by western blot, neither LPS, nor OSM at 0.5 ng/mL resulted in phosphorylation of JNK at 20 min, while 5

ng/mL OSM resulted in a moderate amount of phosphorylation (Fig. 18). When LPS was combined with 0.5 or 5 ng/mL OSM, there was a dose dependent increase in the detectable JNK phosphorylation signal compared to either treatment alone.

#### ***MAAFs – NF- $\kappa$ B phosphorylation***

A known member of the TLR-4 signalling cascade is NF- $\kappa$ B. RelA (p65) is a component of this molecule that acts with p50 to induce transcription once I $\kappa$ B has been phosphorylated. Once inside the nucleus, p65 can be phosphorylated at several sites. Phosphorylation of NF- $\kappa$ B p65 at Ser 536 increases transcriptional activity, and decreases affinity of I $\kappa$ B-alpha for NF- $\kappa$ B [212]. To determine whether the MAAF cells were activating this pathway, MAAF lysates were probed for phosphorylated p65 at Serine 536, and for total p65 (Fig. 18 and 19). LPS stimulation alone or in combination with OSM induced phosphorylation of this motif at 20 minutes (Fig. 18) but not at 4 hours (Fig. 19). At 20 minutes, OSM treatment alone did not induce a phosphorylation signal at this site. At the 4 hour time-point, there was an increase in total p65 upon stimulation with OSM, and so despite the slightly darker bands that resulted from probing for phospho-p65 Ser 536 in OSM treated cells, this could be attributed to an increase in total p65.

#### ***HAoAFs – STAT phosphorylation***

To determine if human cells responded similarly, HAoAF cells were cultured and then stimulated for 20 minutes or 4 hours with media (control), LPS



alone, two different doses of OSM alone, or LPS in combination with the different doses of OSM (Fig. 20 and 21).

OSM was able to stimulate phosphorylation of STATs 1, 3, and 5 alone, and in combination with LPS after 20 minutes (Fig. 20). There was no increase in the phosphorylation signals detected above that achieved with OSM alone at these sites when LPS and OSM were combined. Of the STATs probed, only phosphorylation of STAT-3 at Tyrosine 705 was increased by OSM at 4 hours post stimulation (Fig. 21). The only STAT molecule that was phosphorylated by LPS alone was STAT-3 at Serine 727. This is not surprising, as MAPK activation causes phosphorylation at this site, and LPS caused phosphorylation of ERK1/2 after 20 min.

#### ***HAoAFs – Akt phosphorylation***

Similar to the MAAF<sub>s</sub>, OSM was able to stimulate phosphorylation of Akt at Serine 473 after 20 min, with no additional phosphorylation observable when LPS was combined with OSM (Fig. 20).

#### ***HAoAFs – MAPK phosphorylation***

In terms of the MAP Kinases, at 20 minutes post stimulation there was no phosphorylation of p38 with LPS alone or 0.5 ng/mL OSM alone. When LPS was combined with 0.5 ng/mL OSM, there was phosphorylation that was detectable (Fig. 20). 5 ng/mL OSM alone did produce a signal, however that signal was amplified when combined with LPS (Fig. 20). Therefore, as was seen with

MAAFs, it appears that when LPS is combined with OSM to treat the HAoAFs, there is an amplified p38 signal.

ERK 1/2 phosphorylation levels were regulated in a dose dependent manner by OSM, and were also regulated by LPS treatment, however the effects of the combined treatment was not greater than either treatment alone (Fig. 20). JNK phosphorylation signal was also regulated in a dose dependent manner by OSM, however there was no regulation of phosphorylation by LPS, and the combined treatment did not alter levels of phosphorylation detected compared to OSM alone (Fig. 20). In addition, neither JNK nor ERK 1/2 phosphorylation were detected at the 4 hour time point following stimulation (Fig. 21). Therefore, although there was an amplification of the JNK signal with the combined LPS and OSM treatment in the MAAF, this was not detected in HAoAF cells.

The final protein that was probed for phosphorylation was NF $\kappa$ B p65 at Serine 536. As expected, and as was observed in the MAAF, LPS treatment alone, or in combination with OSM resulted in increased phosphorylation at this site compared to control. OSM treatment had no effect relative to control, and did not amplify the signal when combined with LPS (Fig. 20).

In conclusion, of all the proteins that were probed for phosphorylation in the HAoAFs, only the p38 signal was amplified upon co-stimulation, which correlated with the synergistic cytokine response that was observed upon stimulation of the HAoAFs with LPS combined with OSM.

### **RESULTS PART 3**

There are numerous cytokines that have been implicated in atherosclerosis development and progression, and in reality many are present at the same time within the atherosclerotic lesion. The results described thus far have been focusing mostly on Oncostatin M, a single cytokine. Tests were thus completed to determine whether inhibiting OSM among a milieu of cytokines is effective in modulating HAoAF activity. Peripheral blood mononuclear cells (PBMCs) were isolated from a normal healthy volunteer, plated, and stimulated with 100 ng/mL of LPS. The supernatants were collected after 24 hours and frozen for future use.

#### ***LPS Stimulated PBMCs Produce IFN- $\gamma$ , IL-1 $\beta$ , IL-6, and OSM***

Analysis of the contents of the LPS stimulated PBMC supernatants (Conditioned Medium (CM)) indicated that there were significant levels of (approximate concentrations): IFN- $\gamma$  (300 pg/mL), IL-1 $\beta$  (2300 pg/mL), IL-6 (2900 pg/mL), and OSM (2200 pg/mL) (Fig. 22). In comparison, cultures of PBMCs that were unstimulated did not induce levels of detectable inflammatory molecules (Fig. 22). These LPS stimulated PBMC supernatants provided a milieu of proinflammatory cytokines including OSM.

#### ***Anti-OSM neutralizing antibody does not alter IL-6 in HAoAFs treated CM***

As illustrated in Fig. 23, treatment of HAoAFs with PBMC CM resulted in a robust IL-6 response that was dose dependent as measured by ELISA.

In order to determine the role that OSM was playing in terms of HAoAF cell IL-6 expression, neutralizing antibodies to either the OSM ligand, or the OSM receptor component OSMr- $\beta$  were employed. As a control, 1 ng/mL recombinant OSM was used to stimulate the cells alone, or in combination with a 1:1000 molar ratio of OSM to anti-OSM neutralizing antibody, or mouse IgG control antibody (6.8  $\mu$ g/mL of antibody) (Fig. 24a). This dose of neutralizing antibody resulted in an 80% reduction in recombinant OSM induced IL-6 levels compared to the mouse IgG antibody (Fig. 24a). A dilution of 1:800 of the PBMC CM was chosen for use with antibodies based on the dilution test (Fig. 23), and therefore the concentration of OSM in the CM was diluted from 2200 pg/mL to 3 pg/mL (and the IL-6 in the CM from 3000 pg/mL to 4 pg/mL). The amount of antibody that was added was in a 1:100 molar ratio of OSM ligand: neutralizing or control antibody (1.9 ng/mL of antibody). There was no detectable IL-6 inhibition when the anti-OSM neutralizing antibody was added to the PBMC CM compared to the IgG control antibody (Fig. 24a). This experiment was repeated, and the same results were observed.

***Anti-OSMr- $\beta$  neutralizing antibody inhibits IL-6 production in HAoAFs treated with LPS stimulated PBMC supernatants***

To determine whether a neutralizing antibody to one of the OSM receptor components could inhibit OSM mediated IL-6 levels in HAoAF supernatants, an anti-OSMr- $\beta$  neutralizing antibody was tested. To determine whether the effects of recombinant OSM could be inhibited, recombinant OSM and the anti-OSMr- $\beta$

neutralizing antibody were administered at the same concentrations as described above (1 ng/mL OSM and 6.8 µg/mL of antibody). The OSMr-β neutralizing antibody inhibited IL-6 production 45% compared to mouse IgG levels in cells stimulated with recombinant OSM (Fig. 24b). Using ten times less antibody than was used to inhibit the recombinant OSM, 681 ng/mL of anti-OSMr-β Ab inhibited IL-6 production by HAoAFs stimulated with PBMC CM by 42% compared to HAoAFs treated with CM and an equivalent dose of mouse IgG control antibody (1 800 pg/mL vs. 3 100 pg/mL) (Fig. 24b). This experiment was repeated, and similar results were observed.

## **FIGURES**

**Fig. 1** – MAAF cells were treated for 18 hours, and the supernatants were analyzed by ELISA for IL-6. a) MAAFs were treated with increasing doses of OSM. b) - f) MAAFs were treated with increasing doses of LPS alone or with 0.5, or 5 ng/mL b) OSM, c) LIF, d) CT-1, e) IL-31 or f) IL-11. Note: The limit of detection of the ELISA is 15 pg/mL. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)-f) compared to LPS alone at that concentration.

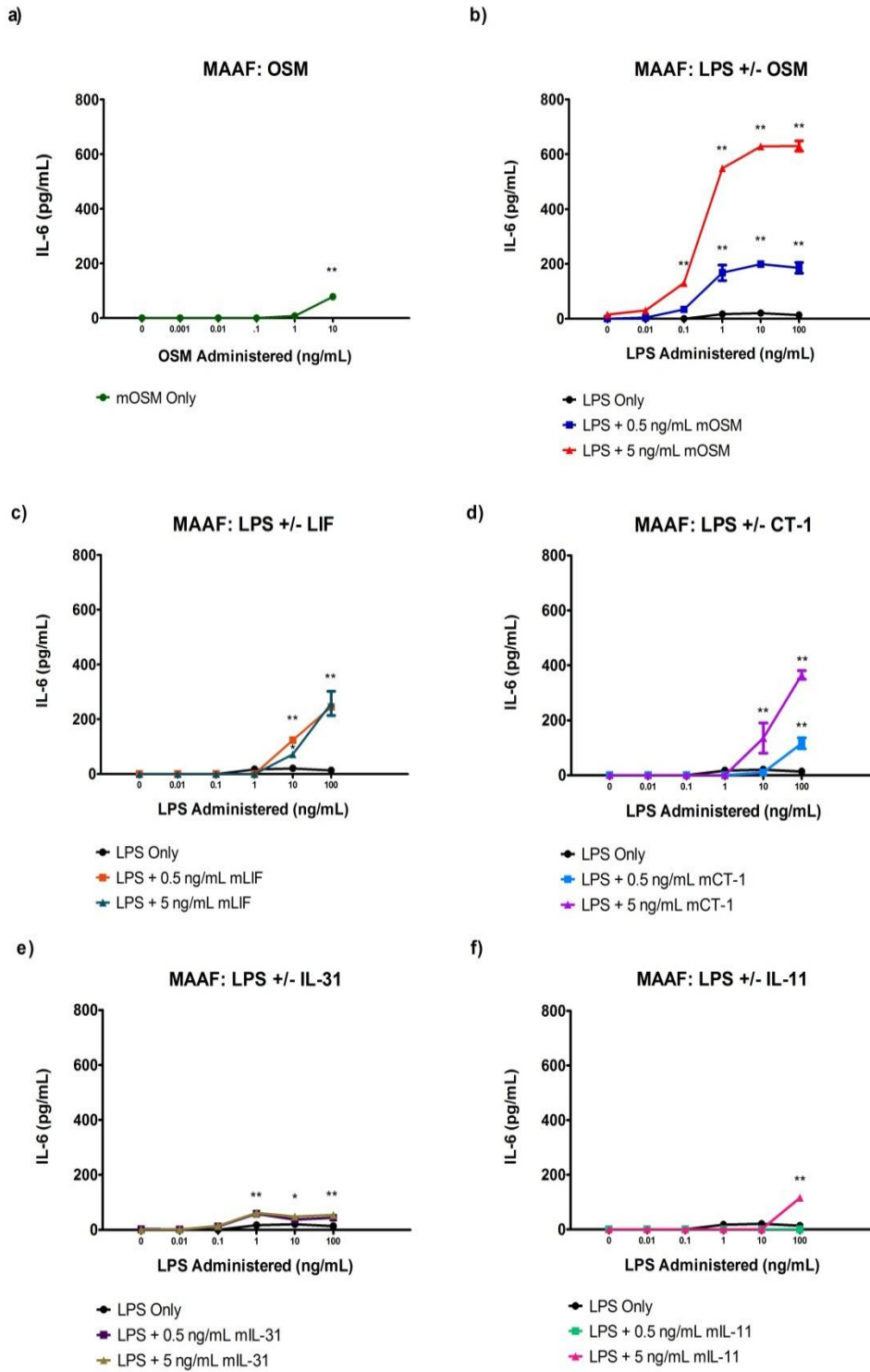


Figure 1

**Fig. 2** – MAAF cells were treated for 18 hours, and the supernatants were analyzed by ELISA for KC. a) MAAFs were treated with increasing doses of OSM. b)–g) MAAFs were treated with increasing doses of LPS alone or with 0.5, or 5 ng/mL: b) OSM, c) LIF, d) CT-1, e) IL-6, f) IL-11, or g) IL-31. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)–g) compared to LPS alone at that concentration.



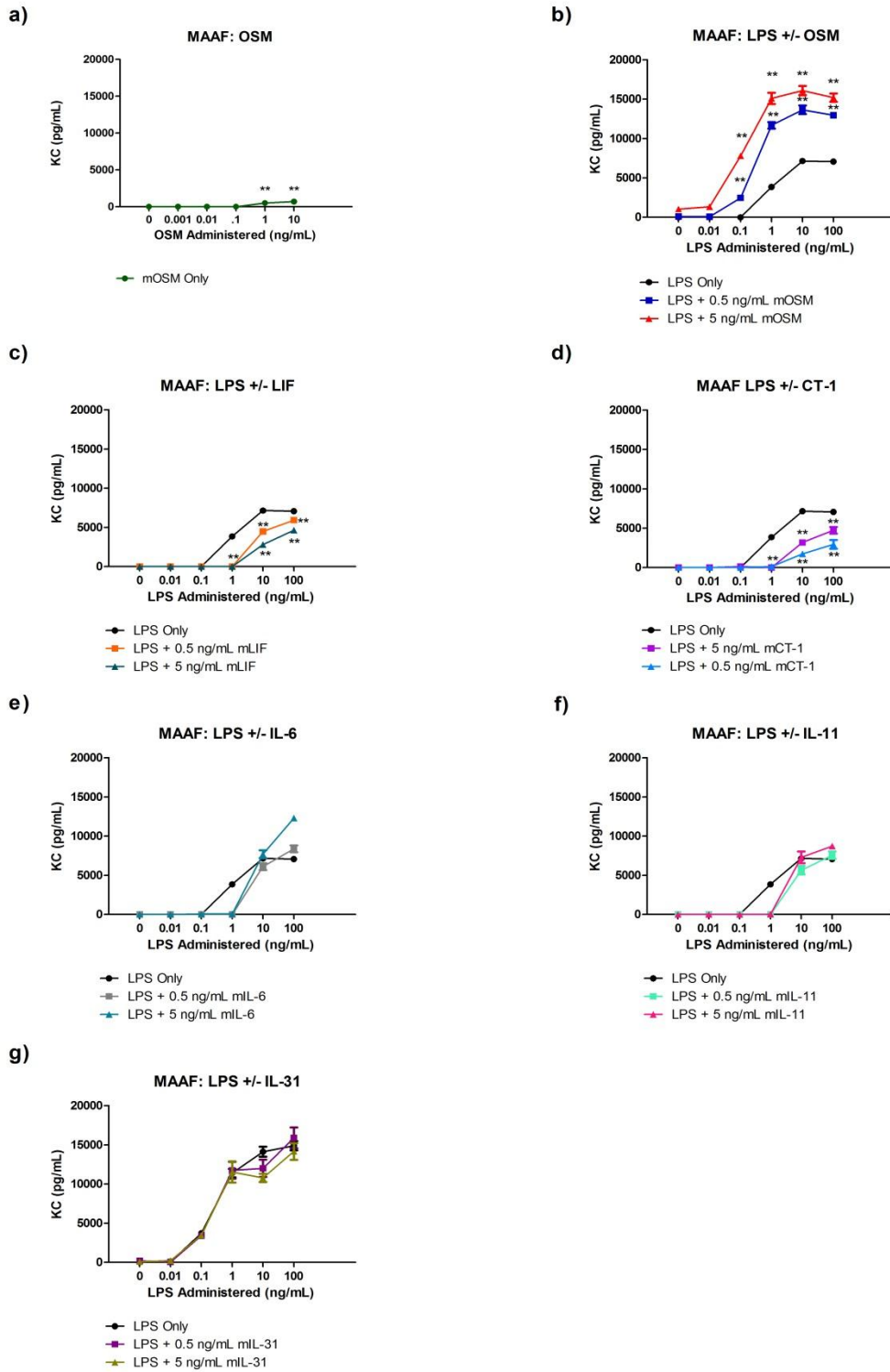
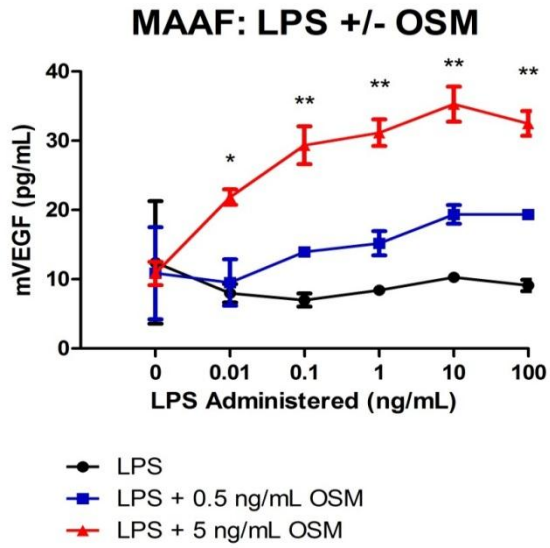


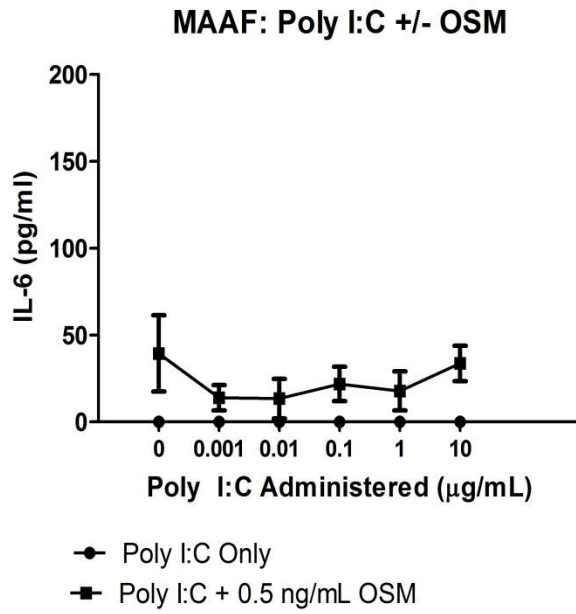
Figure 2

**Fig. 3** – MAAF cells were treated for 18 hours, and the supernatants were analyzed by ELISA for VEGF. MAAFs were treated with increasing doses of LPS alone or with 0.5, or 5 ng/mL OSM. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , compared to LPS alone at that concentration. Note: The limit of detection of the ELISA is 15 pg/mL. MAAFs were also treated with increasing doses of OSM alone, or LPS with 0.5, or 5 ng/mL of LIF, IL-6, IL-31, or IL-11, and none of the VEGF values were above the limit of detection of the ELISA (data not shown).



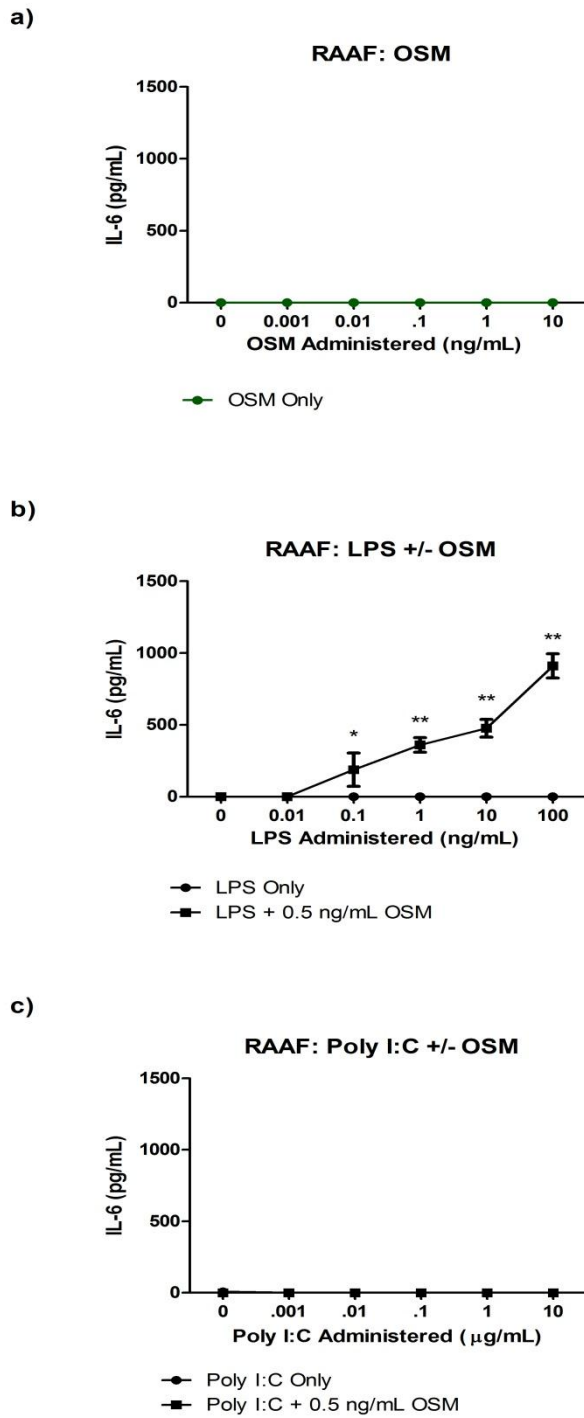
**Figure 3**

**Fig. 4** – MAAF cells were treated for 18 hours, and the supernatants were analyzed by ELISA for IL-6. MAAFs were treated with increasing doses of TLR-3 ligand Poly I:C alone, or with 0.5 ng/mL OSM. Note: The limit of detection of the ELISA is 15 pg/mL.



**Figure 4**

**Fig. 5** – RAAF cells were treated for 18 hours, and the supernatants were analyzed by ELISA for IL-6. a) RAAFs were treated with increasing doses of OSM. b) RAAFs were treated with increasing doses of LPS alone or with 0.5 ng/mL OSM. c) RAAFs were treated with increasing doses of Poly I:C alone or with 0.5 ng/mL OSM. Note: The limit of detection of the ELISA is 125 pg/mL. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , compared to LPS alone at that concentration.



**Figure 5**

**Fig. 6** – Human Aortic Adventitial Fibroblasts (HAoAFs) were treated for 18 hours, and the supernatants were analyzed by ELISA for IL-6. a) HAoAFs were treated with increasing doses of OSM. b)-f) HAoAFs were treated with increasing doses of LPS alone or with b) 0.1, or 0.5 ng/mL OSM, c) 5 ng/mL OSM, d) 5 ng/mL LIF, e) 5 ng/mL IL-31, or f) 5 ng/mL IL-11. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)-f) compared to LPS alone at that concentration.



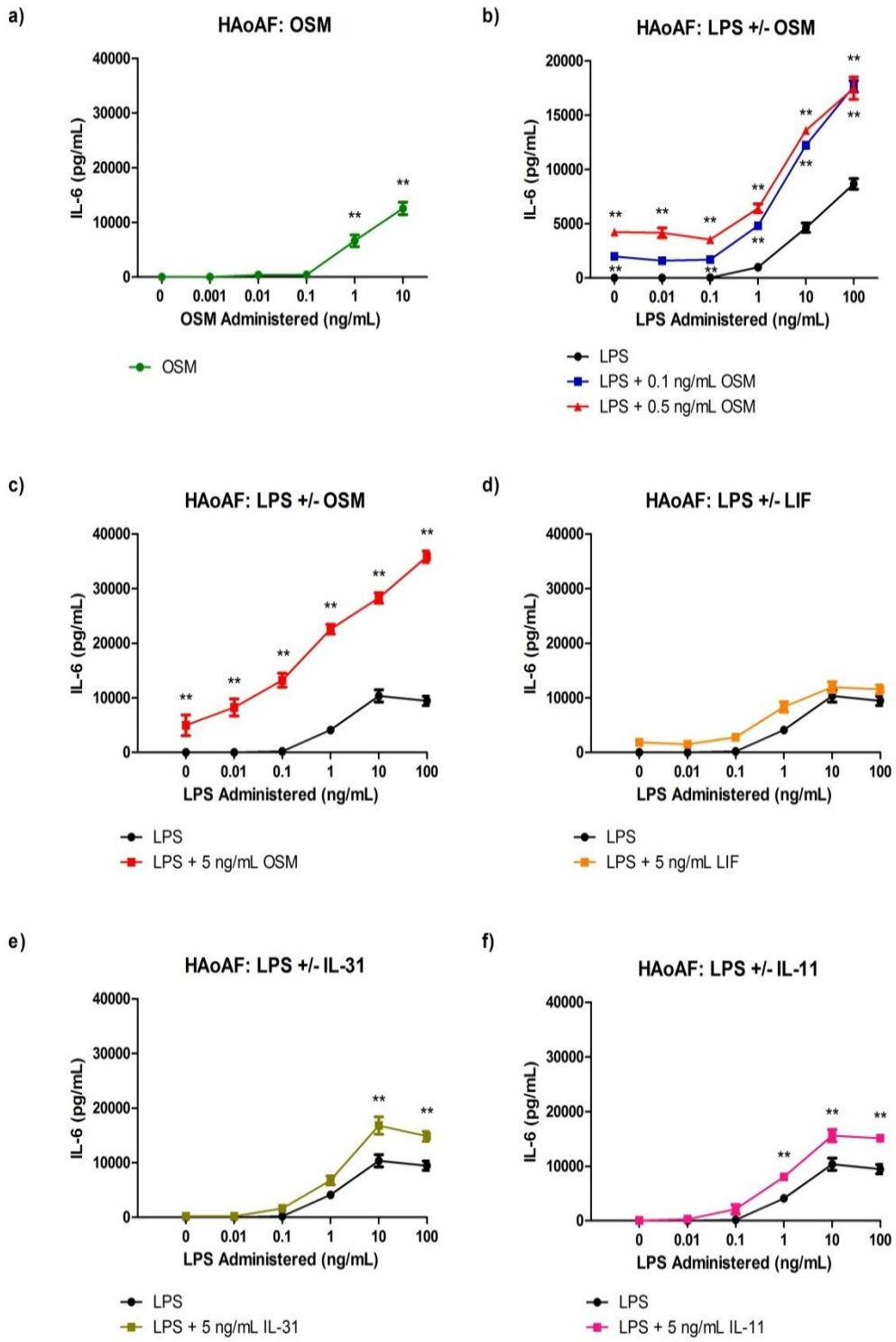


Figure 6

**Fig. 7** – Human Aortic Adventitial Fibroblasts (HAoAFs) were treated for 18 hours, and the supernatants were analyzed by ELISA for IL-8. a) HAoAFs were treated with increasing doses of OSM. b)-f) HAoAFs were treated with increasing doses of LPS alone or with 0.5, or 5 ng/mL b) OSM, c) LIF, d) IL-6, e) IL-31, or f) IL-11. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)-f) compared to LPS alone at that concentration.

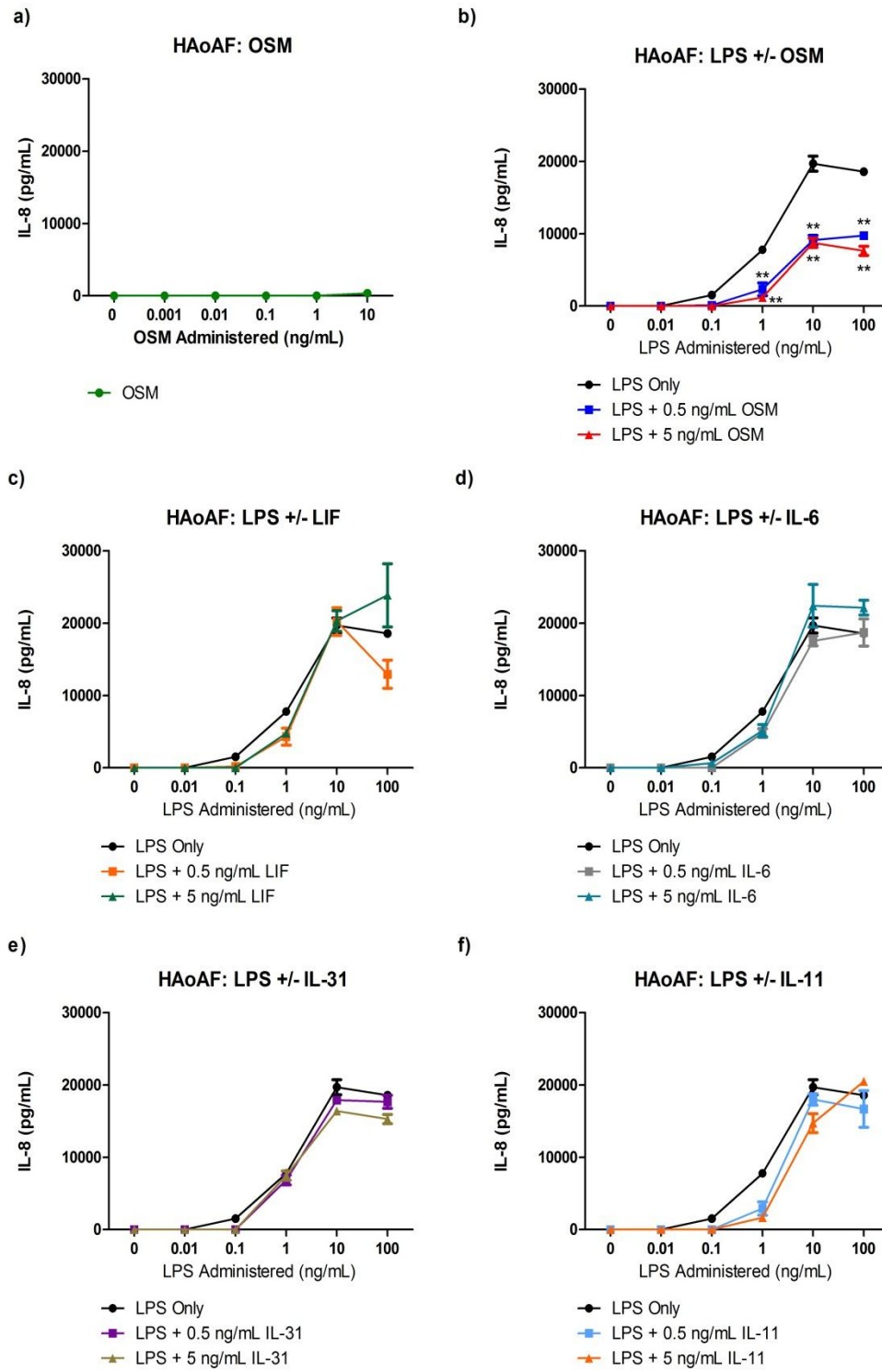


Figure 7

**Fig. 8** – Human Aortic Adventitial Fibroblasts (HAoAFs) were treated for 18 hours, and the supernatants were analyzed by ELISA for VEGF. a) HAoAFs were treated with increasing doses of OSM. b)-f) HAoAFs were treated with increasing doses of LPS alone or with 5 ng/mL b) OSM, c) LIF, d) IL-6, e) IL-31, or f) IL-11. Note: The limit of detection of the ELISA is 30 pg/mL. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)-f) compared to LPS alone at that concentration.

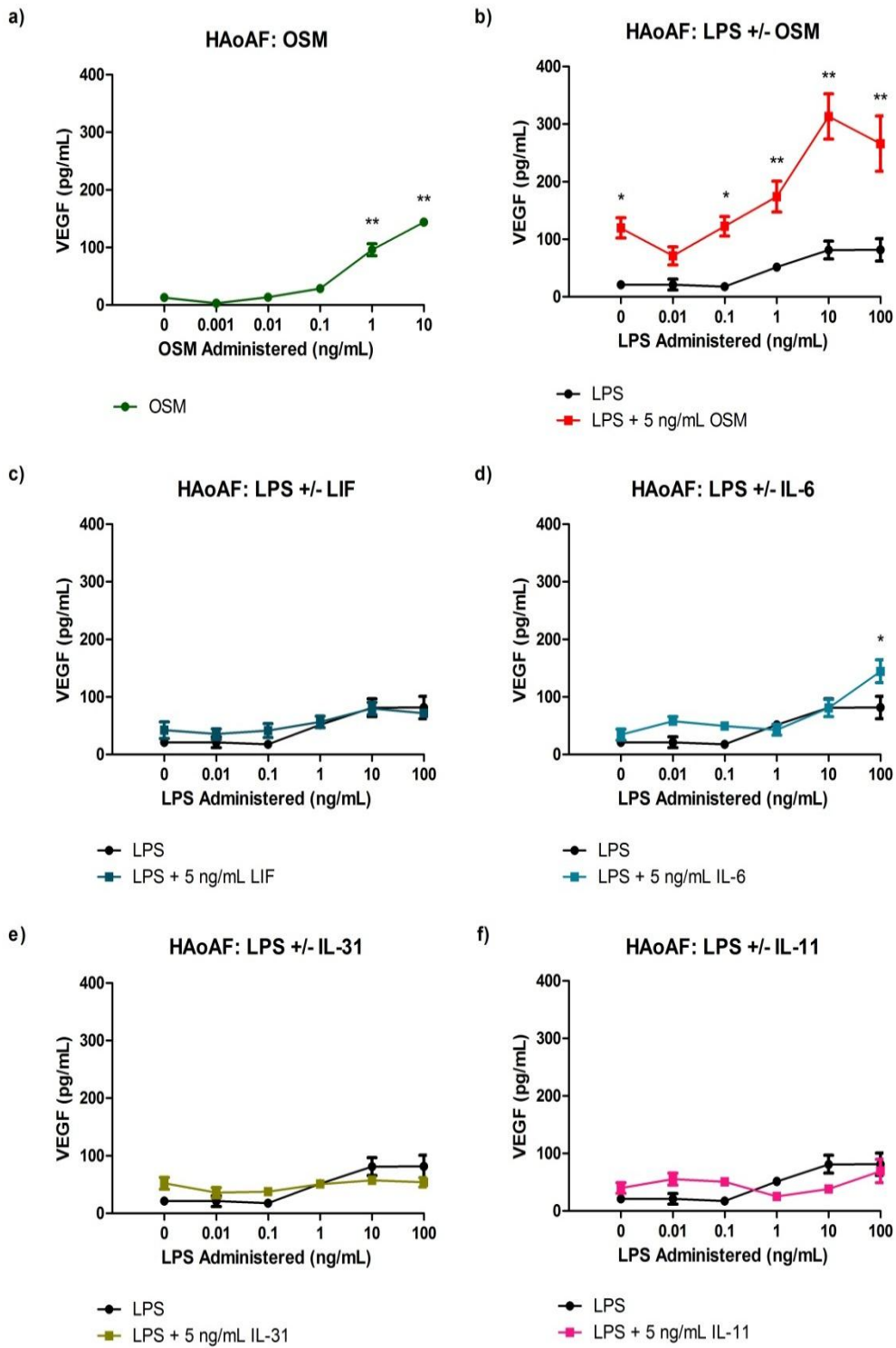


Figure 8

**Fig. 9** – HAoAF cells were stimulated for 2 hours [ a)-d) ] or 6 hours [ e)-h) ] with the treatments listed and then were lysed. The RNA was extracted, and RT-qPCR was performed to quantify the RNA. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ .

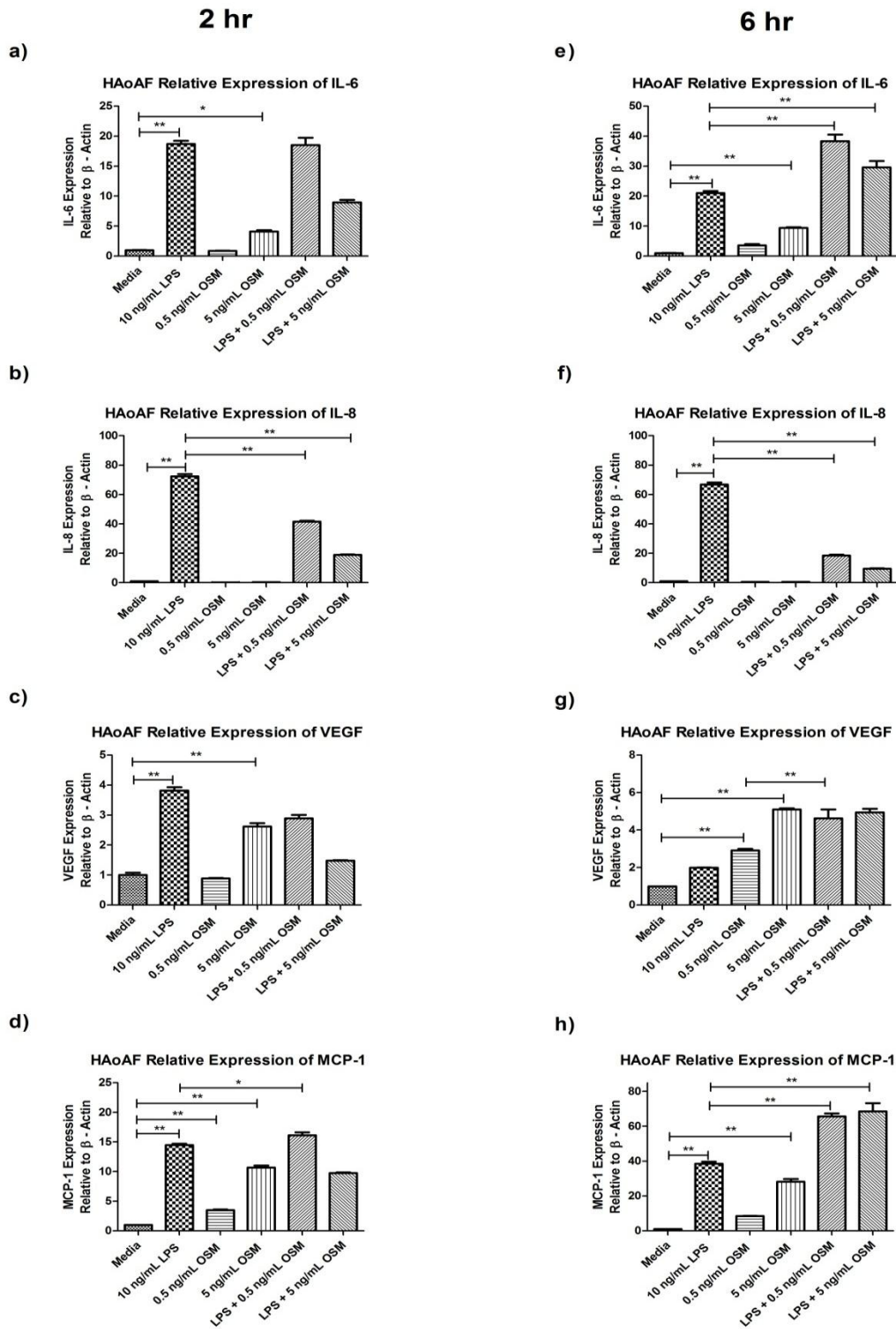
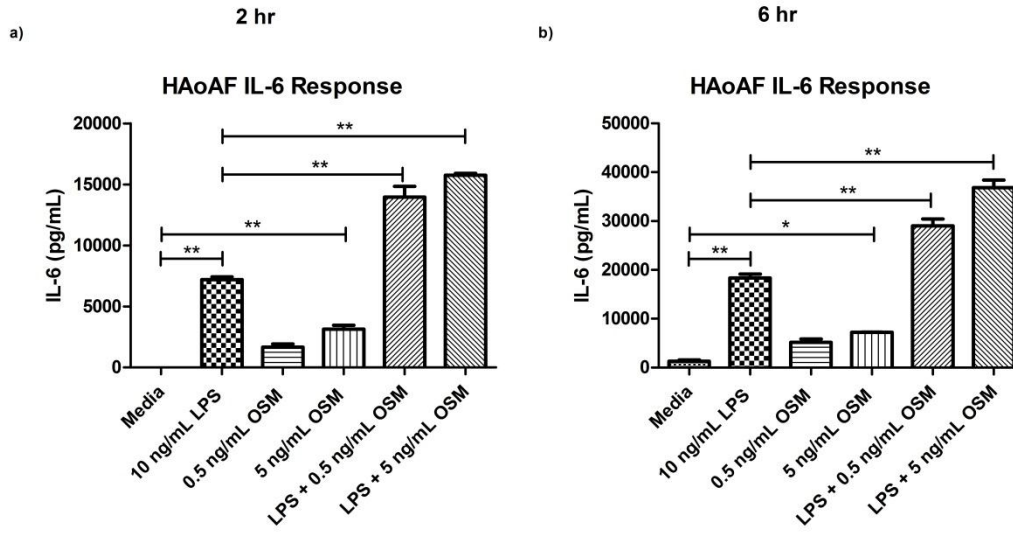


Figure 9

**Fig. 10** – Supernatants of HAoAF cells that were treated for a) 2 hours or b) 6 hours for RNA quantification were collected and analyzed by ELISA for IL-6. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ .





**Figure 10**

**Fig. 11** – HAoSMCs were treated for 18 hours, and the supernatants were collected and analyzed by ELISA for IL-6. a) HAoSMCs were treated with increasing doses of OSM. b) HAoSMCs were treated with increasing doses of LPS alone or with 0.05, or 0.1 ng/mL OSM. c) – f) HAoSMCs were treated with increasing doses of LPS alone or with 0.5 ng/mL c) OSM, d) LIF, e) IL-31, or f) IL-11. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)-f) compared to LPS alone at that concentration.

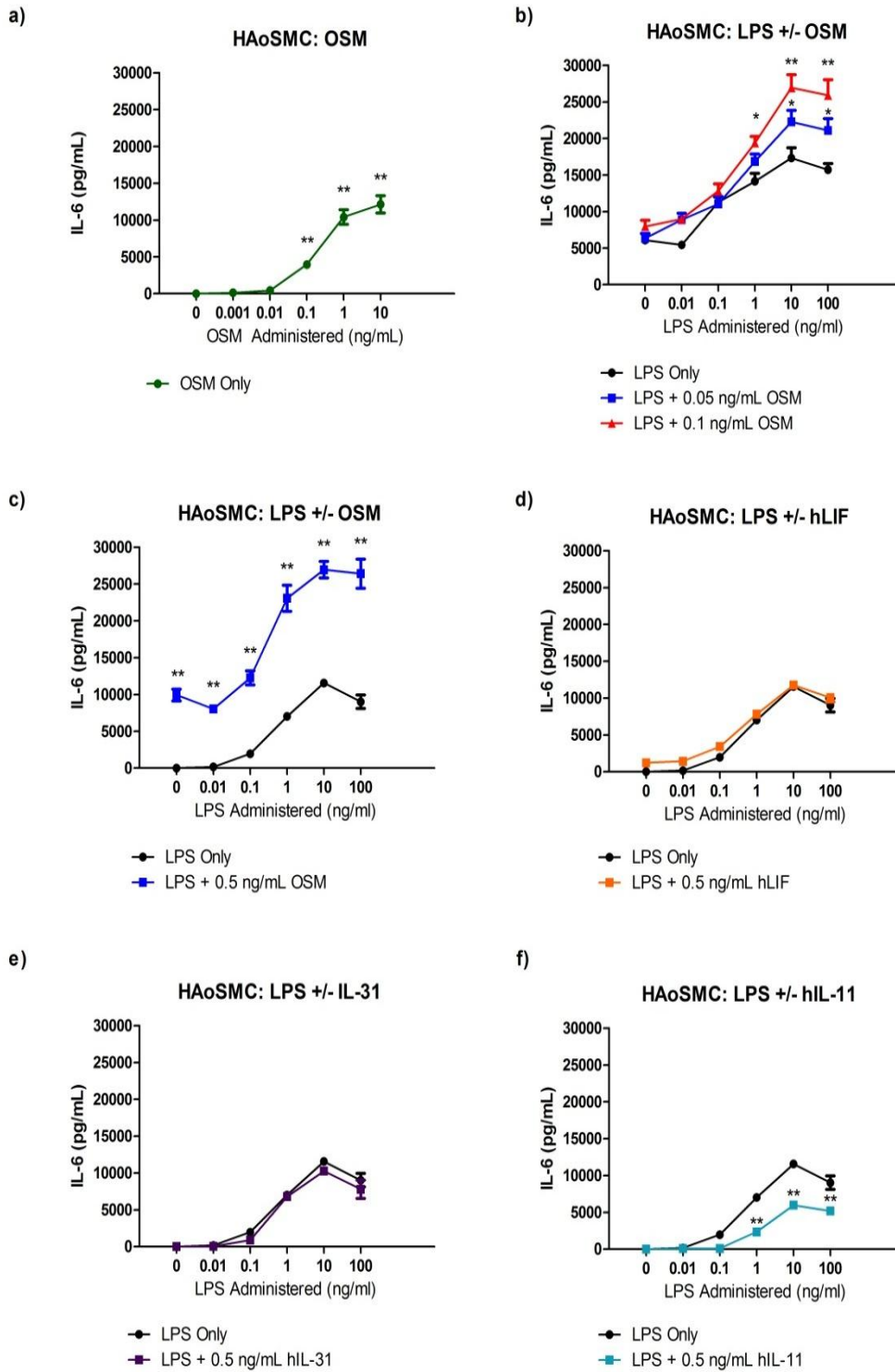


Figure 11

**Fig. 12** – HAoSMCs were treated for 18 hours, and the supernatants were collected and analyzed by ELISA for IL-8. HAoSMCs were treated with increasing doses of a) OSM alone, or HAoSMCs were treated with increasing doses of LPS alone or with 0.5, or 5 ng/mL b) OSM, c) LIF, d) IL-6, e) IL-31, or f) IL-11. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)-f) compared to LPS alone at that concentration.

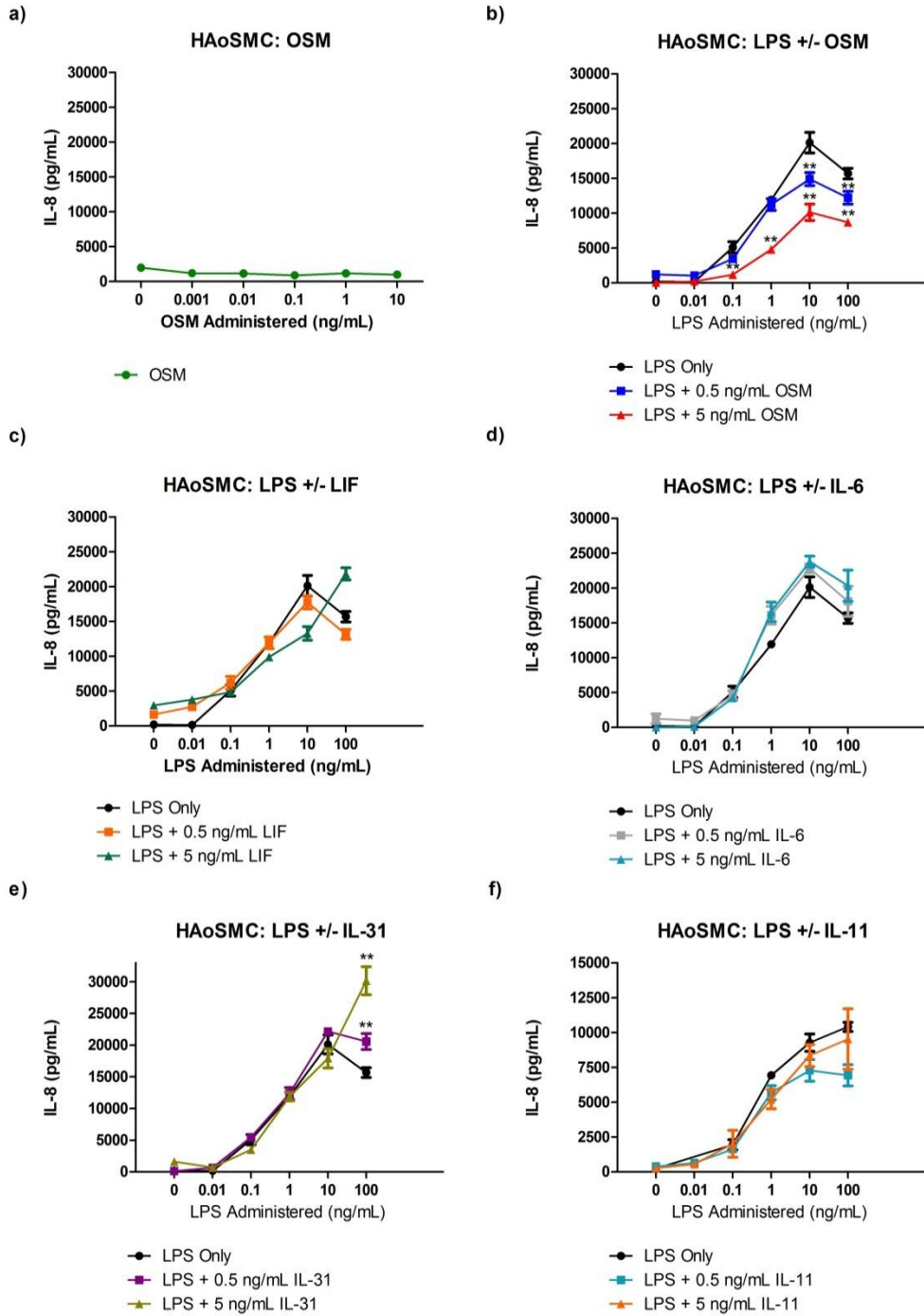


Figure 12

**Fig. 13** – HAoSMCs were treated for 18 hours, and the supernatants were collected and analyzed by ELISA for VEGF. HAoSMCs were treated with increasing doses of a) OSM alone, or HAoSMCs were treated with increasing doses of LPS alone or with 0.5, or 5 ng/mL b) OSM, c) LIF, d) IL-6, e) IL-31, or f) IL-11. Note: the limit of detection of the ELISA is 30 pg/mL. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ , a) compared to control, b)-f) compared to LPS alone at that concentration.

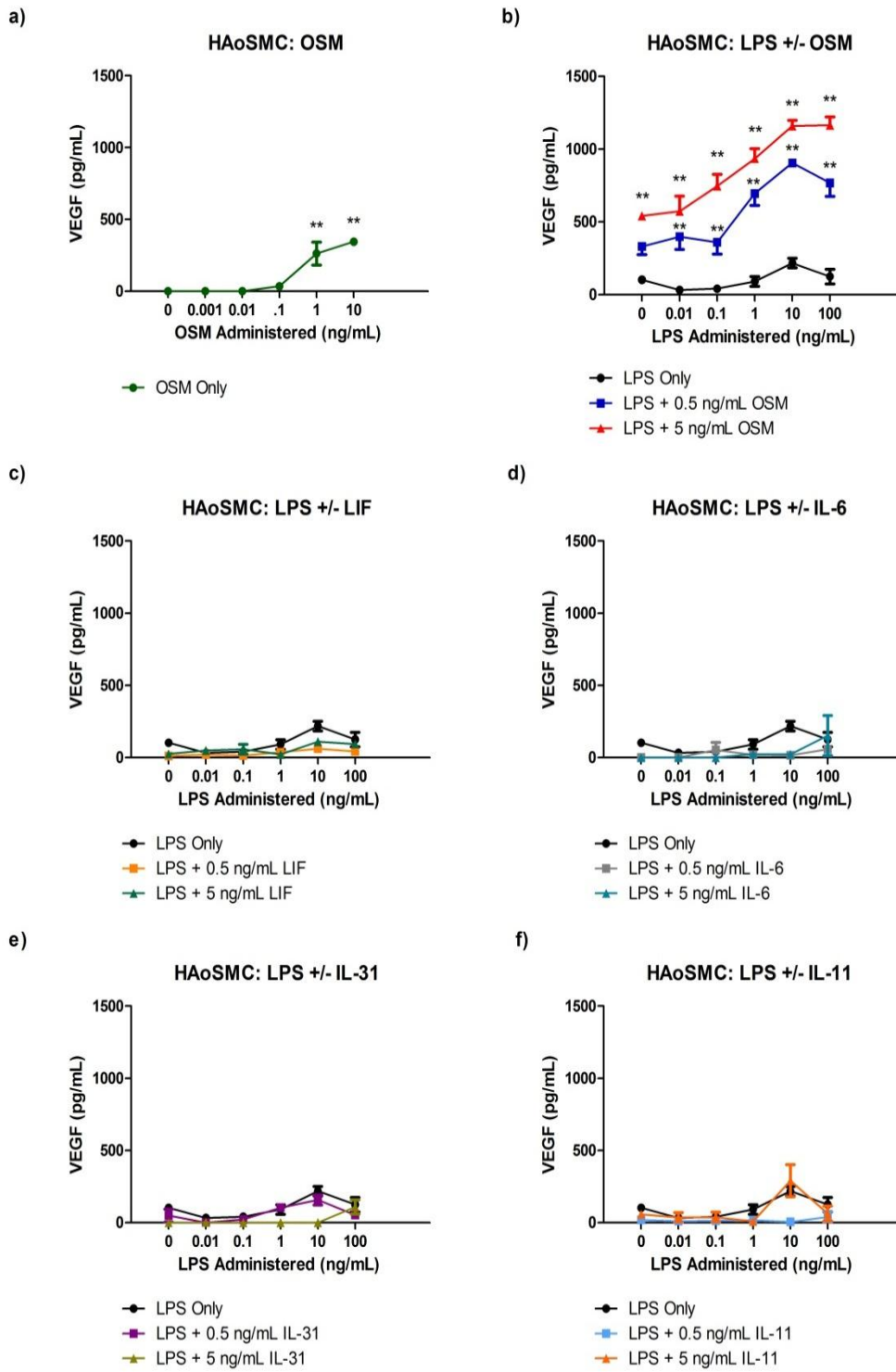


Figure 13

**Fig. 14** – HAoSMCs were stimulated for 6 hours [ a)-d) ] or 18 hours [ e)-h) ] with the treatments listed and then were lysed. The RNA was extracted, and RT-qPCR was performed to quantify the RNA. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ .



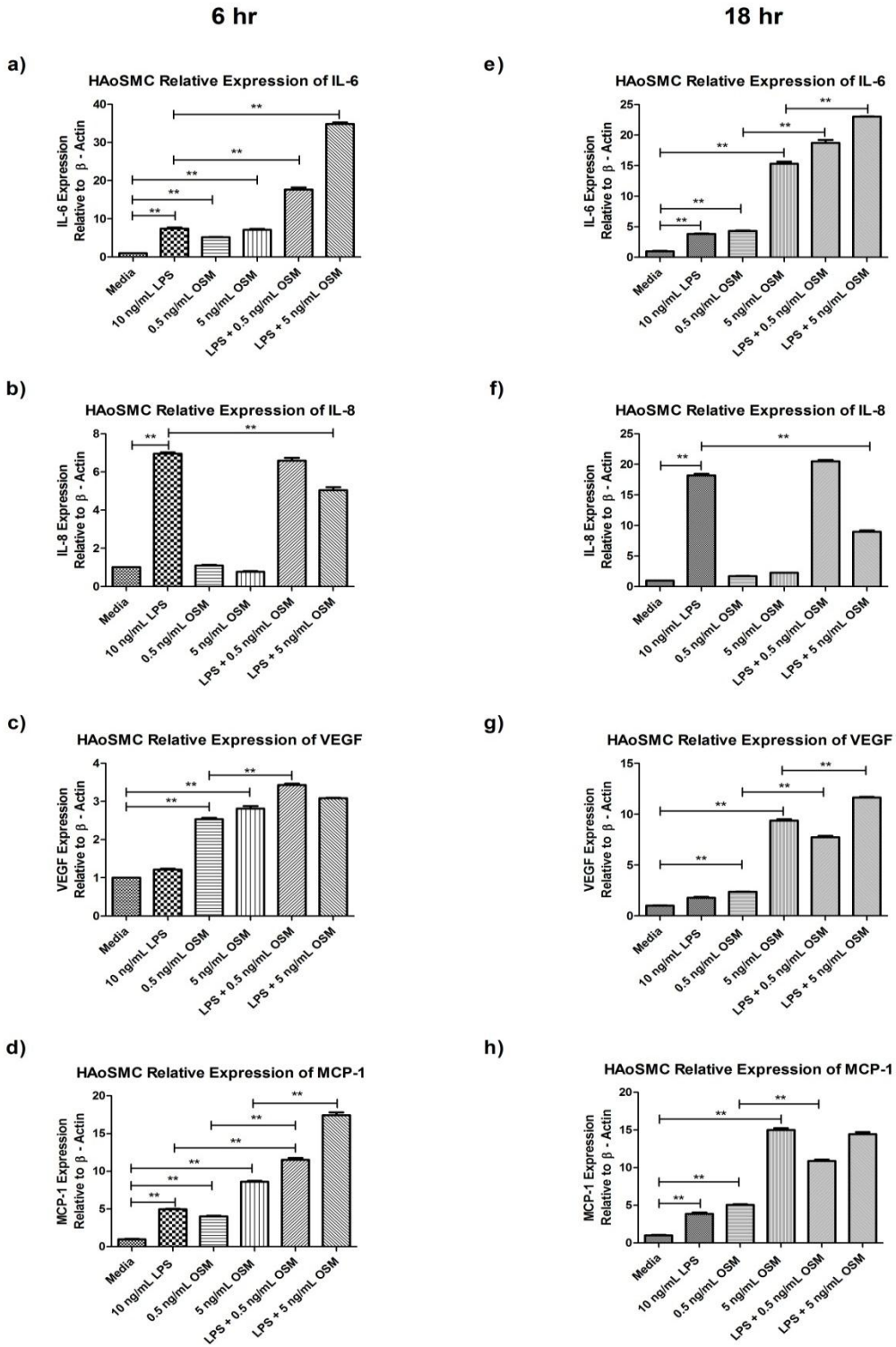
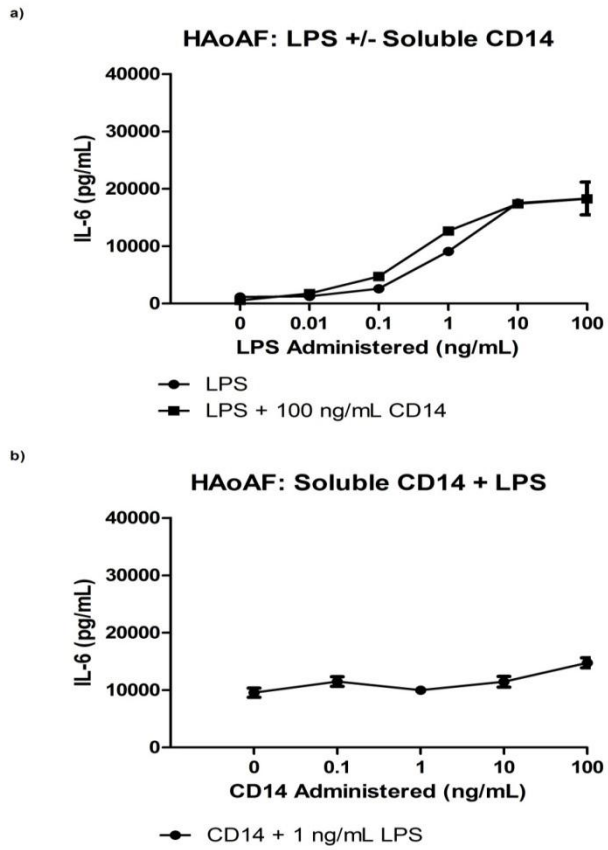


Figure 14

**Fig. 15** – a) HAoAFs were treated with increasing doses of LPS alone or with 100 ng/mL soluble CD14 b) HAoAFs were treated with a constant dose of LPS (1ng/mL) alone or in combination with increasing doses of soluble CD14. Supernatants were collected after 18 hours and analyzed by ELISA for IL-6.



**Figure 15**

**Fig 16** – a) MAAF<sub>s</sub> were treated for 2, 6, or 24 hours with either media (control) or 5 ng/mL of OSM. At the designated time point cells were lifted from the culture dish using 1mM EDTA in PBS. At each time point, the samples were stained with a FITC labelled TLR-4 or isotype control antibody. Mean fluorescence of the samples was measured using flow cytometry. b)-e) HAoAF cells were stimulated for b) 2 or c) 6 hours, and HAoSMCs were stimulated for d) 6 or e) 18 hours with the treatments listed and then were lysed. The RNA was extracted, and RT-qPCR was performed to quantify the RNA. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ .

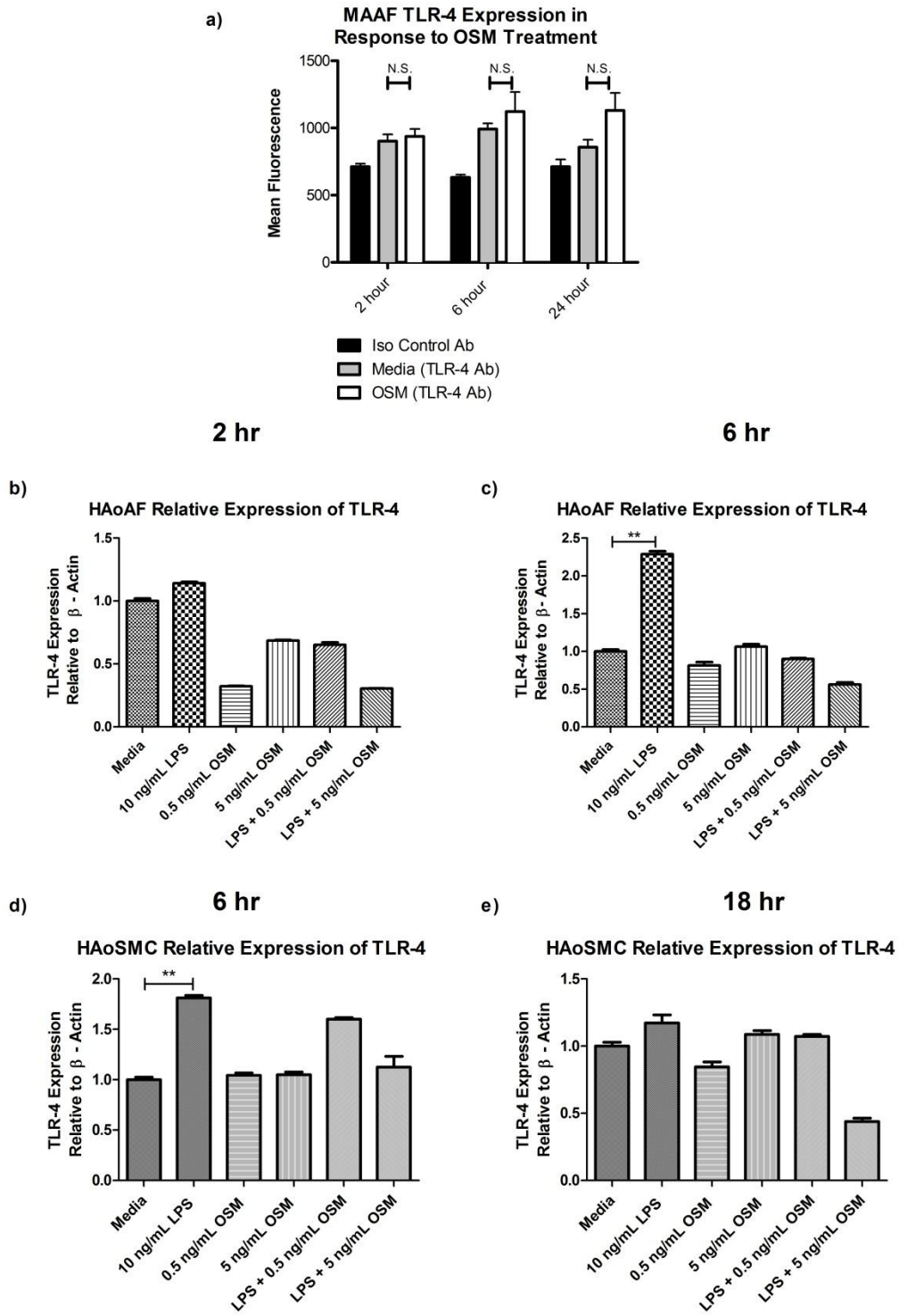


Figure 16

**Fig 17** – HAoAF cells were stimulated for a) 2 or b) 6 hours, and HAoSMCs were stimulated for c) 6 or d) 18 hours with the treatments listed and then were lysed. The RNA was extracted, and RT-qPCR was performed to quantify the RNA. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$ .

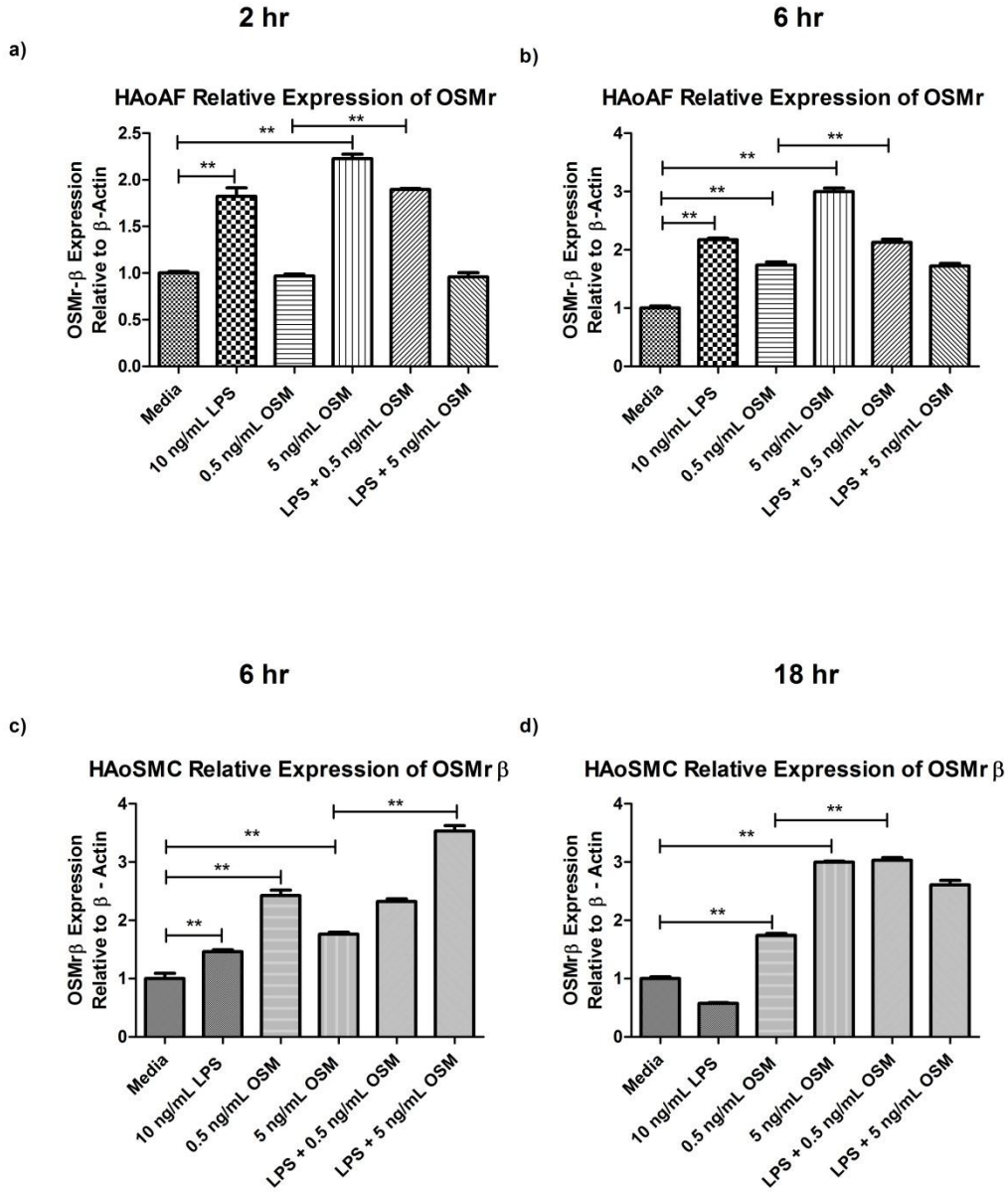
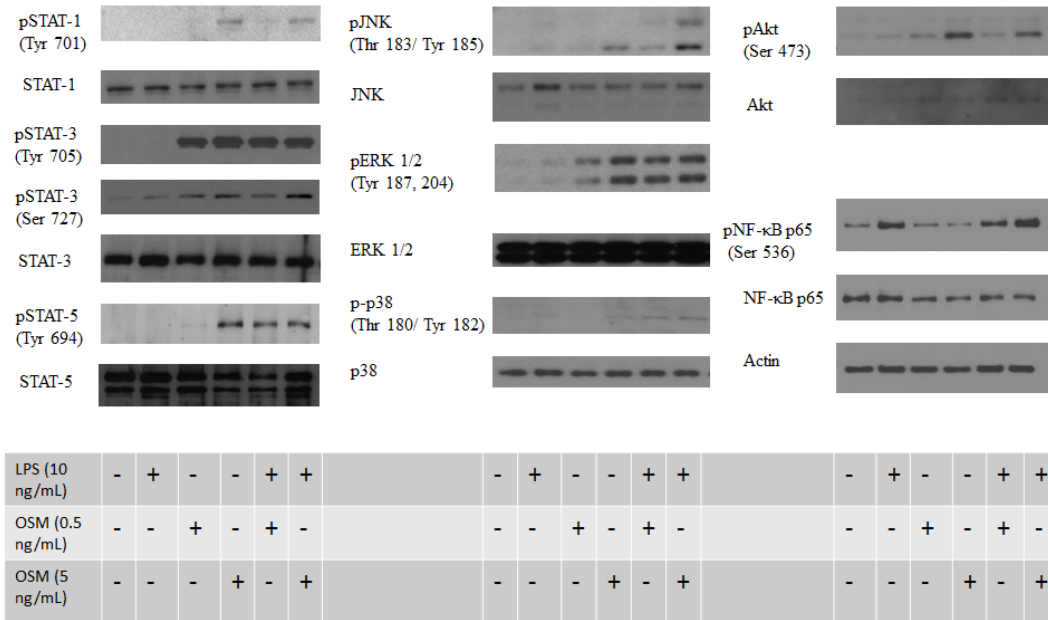


Figure 17

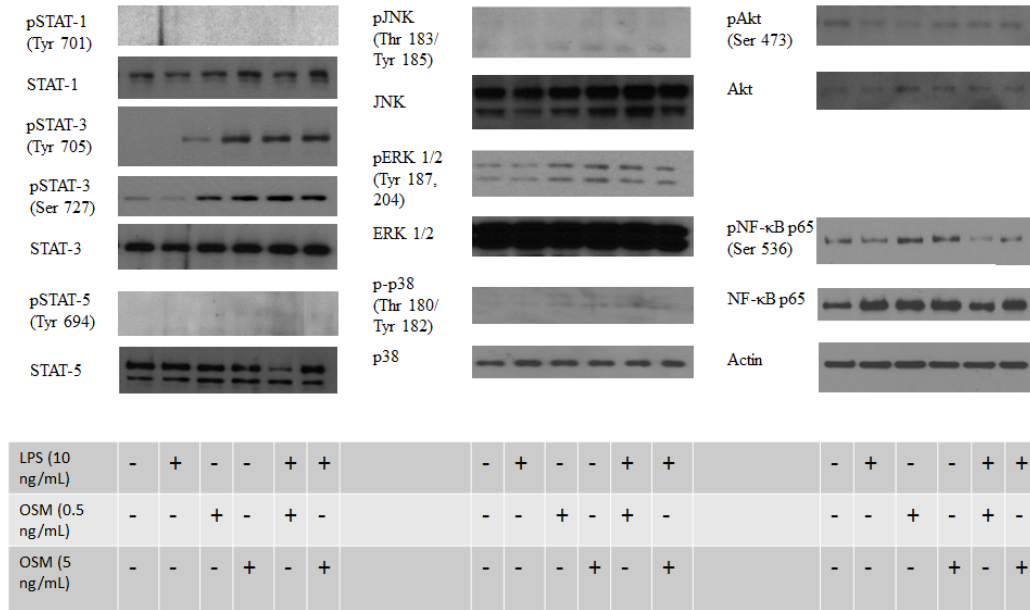
**Fig. 18** – Mouse Aortic Adventitial Fibroblasts (MAAFs) were treated for 20 minutes with media (control), LPS (10 ng/mL), OSM (either 0.5 or 5 ng/mL), or a combination of LPS (10 ng/mL) and OSM (either 0.5 or 5 ng/mL), at which point cells were collected and lysed. Lysates were transferred to a nitrocellulose membrane after separation using SDS-PAGE, and probed with the antibodies listed.





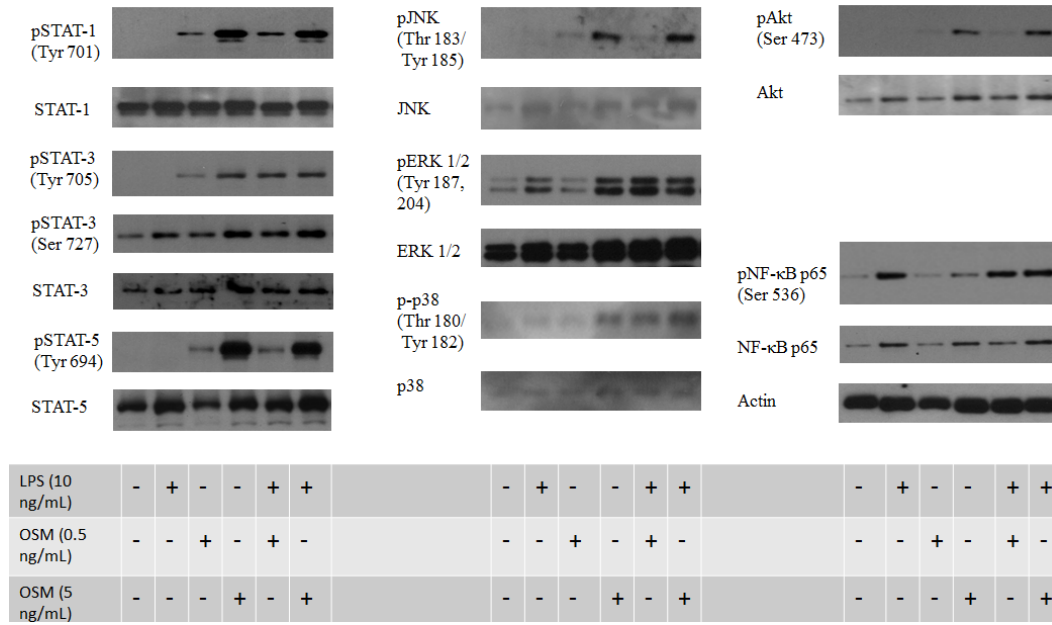
**Figure 18**

**Fig. 19** – Mouse Aortic Adventitial Fibroblasts (MAAFs) were treated for 4 hours with media (control), LPS (10 ng/mL), OSM (either 0.5 or 5 ng/mL), or a combination of LPS (10 ng/mL) and OSM (either 0.5 or 5 ng/mL), at which point cells were collected and lysed. Lysates were transferred to a nitrocellulose membrane after separation using SDS-PAGE, and probed with the antibodies listed.



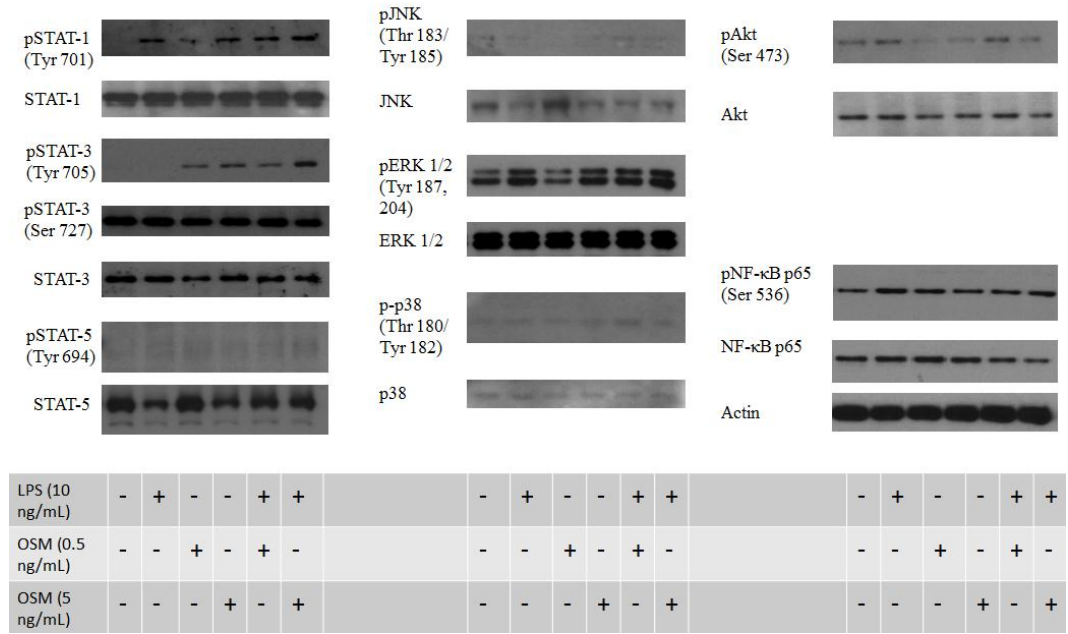
**Figure 19**

**Fig. 20** – Human Aortic Adventitial Fibroblasts (HAoAFs) were treated for 20 minutes with media (control), LPS (10 ng/mL), OSM (either 0.5 or 5 ng/mL), or a combination of LPS (10 ng/mL) and OSM (either 0.5 or 5 ng/mL), at which point cells were collected and lysed. Lysates were transferred to a nitrocellulose membrane after separation using SDS-PAGE, and probed with the antibodies listed.



**Figure 20**

**Fig. 21** – Human Aortic Adventitial Fibroblasts (HAoAFs) were treated for 4 hours with media (control), LPS (10 ng/mL), OSM (either 0.5 or 5 ng/mL), or a combination of LPS (10 ng/mL) and OSM (either 0.5 or 5 ng/mL), at which point cells were collected and lysed. Lysates were transferred to a nitrocellulose membrane after separation using SDS-PAGE, and probed with the antibodies listed.



**Figure 21**

**Fig 22** – PBMCs from a healthy volunteer were isolated, plated, and stimulated with media (control) or 100 ng/mL LPS. Supernatants were collected after 24 hours, and IFN- $\gamma$ , IL-1 $\beta$ , or IL-6 levels were measured by MSD multiplex ELISA. OSM levels were measured by ELISA.



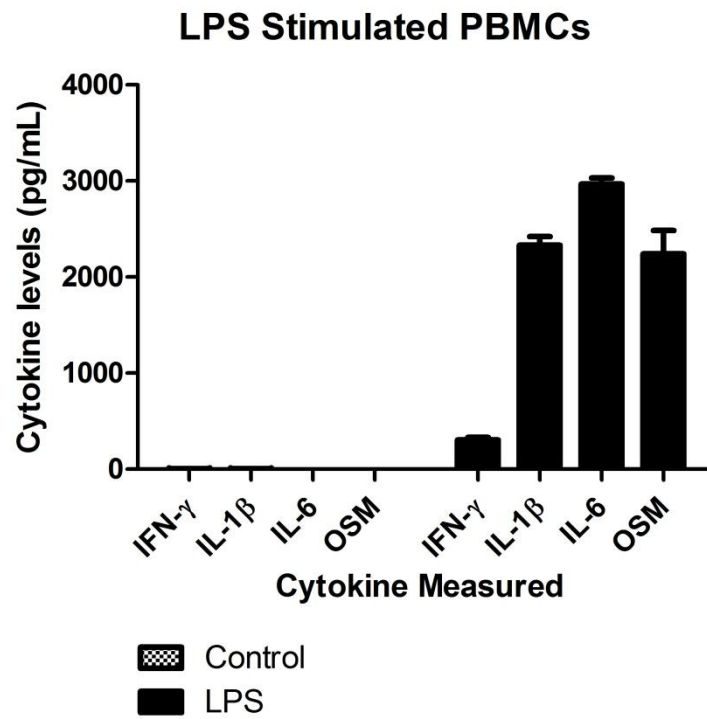
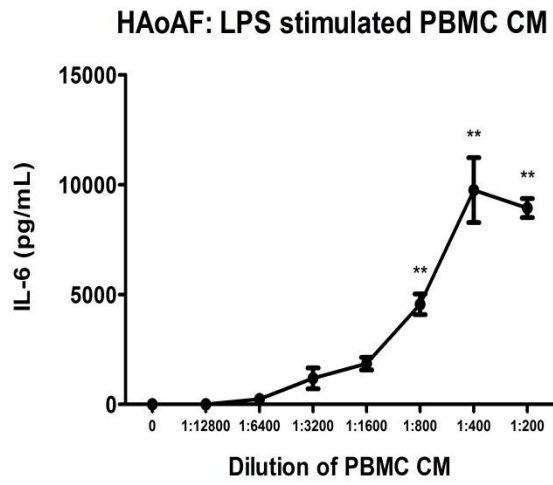


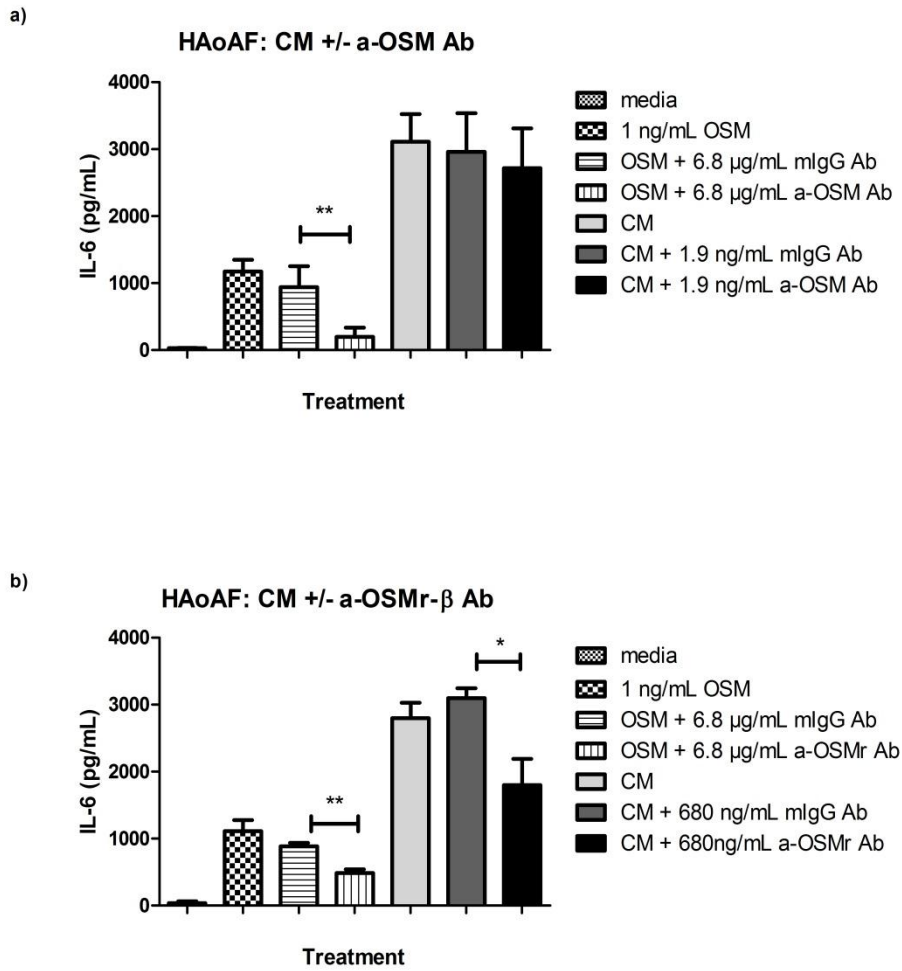
Figure 22

**Fig. 23** – Human Aortic Adventitial Fibroblasts (HAoAFs) were treated with dilutions of LPS stimulated PBMC conditioned medium (CM). After stimulation for 6 hours, the HAoAF supernatants were collected, and were analyzed for IL-6 protein by ELISA. Statistical significance: \*,  $p \leq 0.05$ , \*\*,  $p \leq 0.01$  as compared to control.



**Figure 23**

**Fig. 24 – a)** Human Aortic Adventitial Fibroblasts (HAoAFs) were treated with supernatants from 24 hour LPS stimulated peripheral blood mononuclear cells (CM) alone, with CM and a mouse IgG antibody, or with CM and an anti-OSM antibody. The antibodies were incubated with the CM for 2 hours at 37 °C prior to treatment of the HAoAFs. After 6 hours, the HAoAF supernatants were collected, and were analyzed for IL-6 protein by ELISA. **b)** HAoAFs were treated with CM alone, with CM and a mouse IgG antibody, or with CM and an anti-OSM receptor- $\beta$  antibody. The antibodies were incubated with the HAoAFs 1 hour prior to treatment. After 6 hours the HAoAF supernatants were collected and were analyzed for IL-6 protein by ELISA.



**Figure 24**

## **CHAPTER 4 - DISCUSSION**

Infiltration of inflammatory cells into the vessel wall is a hallmark of atherosclerosis. The earliest lesion is composed of macrophages, some of which contain lipid droplets [213]. Leukocyte adhesion molecules are upregulated on endothelial cells both on the luminal side of the artery and on the vessels that infiltrate the adventitia (vasa vasorum) in areas where these lesions develop [214]. In addition, leukocyte chemo-attractant molecules such as the CC, and CXC chemokines are released by cells within the vessel wall [215]. This results in a further influx of monocytes into the vessel. Inflammatory molecules such as  $TNF\alpha$ ,  $IL-1\beta$ ,  $IL-6$ ,  $IFN\gamma$ , and lipid oxidation products propagate the inflammatory response [16]. Proteases such as MMPs are released, which have the capacity to degrade the fibrous cap and expose the pro-coagulant contents of the plaque to the lumen of the artery [216].

As described above, atherosclerosis is an inflammatory process, and investigations have attempted to characterize stimuli that have the potential to initiate inflammatory responses in the vasculature. Infectious pathogens as well as danger associated host molecules stimulate the innate immune system to respond in a nonspecific, pro-inflammatory manner to cause cell activation and cytokine and chemokine release. Toll-like Receptors (TLRs) are involved in this response, and are receptors that bind specific exogenous and endogenous molecules [146]. TLR-4 is a well characterized member of this family that binds various ligands including the bacterial component LPS, and the endogenous molecules heat shock

protein 60 (HSP60), fibrinogen, heparan sulfate, fibronectins, and oxidized LDL [217,149]. TLR-4 signals through NF- $\kappa$ B as well as the MAPK family to induce the expression of inflammatory molecules including IL-6 [75,89,225,227,228], and TNF $\alpha$  [218] in numerous cell types, and IL-12 primarily in phagocytes and lymphocytes [219]. TLR-4 has been studied with respect to its potential role in atherosclerosis. It is expressed on various cells within the atherosclerotic plaque [65,75], and when knocked out of ApoE  $-/-$  mice, there is a protective effect in terms of atherosclerotic lesion development [78]. Most studies examine TLRs with respect to innate immune cells such as macrophages or natural killer cells. There has been less emphasis on understanding the TLR responses of vessel wall cells of the aorta including smooth muscle cells (SMCs) and adventitial fibroblasts. The effects of TLR-4 signaling in these cells merits further investigation, especially in the context of other inflammatory stimuli.

OSM is a member of the gp130 cytokine family that has also been implicated in chronic inflammatory diseases. OSM is expressed, and participates in disease processes of the lungs of idiopathic pulmonary fibrosis [220] and asthmatic patients [221], and the joints of individuals with rheumatoid arthritis [163]. There is also evidence that one of the roles of OSM in the local tissue inflammatory response is to amplify the effects of other mediators of inflammation. Specifically, OSM in combination with TNF $\alpha$ , IL-1 $\alpha$  or with IL-17 was shown to cause synergistic breakdown of cartilage in explant models [222,223,224]. In addition, OSM combined with IL-1 *in vivo* to mediate joint

destruction in mice [225]. In human airway smooth muscle cells, the synergistic actions of OSM with IL-1 $\beta$  resulted in increased MCP-1, VEGF, and IL-6 [240]. Also in lung tissue, however this time in fibroblasts, OSM synergized with IL-4 in inducing eotaxin elevation [120]. As a result, OSM has been shown to not only elevate the levels of inflammatory factors alone, but evidence suggests that it can synergize with various mediators of inflammation. Various cell types express OSM including macrophages [226], T cells [99], eosinophils [101], dendritic cells [103] and neutrophils [104]. Thus inflammatory responses in local tissues that are infiltrated by such cells could show an increased load of OSM.

OSM has also been implicated in atherosclerosis. It has been detected both in human [175] and mouse (Richards, C.D., unpublished) atherosclerotic plaques. As detected by immunostaining, OSM localizes to macrophages, which can be detected in all layers of the vessel, as well as SMCs [175]. Endothelial cells have been shown to increase their expression of leukocyte adhesion molecules ICAM-1, VCAM-1, P-, and E-selectin upon stimulation with OSM [168], as well as endothelial cell proliferation and migration [167]. The presence of OSM in plaques, its ability to enhance leukocyte recruitment through expression of cell adhesion molecules and chemotactic factors, and its capacity to enhance the levels of pro-inflammatory molecules in connective tissue cells serve as evidence that it may play a role in the atherosclerotic process. This, combined with its ability to amplify inflammation in the context of other inflammatory mediators led to the



goal of this thesis work: to characterize the effects of OSM and LPS in vascular wall cells of the aorta, a well established site of atherosclerosis.

### **Interleukin-6**

OSM has been shown to elevate levels of IL-6, another member of the gp130 cytokine family, in numerous cell types including endothelial cells [171], astrocytes [172], peritoneal mesothelial cells [173], lung fibroblasts [211], and human aortic smooth muscle cells [174]. In the current study, the results of Bernard *et al.* [174] were reproduced, as OSM was able to elevate levels of IL-6 in HAoSMC supernatants in a dose dependent manner (Fig. 11a). In addition, MAAFs and HAoAFs were shown to elevate levels of IL-6 in response to OSM (Fig. 1 a and 6 a). The mRNA steady state levels of IL-6 were increased in the HAoAF cells as well at 2 and 6 hours in response to OSM (Fig. 9 a and e). To the best of our knowledge, this is the first time that aortic adventitial fibroblasts, either in mice or in humans, have been shown to express IL-6 in response to OSM.

LPS has also been shown to induce IL-6 in various cells including alveolar macrophages [227], lung fibroblasts [227], blood monocytes [225,227], umbilical vein endothelial cells [228], coronary artery smooth muscle cells [75], and IL-6 mRNA in human aortic adventitial fibroblasts [89]. LPS induced production of IL-6 has been shown to be mediated by the transcription factors NF-IL-6, NF- $\kappa$ B, and AP-1 [229]. Figures 6 and 11 indicate that IL-6 levels were elevated in HAoAF and HAoSMC supernatants respectively in a dose dependent manner

upon stimulation with LPS. In terms of steady state RNA levels that were detected, stimulation of HAoAFs and HAoSMCs with LPS resulted in elevated levels of IL-6 RNA compared to control values at two different time points (Fig. 9 a and e, 14 a and e).

Intriguingly, when LPS was used in combination with OSM to stimulate aortic adventitial fibroblasts from three different species (mouse, rat, and humans), there was a synergistic elevation of IL-6 that was detected in the supernatants (Fig. 1b, 5b, 6 b and c). This effect was also observed in HAoSMCs (Fig. 11 b and c). It can be inferred that in the human cells OSM was mediating its effects through the gp130:OSMr- $\beta$  complex and not the gp130:LIFr- $\beta$  complex, as LIF only signals through the gp130:LIFr- $\beta$  complex and did not produce the same effects as OSM (Fig. 6 d, 11 d). A recent study demonstrated that IL-6 was synergistically enhanced in astrocytes in response to TNF $\alpha$  and a  $\beta$ 2-adrenergic receptor agonist 'isoproterenol' [230]. This synergistic response was shown to be mediated by NF- $\kappa$ B, cAMP response element-binding (CREB) protein, and CREB binding protein (CBP) transcription factors [230]. TNF $\alpha$  alone activated NF- $\kappa$ B, and isoproterenol alone activated CREB binding to the IL-6 promoter respectively [230]. When the two treatments were combined, there was a synergistic increase in CBP that was bound to the IL-6 promoter [230]. CBP is a cofactor that binds numerous transcription factors including CREB, NF- $\kappa$ B, AP-1, and E2F, and can enhance transcription in numerous ways including acting as a link between transcription factors and transcription machinery, recruiting acetyl transferases to

the region, or acting as an acetyl transferase itself (reviewed in by Shiama [231]). CBP has been shown in numerous studies to mediate synergistic transcription. One such study demonstrated CBP induced synergy between NF- $\kappa$ B and STAT-1, activated by TNF $\alpha$  and IFN- $\gamma$  respectively, to increase transcription of CXCL9 (MIG)[232]. The elevated CBP binding the target promoter region in both of the studies discussed was shown to be correlated with an increase of RNA polymerase II binding to the promoter of the gene being transcribed (IL-6 [230] or MIG [232]). In the results presented in this report, LPS alone resulted in an increased phosphorylation signal of NF- $\kappa$ B p65, while OSM alone resulted in an increased phosphorylation signal of STAT-1 (Fig. 18 and 20). Furthermore, CBP has been shown to bind transcription factors including the AP-1 components c-fos and c-jun which can be activated by the MAPK pathway [231], and AP-1 has been demonstrated to be required for certain stimuli to cause IL-6 transcription [233]. The immunoblots performed in the current study (Fig. 18 and 20) indicate that there is a phosphorylation signal detected for the MAPK pathways implying activation of these pathways. Furthermore, there is a slight but detectable increase in the phosphorylated p38 signal in MAAFs and HAoAFs with the combined LPS and OSM treatment above either treatment alone. It is thus possible that CBP is enhancing transcription mediated by NF- $\kappa$ B in combination with either STAT-1 or one of the transcription factors downstream of the MAPK pathway, resulting in the IL-6 synergy observed in the vascular wall cells presented in this report. To further support this, one study demonstrated that LPS mediated IL-6 induction

was almost completely abrogated when the promoter region for NF- $\kappa$ B was mutated, indicating that LPS alone relies primarily on one transcription factor to induce IL-6 [233]. Should a second stimulus (OSM in this case) activate a different transcription factor to bind the promoter, this could enhance co-factor binding, thus increasing transcription.

The increased levels of IL-6 detected in the supernatants of the adventitial fibroblasts and SMCs could have important implications for atherosclerosis. Mice prone to atherosclerosis that have been injected with IL-6 have larger lesions compared to control animals [190]. Furthermore these mice express increased plasma levels of the pro-inflammatory TNF $\alpha$  and IL-1 $\beta$ , and have elevated levels of fibrinogen [190]. In a separate study, ApoE deficient mice infected with *P. gingivalis* showed increased levels of serum IL-6, and IL-6 levels correlated both with infection and with lesion size [234]. IL-6 has been shown to increase the expression of monocyte chemoattractants in endothelial cells [193,194], which could recruit monocytes to the vessel wall. If OSM and TLR-4 activation synergistically enhance IL-6 levels *in vivo* as they do *in vitro*, this could be a biologically relevant pathway mediating vascular inflammation. It should be noted that the role of IL-6 in atherosclerosis requires further investigation, as LDLr  $-/-$  mice crossed with IL-6  $-/-$  mice experienced only modest decreases in lesion size compared to LDLr  $-/-$  mice alone [235].

### **Vascular Endothelial Growth Factor (VEGF)**

There are 4 human VEGF proteins (VEGF A-D), with VEGF A (from now on 'VEGF') being the best characterized. VEGF was originally given the name 'vascular permeability factor' due to its ability to rapidly increase the permeability of microvasculature upon release from tumors [39]. It was later found to be a mitogen for endothelial cells [236], and to induce angiogenesis [237]. The VEGF gene is located on human chromosome 6p12, and has 8 exons separated by 7 introns [238]. VEGF is able to bind two receptors: VEGFr-1 and VEGFr-2, both of which are present on vascular endothelial cells (VEGFr-2 is also present on lymphatic endothelial cells) [238], while VEGFr-1 is present on mononuclear leukocytes [239].

OSM has been shown to induce VEGF expression in human airway smooth muscle cells [240], and in coronary and aortic smooth muscle cells of atherosclerotic patients [146]. The latter finding that OSM induces elevated levels of VEGF in HAoSMC supernatants was reproduced here (Fig. 13a). However to the best of our knowledge the data presented in this report is the first time HAoAFs have been shown to elevate expression of VEGF in response to OSM or LPS alone, at the protein (Fig. 8 a) or mRNA levels (Fig. 9 c and g). It can be inferred that in the human cells OSM was mediating its effects through the gp130:OSMr- $\beta$  complex and not the gp130:LIFr- $\beta$  complex, as LIF only signals through the gp130:LIFr- $\beta$  complex and did not produce the same effects observed with OSM (Fig. 8 c, 13 c).

Interestingly, it was demonstrated that macrophages were unable to produce detectable levels of VEGF upon stimulation with LPS alone, however in combination with adenosine A2A receptor (A2AR) agonists was able to synergistically enhance the expression of VEGF [241]. The hypoxia response element (HRE) of the VEGF promoter, which is the binding site for the transcription factor hypoxia inducible factor-1 (HIF-1), was found to be necessary for the synergistic response [241]. This transcription factor is composed of two proteins that form a dimer: HIF-1 $\alpha$  and HIF-1 $\beta$ , and while HIF-1 $\beta$  is stably expressed, HIF-1 $\alpha$  is extremely unstable at normal oxygen levels [241]. Several growth factors and hormones have been shown to increase HIF-1 $\alpha$  steady state expression, and Ramanathan *et al.* [241] demonstrated that the macrophages increased transcription of HIF-1 $\alpha$  in response to LPS. This increase in HIF-1 $\alpha$  mRNA was dependent on NF- $\kappa$ B activation. NF- $\kappa$ B was found to have an increased phosphorylation signal in the findings presented here in response to LPS (Fig. 18 and 20) suggesting activation of NF- $\kappa$ B. It is thus possible that this same mechanism was mediating the synergistic VEGF response observed in MAAF $s$  (Fig. 3 b), HAoAF $s$  (Fig. 8b), and HAoSMC $s$  (Fig. 13 b) when LPS was combined with OSM, as LPS alone did not elevate VEGF in the supernatants of either MAAF $s$  or HAoSMC $s$ , OSM did elevate VEGF levels, and in combination there was a synergistic response.

Despite the fact that elevated levels of VEGF protein were detected in response to the combined treatment of LPS and OSM in all three cell types, the

mRNA data for both HAoAFs and HAoSMCs did not indicate an increase in steady state VEGF mRNA levels with the combined treatment compared to the treatments alone (Fig. 9 c and g, 14 c and g). One possible explanation for the incongruence between the mRNA and ELISA data is that the time points chosen may not have been optimal for detecting augmented VEGF RNA in the combined treatment compared to the treatments alone. In a time course experiment performed by Martin *et al.* [242] where mouse endothelial cell VEGF mRNA was measured at various time points post stimulation with IL-8, maximal VEGF mRNA was expressed at 12 hours post stimulation compared to control.

Alternatively, it is possible that events post-transcription were responsible for an increase in the protein detected, without increases in mRNA levels. For example, the phosphorylated form of the eukaryotic initiation factor eIF-4E has been shown to have 3-4 fold higher affinity for the 7-methylguanosine-containing cap structure of mRNA than the non-phosphorylated form [243], which can result in increased protein synthesis [244]. eIF-4E phosphorylation by LPS could explain why despite not having elevated steady state mRNA levels, there were increased levels of VEGF protein detected in the supernatants, as the VEGF mRNA induced by OSM could be translated more efficiently. However it is unknown if this mechanism is likely to occur, as LPS has been shown to increase the binding of eIF-4E to 4E-BP-1, forming an inactive complex, which results in decreased protein synthesis [245].

There is evidence to suggest that VEGF could play a role in the development of atherosclerosis. It has been shown to increase the permeability of endothelial cells [39], which could facilitate monocyte entry into the vessel wall. Supporting this, VEGF can induce monocyte chemotaxis *in vitro* [38]. VEGF has also been detected in atherosclerotic plaques, but not in normal arteries, and was found to localize to endothelial cells, macrophages, and SMCs, and within microvessels of advanced lesions [246]. Mice deficient in ApoE and ApoB100 that were treated with low doses of VEGF were shown to have larger lesions that contained more macrophages compared to control mice [247].

One of the roles of VEGF is in promoting angiogenesis [236,237]. Intimal capillaries have been shown to have higher levels of leukocyte adhesion molecules VCAM-1, ICAM-1 and E-selectin than are present at the artery surface [248], which would have the potential to mediate increased extravasation of monocytes into the vessel wall. Furthermore, enhanced vasa vasorum was found in plaque regions, but not in areas of healthy vessels, and administration of an inhibitor of angiogenesis reduced the plaque size and vasa vasorum of Apo-E deficient mice [249]. Moreover, the presence of microvessels co-localized with VEGF within the plaque, and these vessels have been correlated with plaque instability and ischemic symptoms [250]. The findings presented in this report that LPS and OSM can synergize to enhance VEGF levels in the supernatants of MAAFs, HAoAFs, and HAoSMCs suggest that this is another mechanism by which these two factors could influence inflammation in the vascular wall.



### **KC and IL-8**

Keratinocyte-derived cytokine (KC) was originally discovered to be released by 3T3 cells in response to PDGF [251] and has been found to be a potent neutrophil chemoattractant [252,253]. KC is a member of the murine CXC chemokine family and like other members of this family, it is located on mouse chromosome 5 [201]. Increased levels of KC have been shown to be expressed in response to both OSM and LPS. KC has been shown to be expressed by murine macrophages [254], and porcine [255] and murine [256] endothelial cells in response to LPS. OSM stimulation of murine cardiac fibroblasts resulted in KC expression by these cells [200]. The findings presented in this report indicate that LPS alone increased KC levels significantly, while the higher doses of OSM alone also elevated the levels of KC in MAAF supernatants (Fig. 2 a and b).

In the mouse, KC has been implicated in atherosclerosis. In one study the authors transplanted bone marrow from KC receptor CXCR-2 deficient or CXCR-2 competent mice into LDLr <sup>-/-</sup> mice, and both groups received a high fat diet for 16 weeks. The lesions of the CXCR-2 competent mice were significantly larger after this time period, and while KC was found in the lesions of both groups of mice, CXCR-2 was only found in the lesions of CXCR-2 competent mice [257]. In an *ex vivo* model of hypertension, KC was one of the mediators of monocyte adhesion to murine carotid arteries [199]. These studies suggest a role for both the KC ligand and its receptor in mouse models of atherosclerosis. The combined treatment of MAAF cells with LPS and OSM resulted in synergistically elevated

levels of KC, which was a unique property of OSM compared to the other gp130 cytokines tested (Fig. 2). Should these two stimulants be present in the murine vascular wall, it is possible that they could participate in atherosclerosis in mice through elevation of KC.

In terms of KC gene expression, several potential transcription sites have been located at the 5' end of the KC gene, and mutation of two  $\kappa$ B binding sites significantly inhibited KC expression in response to LPS stimulation [258]. Both of these sites were shown to bind NF- $\kappa$ B proteins p50, p65, and c-Rel [258]. As observed in the findings presented in this report (Fig. 18), the phosphorylation signal of p65 was elevated in response to LPS, and so it is possible that p65 was mediating transcription of KC in this system as well. OSM mediated KC expression was shown in cardiac fibroblasts to be dependent on the PI3K/Akt pathway [200]. OSM was able to increase the phosphorylated Akt signal detected in MAAF's (Fig. 18), suggesting that this pathway may be necessary for the OSM induced elevation in KC detected in the supernatants. Therefore it is also possible that the combined activation of the NF- $\kappa$ B pathway and the Akt pathway that were activated separately by LPS and OSM respectively, were mediating the synergistic elevation in KC detected when the two treatments were combined.

There are numerous similarities between KC and murine MIP-2. These chemokines have amino acid sequences that were shown to be 63% similar [259], and they were shown to bind the same receptor, CXCR-2 [253]. MIP-2 was released by macrophages in response to endotoxin, and was shown to be a potent

chemoattractant for PMNCs [260]. The closest human protein that was associated with MIP-2 was that of human growth regulated oncogene (Gro), and they were 62.5% identical [260]. KC was shown to have a closely related amino acid sequence to the human Gro cytokine as well, as the sequences exhibit 65.6% similarity [260]. In addition, KC and MIP-2 were shown to respectively have 41 and 36% similarity to the human IL-8 amino acid sequence [259]. There appears to be a consensus in the literature that human IL-8 does not have a direct homolog in mice. However, there is uncertainty in the field as to whether KC and/or MIP-2 is/are the *functional* homolog(s) of IL-8 or Gro.

IL-8 is a member of the CXC chemokine family, and has a well established role as a neutrophil chemo-attractant [73]. It is expressed as a 77 and 72 amino acid long protein in non-immune and immune cells respectively. IL-8 signals through either CXCR-1 or CXCR-2, two G-protein coupled cell surface receptors. While CXCR-1 is able to bind IL-8 and granulocyte chemotactic protein-2, CXCR-2 can bind numerous CXC chemokines such as IL-8, neutrophil activating peptide, granulocyte chemotactic protein-2, and the growth-related oncogenes (GRO $\alpha$ ,  $\beta$ , and  $\gamma$ )(reviewed by Waugh and Wilson [261]). The transcription factors NF-IL-6 and the p65 component of NF- $\kappa$ B have been shown to be important in mediating hIL-8 gene expression [262].

In terms of atherosclerosis, foam cells have been shown to express high levels of IL-8 [263], and IL-8 has been found inside human coronary atherectomy tissues [264]. IL-8 is able to induce vascular smooth muscle cell proliferation

[264], and has been demonstrated *in vitro* to cause monocyte adhesion to endothelial cells, suggesting a role in monocyte recruitment [265]. However the role of IL-8 in atherosclerosis is not entirely clear, as it has been demonstrated that IL-8 was able to decrease VCAM-1 expression on endothelial cells in an *in vitro* model mimicking athero-prone blood flow [266], which would result in decreased monocyte recruitment. In addition, IL-1 $\beta$  induced NF- $\kappa$ B activity and p38 phosphorylation was inhibited by IL-8 [266]. Thus although the role of IL-8 in atherosclerosis is not completely defined, it appears to participate in the process in a pro-inflammatory manner. The results described in this report that demonstrate IL-8 to be suppressed in human vascular wall cells in response to LPS combined with OSM compared to LPS alone, suggest that if these results are representative of what occurs in the atherosclerotic plaque, OSM may have diverse inflammatory roles within the vessel wall.

The inhibition of IL-8 levels that were detected when OSM was combined with LPS to treat cells appears to be occurring prior to translation of the IL-8 transcript, as IL-8 mRNA steady state levels are lower in HAoAFs (Fig. 9 b and f) and HAoSMCs (Fig. 14 b and f) in response to the combined treatment compared to LPS stimulated levels alone. One study has demonstrated that the IL-8 that was induced upon exposure of airway epithelial cells to TNF $\alpha$  was suppressed in response to glucocorticoid receptor upregulation. This upregulation of the receptor resulted in decreased histone H4 acetylation at the IL-8 promoter region [267]. This in turn would increase the attraction between the histone and DNA,

inhibiting transcription. OSM has indirectly been shown to activate histone deacetylases (HDACs), as treatment of cartilage samples *in vitro* with HDAC inhibitors decreased the OSM mediated cartilage proteoglycan and collagen proteolysis and MMP upregulation [268]. OSM causing decreased histone H4 acetylation by activating HDACs is a possible mechanism that could explain why IL-8 levels were suppressed with the combined LPS and OSM treatment. In another study, OSM inhibited the expression of IL-8 that was induced when human lung or synovial fibroblasts were stimulated with IL-1 [269]. This occurred concurrently with an enhancement in IL-6 levels that were observed with the IL-1 and OSM combined treatment [269]. It was postulated that OSM stimulation of cells could affect the AUUUA motif of the IL-8 mRNA which has been demonstrated to confer instability in the 3' untranslated region [269,270]. However it was also noted that this motif is expressed on the IL-6 transcript as well, and therefore how OSM would affect this motif of one transcript but not the other is unclear [269]. Another study demonstrated that OSM was also able to suppress IL-8 that was induced by IL-1 in human peritoneal mesothelial cells, while at the same time synergizing with IL-1 to cause the elevation of IL-6 and MCP-1 [173]. The authors suggested that OSM may inhibit neutrophil recruitment while at the same time attracting mononuclear leukocytes to sites of inflammation [173]. This observation may apply to atherosclerosis, as historically neutrophils have not been detected in developing lesions, although recently this view has come into question (reviewed by Soehnlein [271]).

It is interesting to note that besides being a leukocyte chemo-attractant, IL-8 also participates in angiogenesis [264, 272]. Data in this report indicates that while OSM is able to induce one potent angiogenic factor (VEGF) both in protein and mRNA expression alone and in combination with LPS, it markedly inhibits another angiogenic factor (IL-8) that is induced by LPS.

The fact that OSM suppresses IL-8 expression in HAoSMCs and in HAoAFs but not KC in MAAF's may be explained by the fact that KC and IL-8 are not direct homologs, and despite sharing some functional characteristics, appear to differ significantly in others. It would be interesting to determine the role of OSM on MIP-2 levels in the MAAF supernatants, as if MIP-2 is suppressed, this may contribute to the evidence that MIP-2 is more of a functional homolog of hIL-8 than KC.

### **MCP-1**

MCP-1 is a member of the CC chemokine family, and is 13 kDa in size. MCP-1 binds to the CCR2 receptor, and the N-terminal region of the protein is necessary for this to occur [273]. MCP-1 was first isolated from murine embryonic fibroblasts [251], and shortly after in human tumours [274]. It is released by numerous cell types, and is best known as a potent monocyte chemoattractant, but also recruits T cells and dendritic cells to sites of tissue damage and infection (reviewed by Yadav *et al.* [275]).

The discovery that MCP-1 was present in human atherosclerotic plaques [276], that monocytes/macrophages were present in atherosclerotic plaques [276],

and that MCP-1 caused monocyte/macrophage chemoattraction and extravasation [277] led to hypothesis that MCP-1 was involved in the atherosclerotic process. Mice that were LDLr  $-/-$  were crossed with mice that were MCP-1  $-/-$ , and these mice presented with 83% less lipid accumulation in aortic plaques compared to LDLr  $-/-$  mice that were wild-type for the MCP-1 alleles [278]. Furthermore, mice on high fat diets that were deficient in the ApoE gene and the MCP-1 receptor CCR2 gene had significantly fewer macrophages in the aorta and markedly smaller lesions than control ApoE deficient mice [279]. These and other studies indicate that MCP-1 plays a central role in the atherosclerotic process [16].

MCP-1 is induced in several cell types including human peritoneal mesothelial cells [173], human airway smooth muscle cells [240], and osteosarcoma cells [280] in response to OSM. The mRNA data presented in this report is based on samples from one experiment at each time point listed. Despite this, an effect observed in multiple cell types at multiple doses at multiple time points allows trends to be observed and conclusions to be drawn. The steady state MCP-1 mRNA levels were elevated in response to OSM alone in HAoAFs (Fig. 9 d and h) and HAoSMCs (Fig. 14 d and f). In addition, in both HAoAFs and HAoSMCs at the 6 hour time point, LPS in combination with OSM caused a marked elevation of MCP-1 steady state mRNA levels compared to either of the treatments alone (Fig. 9 h and 14 d). In an osteosarcoma cell line, through the use of inhibitors it was demonstrated that OSM induced MCP-1 through AP-1 that was activated by STAT-3, and ERK 1/2 [280]. The findings of the current report

show that OSM was able to induce STAT-3 and ERK 1/2 phosphorylation after 20 minutes in HAoAF cells (Fig. 20). Although specific inhibitors of intracellular signaling molecules were not used on the HAoAFs, it is possible that both STAT-3 and ERK 1/2 participate in the elevated detection of MCP-1 RNA in this system as well.

These findings provide further evidence that TLR-4 activation in the presence of OSM in vascular wall cells could participate in an inflammatory process such as atherosclerosis. The findings that in the presence of LPS and OSM, MAAF and HAoAFs synergistically increased IL-6 and VEGF detected in the supernatants, and elevated levels of steady state MCP-1 mRNA in the HAoAFs, provide support for the idea that fibroblasts of the adventitia are capable of participating in inflammatory responses. These observations, as well the work done by others [83,84,86,88,89] suggest that the role of the adventitia should be closely examined in relation to the development of atherosclerosis.

### **TLR-4 Expression**

After determining in MAAF, HAoAF, and HAoSMCs that the combined treatment of LPS with OSM was able to induce synergistic effects in certain endpoints, potential mechanisms involved in mediating these effects were of interest. It was originally hypothesized that OSM was increasing the expression of one of the TLR-4 receptor components, as OSM has been shown in other systems to synergize with molecules by increasing the expression of that molecule's receptor. For example in mouse lung fibroblasts OSM is able to synergize with



IL-4 and IL-13 to produce eotaxin by markedly elevating the IL-4R $\alpha$  and the IL-13R $\alpha$ 1 [204]. Furthermore in human airway smooth muscle cells, OSM synergized with IL-1 $\beta$  to augment VEGF levels *in vitro* by increasing the expression of the IL-1 receptor [240]. Thus experiments were first performed to determine whether increased levels of the TLR-4 associated receptor component CD14 could reproduce effects seen with the combination treatment of LPS and OSM. As illustrated in figure 15, stimulating cells with sCD14 in combination with LPS did not enhance the levels of IL-6 detected in the supernatants compared to LPS alone. Thus soluble CD14 did not appear to be a rate limiting step in the production of IL-6 in these cells. It is possible that lipopolysaccharide binding protein (LBP) is required in conjunction with soluble CD14 to mediate IL-6 increases, and it is also possible that there is sufficient soluble CD14 in the serum [207] (included in the media) to induce maximal LPS/CD14/TLR-4 signalling.

The results of these experiments indicate that even if OSM was causing upregulation of CD14, it is unlikely to be resulting in the observed IL-6 synergy as increasing the levels of sCD14 available to the HAoAFs had little effect on IL-6 detected in the supernatants. The next question that was of interest was whether TLR-4 itself was being upregulated when the cells were stimulated with OSM.

In MAAF cells it was determined by flow cytometry that at several time points post stimulation, OSM did not cause a detectable increase TLR-4 expression on the cell surface (Fig. 16 a) . Similarly, in the HAoAFs and HAoSMCs, OSM did not cause increases in steady state levels of TLR-4 mRNA

compared to control levels (Fig 21 b-e). To summarize the TLR-4 expression experiments, results in all three cell types suggest that at the indicated time points OSM is not causing detectable increases in TLR-4 expression. It is therefore unlikely that an increase in TLR-4 upon treatment with OSM is causing the synergistic pro-inflammatory cytokine responses observed upon treatment of these cells with OSM and LPS.

### **OSMr- $\beta$ Expression**

The results for experiments on either the TLR-4 itself or the associated receptor component CD14 indicated that it was unlikely that their up-regulation was responsible for the effects observed with the combined stimulation of the vascular wall cells with LPS and OSM. It was hypothesized that alternatively, LPS could be increasing the expression of one of the OSM receptor components, thus increasing the ability of the cells to respond to OSM.

LPS was able to increase OSMr- $\beta$  mRNA steady state levels approximately 2 fold compared to control levels in three out of the four time points analyzed, however OSM treatment also resulted in an increase in the OSMr- $\beta$  mRNA steady state levels, and these effects in general were not additive with the combined treatment (Fig. 17). It has been demonstrated in human monocytes that stimulation with LPS can result in the increased expression of OSMr- $\beta$  [208]. Due to the results discussed, and as LPS has been shown previously to increase OSMr- $\beta$  RNA levels [208] it remains a possibility that the

synergy observed with some of the endpoints is mediated by LPS increasing the expression of the OSMr.

### **Cell Signaling**

In addition to measuring the expression levels of receptors, the phosphorylation signals of various proteins downstream of either the LPS or the OSM signaling pathways were analyzed. There are several different mechanisms by which the combined LPS and OSM treatment could result in the synergistic response that was observed in some endpoints. First of all, it is possible that one ligand binding its receptor activates one (or more) transcription factor(s) that bind to a promoter for the gene in question. Should the other ligand, upon binding its receptor, cause the activation of a different transcription factor which can bind the same or a close-by region of the promoter that was the target of the first ligand, increased transcription could occur. As described above, it is possible that a cofactor such as CBP can link the two transcription factors together and stabilize them on the promoter [230]. Another example of this principle was demonstrated when TNF- $\alpha$  was applied to endothelial cells. Stimulation with TNF- $\alpha$  resulted in activation of p38 and NF- $\kappa$ B, and when a plasmid containing p300 (a co-factor similar to CBP) was transfected into endothelial cells stimulated with TNF- $\alpha$ , there was increased transcription of MCP-1. However upon application of a p38 inhibitor, the p300 enhanced induction of MCP-1 in these cells was completely abrogated, indicating that co-factors can enhance transcription, but may require activation of two distinct pathways [281]. Furthermore, cooperating transcription

factors can recruit more RNA Polymerase II to the promoter site [230], increasing transcription compared to either ligand alone. Assuming that the detection of a phosphorylation signal means that a given pathway is activated, there were several pathways that were activated by only one of the ligands in the immunoblots presented in this report (Fig. 18 and 20). LPS induced NF- $\kappa$ B p65 phosphorylation in MAAFs and HAoAFS, while OSM induced STATs 1, 3, and 5 phosphorylation. Although CBP has been shown to bind NF- $\kappa$ B and STAT-1, it is possible that another co-factor is able to bind a different STAT and NF- $\kappa$ B. For example, the 'steroid receptor coactivator 1', (NcoA/SRC1) has been demonstrated to bind STAT-3 and increase its transcriptional capabilities [282], and separately has been shown to enhance the transcriptional capabilities of NF- $\kappa$ B [283], AP-1 [284], and STAT-6 [285]. Thus co-factors acting on transcription factors activated by either LPS or OSM could be mediating the synergistic response.

Apart from acting as a bridge between the transcription factor and the transcription machinery, some co-factors act as histone acetyltransferases. This allows increased access to the DNA, and thus increased transcription. Both NcoA/SRC1 [282] and CBP [230] have acetyltransferase capabilities. Activation of one pathway by one ligand (example NF- $\kappa$ B by LPS) may result in acetyltransferase activation, which could allow members of a separate pathway activated by the other ligand (example: STAT-3 by OSM) to more efficiently carry out transcription. The alternative is also possible: one of the stimulants

could decrease the activity of a histone de-acetylase (HDAC). LPS has been shown to modulate histone de-acetylase activity in macrophages [286]. If this decrease in HDAC by LPS occurred in the MAAFs, HAoAFs, and HAoSMCs, the OSM stimulated transcription factors could transcribe their target gene with increased efficiency. This increased transcription by the OSM stimulated transcription factor when LPS is also used to treat the cells compared to when OSM alone is applied could explain the synergistic protein detection in the supernatants.

Alternatively, the convergence of shared signaling pathways activated by both LPS and OSM could result in increased phosphorylation of a kinase or transcription factor compared to either treatment alone. This could result in increased activity of this molecule, resulting in increased transcription. In the current report, there was a slight but detectable increase in the signal detected for the phosphorylation of p-38 in both HAoAFs and MAAFs. This correlated with the synergistic protein production in several endpoints that were detected by ELISA. In addition, p38 or one of its downstream targets has been shown to mediate IL-6 [287], MCP-1 [288], and VEGF [289] induction. Thus there are several possible mechanisms which could explain how the synergistic protein response was occurring.

### **Neutralizing Antibodies to OSM and OSMr- $\beta$**

Both endogenous and exogenous TLR-4 ligands have been found inside the adventitia of atherosclerotic arteries [292,296]. In addition, PBMCs can

infiltrate into the adventitia through the vasa vasorum. It was of interest to determine whether the cytokine milieu that resulted from LPS stimulated PBMCs (conditioned medium (CM)) could regulate cytokine levels in the supernatants of human aortic adventitial fibroblasts. It was determined that several pro-inflammatory molecules including IFN- $\gamma$ , IL-1 $\beta$ , IL-6, and OSM were present in the CM, but not in control medium (Fig. 22). Upon treatment of HAoAFs with dilutions of the CM there was a strong dose dependent response of IL-6 detected in the supernatants (Fig. 23). This can be attributed to the IL-6 that originated from the HAoAFs as opposed to IL-6 that was already present in the CM, as dilutions of CM to 1 in 400 would have only contained approximately 8 pg/mL of IL-6, while approximately 10 ng/mL was detected in the supernatants upon stimulation with the CM. This finding illustrates the potential role of adventitial fibroblasts in inflammatory responses. These fibroblasts have the capacity *in vitro* to actively participate in an inflammatory response to a physiologically relevant mixture of cytokines, and in this system are not passive bystanders.

As OSM was present in tandem with several other pro-inflammatory molecules in the CM, tests were completed to determine whether inhibiting OSM among a milieu of cytokines was effective in modulating HAoAF activity. Interestingly, application of the anti-OSMr- $\beta$  antibody in combination with the CM caused a 42% reduction in the IL-6 levels that were detected in the HAoAF supernatants (Fig. 24 b). The anti-OSM antibody was not able to produce the same results (Fig. 24 a). A possible explanation for this difference is that OSM

produced by cells that secrete it naturally can be glycosylated [290], while the neutralizing antibodies specific to OSM are developed to bind to recombinant, non-glycosylated OSM. This glycosylation could hide epitopes necessary for antibody binding, either directly, or by causing an altered conformation of the protein.

Given that other studies have indicated that OSM could synergize with IL-1 $\beta$  in different systems [223,240], and as IL-1 $\beta$  was one of the molecules detected in the CM, it is possible that OSM in this system was synergizing with LPS and IL-1 $\beta$  to produce IL-6. If this were the case, it should not be surprising that neutralizing the activation of the OSMr- $\beta$  signaling pathway would decrease the IL-6 levels detected in the supernatants. It should be noted that these experiments were repeated twice, both showing similar results. However the results still must be interpreted with caution as the CM was obtained from one volunteer's PBMCs, and experiments were performed on one individual's HAoAFs. Based on these experiments, the possibility remains that OSM, even in the context of other pro-inflammatory cytokines, has the capacity to significantly effect the regulation of HAoAFs.

### **SUMMARY AND CONCLUDING REMARKS**

TLR-4 has been shown to be expressed in various sites in the atherosclerotic plaque, including the adventitia. In addition, immunofluorescent staining and immunoblotting found TLR-4 to be present on HAoAFs [89]. TLR-4 activation has been suggested to be a link between either pathogen based or endogenous inflammation and atherosclerosis [65,89,291]. There is evidence that the adventitia plays a role in this TLR-4 mediated inflammation. *In vivo* studies have shown that a luminal injury can result in the accumulation of fibronectin in the media and adventitia [292], and that these same splice variants of fibronectin can stimulate TLR-4 activation [293]. Another endogenous molecule that can activate TLR-4 is HSP60 [294], which has been shown to be present in the intima and media, and on macrophages of atherosclerotic plaques, while not in control arteries [295]. In addition, *C. Pneumoniae*, a gram negative bacterium that expresses LPS, has been detected in the adventitia of atherosclerotic lesions, and is typically found inside macrophages in this site [296]. Infected macrophages may localize to the artery and enter the wall by way of the vasa vasorum [296], or through the luminal side of the artery. There are therefore numerous potential TLR-4 ligands that have been shown to be present in atherosclerotic plaques.

The findings that HAoSMCs and HAoAFs can respond to the TLR-4 ligand LPS to increase detectable levels of IL-6, MCP-1 and IL-8 [75,89,297] have been reproduced and presented in this report. In addition, the findings that vascular SMCs can elevate levels of detectable IL-6 and VEGF in response to



OSM alone have also been reproduced [174,146]. To the best of our knowledge, this is the first time that OSM alone has been shown to induce MCP-1 steady state mRNA expression in HAoSMCs or HAoAFs. Furthermore, the synergistic induction of IL-6 and VEGF levels in HAoAF, HAoSMC, and MAAF cell supernatants, and MCP-1 mRNA levels in HAoAFs and HAoSMCs in response to LPS in combination with OSM are, to the best of our knowledge, novel findings, as is the suppression of LPS induced IL-8 levels by OSM in the human vascular wall cells assayed. It was determined that these effects were not correlated with an increase in TLR-4 expression in response to OSM at the time points measured, but that they did correlate with an increased phosphorylation signal of the MAPK p38 in response to the combined LPS and OSM treatment compared to either treatment alone. Moreover, a neutralizing antibody to the OSMr- $\beta$  was able to inhibit HAoAF IL-6 responses to PBMC conditioned medium. Together, these findings indicate that OSM and LPS can synergize *in vitro* to induce the production of inflammatory factors in vascular wall cells, helping to characterize the potential role of OSM, TLR-4 ligands, and adventitial fibroblasts in vascular inflammation.

Based on the current literature and results described here, a model of exacerbation of atherosclerosis could be as follows: The TLR-4 ligands within the plaque can induce the expression of chemotactic factors (for example MCP-1 [75,194]), and leukocyte adhesion molecules (for example P-selectin [298]) by vascular wall cells. Monocytes are thus attracted to these sites, enter the arterial

wall, increase their expression of scavenger receptors, and uptake ox-LDL [15,16]. Macrophage activation results in pro-inflammatory cytokines such as IL-1, TNF $\alpha$ , and OSM production [103], which in turn amplify the inflammatory response. Cells throughout the arterial wall respond to the TLR-4 ligands, OSM, and other pro-inflammatory factors by: releasing cytokines such as IL-6 (described in this report, and [75,227,228]); releasing chemoattractants such as MCP-1 to recruit monocytes to the lesion (described here and [75]); upregulating the expression of leukocyte adhesion molecules [168,298] which also serves to recruit additional inflammatory cells to the site of inflammation; expressing growth factors such as VEGF which can increase the permeability of the endothelium [39], vascularize, and disrupt the plaque (described here and [175,146,246]); and releasing MMPs which, in the later stages of the disease, can degrade the fibrous cap [86,164,216].

Upon release from infiltrating leukocytes, the role of OSM in this model could be to activate endothelial cells [171], SMCs, and adventitial fibroblasts to express pro-inflammatory cytokines and chemokines alone, and synergistically with other stimuli present within the vascular wall. Moreover OSM could induce the expression of leukocyte adhesion molecules [168], MMPs [164], and pro-coagulant factors [299].

To test the overall function of OSM *in vivo*, transgenic mice could be developed that over-express OSM in a mouse model of atherosclerosis. Alternatively, the activity of OSM could be neutralized, either through

administration of a neutralizing antibody, or gene targeting could be employed to create double knock out ApoE and OSM deficient strains of mice, as ApoE deficient mice are inherently prone to atherosclerotic lesions.

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