OSM REGULATION OF RESPONSES TO TLR-LIGANDS IN HASMC

OSM REGULATION OF RESPONSES TO TLR-LIGANDS IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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-ABSTRACT-

Allergic atopic asthma is a respiratory condition that involves immune responses to specific allergens resulting in coughing, wheezing, shortness of breath and tightness in the chest. During an atopic asthmatic attack, the immune system initiates cellular infiltration of lymphocytes and eosinophils, airway hyper-responsiveness and ECM remodeling, which manifests in lung dysfunction in chronic disease. ASMC have recently been shown to play a role in the inflammatory processes of asthma through the production of inflammatory mediators. Various cytokines and chemokines serve as stimulants for these pathways and therefore require further attention to examine inflammatory signaling. OSM, a member of the gp130 family of cytokines, is secreted by inflammatory cells and has been detected in the sputum of asthmatics. Previous findings have established the potential of OSM in induction of lung inflammation, its role in increasing ECM, and its potential role in asthma.

Viral or bacterial infections cause asthma exacerbations which result in increased severity of symptoms. The innate immune system relies on pattern recognition receptors including the TLRs to recognize invading pathogens and activate cells such as macrophages and natural killer cells. Although there are a number of these TLRs, this project will focus on the role of TLR3 and TLR4 in ASMC. I generally hypothesized that ***OSM markedly increases lung cell airway smooth muscle cell responses to external stimulae, such as products of bacteria or viruses that activate toll-like receptors. This***

exacerbates inflammation and extracellular matrix remodeling which contributes to pathology in asthmatic patients.

Findings in this thesis have demonstrated that OSM stimulation increases the production of various cytokines and chemokines and growth factors seen in asthma. Co-stimulations with OSM and TLR-ligands augmented the production of a variety of these inflammatory mediators in comparison to ligands alone. TLR responses were shown to be associated with TLR expression, at both the mRNA and protein level, as well through the activation of the JAK-STAT and NFκB pathways. These findings implicate ASMC in immunomodulatory roles in response to TLR-ligands and OSM, and could play a role in the increased severity of asthma seen during exacerbations.

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Anti-OSM	OSM Ligand Antibody
Anti-OSMR	OSM Receptor Antibody
ASM	Airway Smooth Muscle
ASMC	Airways Smooth Muscle Cells
BAL	Bronchoalveolar Lavage
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CC	Beta Chemokine
CD	Cluster of Differentiation
CM	Conditioned Media
CNTF	Ciliary Neurotrophic Factor
CREB	cAMP Response Element Binding
CT-1	Cardiotrophin 1
CTGF	Connective Tissue Growth Factor
CXC	Alpha Chemokine
DD	Death Domain
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECM	Extracellular Matrix Remodeling
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FMO	Fluorescence Minus One
GE	General Electric Preparation of POLY I:C
Gp130	Glycoprotein 130
HASMC	Human Airway Smooth Muscle Cells
HRP	Horseradish Peroxidase
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IgG	Immunoglobulin G
IKK	I-Kappa-B Kinase

IL	Interleukin
IP-10/CXCL10	IFN Gamma Induced Protein-10/C-X-C Motif Chemokine-10
IRAK	Interleukin Receptor Associate Kinase
IRF	Interferon Regulator Factor
ISRE	Interferon Stimulated Response Element
IκB	I-Kappa-B
JAK	Janus Kinase
JNK	c-Jun N Terminal Kinase
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated Protein Kinase
MAPKK	Mitogen-activated Protein Kinase Kinase
MAPKKK	Mitogen-activated Protein Kinase Kinase Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MDC	Macrophage-derived Chemokine
MIP-1β	Macrophage Inflammatory Protein-1beta
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MSD	Mesoscale Discovery
MyD88	Myeloid Differentiation Primary Response Gene
NFκB	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells
NNT-1/BSF-3	Novel Neurotrophin-1/B Cell Stimulating Factor-3
OSM	Oncostatin M
OSMR	Oncostatin M Receptor
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PIGF	Phosphatidylinositol-glycan Biosynthesis Class F Protein
PMSF	Phenylmethylsulfonyl Fluoride
POLY I:C	Polyinosinic:Polycytidylic Acid
PRR	Pattern Recognition Receptor
RANTES	Regulated Upon Activation Normal T Cell Expressed and Secreted
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction

SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
sFIT-1	Soluble VEGF A Receptor
SRE	Serum Response Element
STAT	Signal Transducer and Activator of Transcription
TAK1	Transforming Growth Factor-Beta Activated Kinase
TARC	Thymus and Activation Regulated Chemokine
TBK-1	Tank Binding Kinase-1
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with Tween 20
TGF-β	Transforming Growth Factor Beta
TH	T-Helper Cells
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
TIR	Toll Interleukin-1 Receptor
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor-Alpha
TRAF6	Tumor Necrosis Factor Receptor Associated Factor-6
TRIF	Toll IL-1 Receptor Domain Containing Adapter Inducing IFN-Beta
TYK	Tyrosine Kinase
VCAM-1	Vascular Cell Adhesion Protein-1
VEGF	Vascular Endothelial Growth Factor

-CHAPTER 1-

INTRODUCTION

1.1 Asthma

Asthma is a variable respiratory condition that affects approximately 300 million people worldwide [1]. In asthma, contact with a stimulus, allergic or non-allergic, results in coughing, wheezing, shortness of breath and tightness in the chest. These symptoms present as a result of airway inflammation, cellular infiltration, mucus hyper-secretion, airway hyperresponsiveness (AHR) and extracellular matrix (ECM) remodeling [2, 3]. In sensitized individuals, upon exposure to a specific allergen, T-helper (Th) cells are recruited to atopic asthmatic airways and secrete Th2 associated cytokines, such as interleukin (IL)-4, IL-5 and IL-13 [4]. The production of these cytokines as well as other inflammatory mediators induces mast cell stimulation, eosinophilia, leukocytosis, and B cell antibody production, all of which contribute to the lung dysfunction observed during an asthmatic attack [4, 5]. AHR is a characteristic feature of asthma and occurs when the smooth muscle that surrounds the airways contracts (bronchoconstriction), causing excessive narrowing of the airways and therefore reduced lung capacity [6]. In asthma, the involuntary bronchoconstriction is augmented by asthmatic stimulae leading to further shortness of breath, chest tightness and coughing. Airway smooth muscle (ASM)

has been shown to not only play a role in the structural integrity of the lung and airway caliber, but can also participate in various immune/inflammatory processes [7]. A definitive mechanism of the development of this hyper-responsiveness has yet to be determined however genetic predisposition, airway inflammation, abnormalities/dysfunctions in ASMCs, and mucus hyper-secretion have all been found to impact this manifestation [8].

1.2. Asthma Exacerbations

Acute asthmatic exacerbations precipitate the induction and severity of AHR as well as other characteristics of asthma and are currently the major cause of morbidity and mortality in asthmatics [9]. Recent findings have identified a positive correlation between upper respiratory tract infections and asthmatic exacerbations when comparing patient nose/throat swabs to decreases in peak expiratory flow rate [10] . Furthermore, previous findings have suggested that viral infections are associated with 50% of asthmatic exacerbations in adults and up to 80% in children [9, 11]. Numerous respiratory pathogens have been implicated in asthmatic exacerbations including parainfluenza/influenza virus, respiratory syncytial virus and coronavirus, however rhinovirus has been identified as the most common viral pathogen linked to exacerbations [12, 13]. Although to a lesser extent than viral infections, bacterial infections such as Chlamydia pneumoniae and Mycoplasma pneumoniae have also been shown to be present during acute asthmatic exacerbations, however their roles in inducing these exacerbations is not fully understood [13, 14].

Current therapies employed for treating and preventing asthma symptoms such as inhaled steroids have not been shown to be effective against preventing acute exacerbations of asthma caused by viral and bacterial pathogens[9]. These findings suggest that the biological mechanisms of asthmatic exacerbations are different than the mechanisms of asthma itself, proposing the need for acute asthma exacerbation targeted therapies [9]. In order to establish optimal therapies for pathogen induced exacerbations, the pathogenesis of viral/bacterial induced exacerbations must first be better identified.

1.3 Role of Airway Smooth Muscle Cells in Asthma

Asthma is a chronic inflammatory disease that results in structural changes within the walls of airways due to ECM protein deposition, altered ASMC mass and increased numbers of fibroblasts [15]. The release of numerous pro-inflammatory cytokines and chemokines cause initiation and maintenance of inflammation, contributing to hypersensitive immune responses, which is the foundation for the characteristics of asthma. As a result, manifestations of asthma include airflow obstruction, bronchial hyper-responsiveness, airway inflammation and ECM remodeling [4].

ASMC's have recently become an area of interest for asthma as they have been found to not only be involved in the structural integrity of the lung and airway caliber but also in inflammatory processes [16]. Numerous immune modulators have been shown to be secreted by ASMCs including cytokines, chemokines, and growth factors. Previous findings have established ASMCs as a source of the chemoattractant eotaxin, which aids in the accumulation and activation of eosinophils, a novel feature of allergic asthma [7].

Regulated upon activation, normal T cells expressed and secreted (RANTES), is also a chemoattractant for eosinophils, as well as monocytes and memory T cells, and has been recognized to be released from human ASMCs (HASMC) [8]. Expression of adhesion receptor molecules, ICAM-1, VCAM, and CD44 are found on HASMC, implicating a role in T cell activation, asthmatic cellular infiltration and thus airway inflammation [17, 18]. Growth factors released from HASMC include vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, and platelet derived growth factor (PDGF), all of which may play a role in ECM remodeling [8, 19] . Cytokines are essential in asthmatic immune responses as they alter pro-inflammatory gene expression in accordance to stimulae [20]. ASMCs can secrete cytokines and chemokines such as IL-5, Interferon (IFN)-β, monocyte chemoattractant protein (MCP)-1,2 and 3, IL-8, and IL-17 [8, 21]. IL-6 and leukemia inhibitory factor (LIF), members of the gp130 family of cytokines, can also be released by ASMCs [21].

1.4 Extracellular Matrix Remodeling in Asthma

The ECM of the lung is comprised of collagens, elastin, proteoglycans and noncollagenous glycoproteins [22, 23]. These structural components aid in the ECM's role to maintain mechanical support as well as airway function and structure [24]. However, the ECM also has the capability to influence a variety of cellular functions. The nature of ECM may affect migration, differentiation and proliferations of various cells, and interact with inflammatory cells through cytokines, chemokines and growth factors [24, 25]. Alterations in ECM proteins and stromal cells such as, ASMCs and fibroblasts,

contribute to the pathogenesis of asthma [4, 24]. In addition to cytokines, chemokines and growth factors, ASMCs have also been recognized to express ECM proteins, matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs), all of which play important roles in ECM remodeling [24]. In a healthy lung, the homeostasis of ECM in airways and alveoli enable normal gas exchange, however the ECM remodeling that occurs in asthmatic lungs obstructs breathing capacity and thus gas exchange [26]. Previous findings in the lab have identified that increased amounts of collagen in the parenchyma of the lung in mice is associated with increased airway reactivity, tissue resistance and tissue elastance [27]. In relation to the impact ECM remodeling can have on the immune response, changes in ECM proteins, such as fibronectin and collagen induction, can intensify the inflammatory response through the secretion or inhibition of cytokines and chemokines which can alter ASMC responses [28].

Alterations in the ECM are dependent on increases in ECM proteins, such as collagen and elastin, and the balance between the activities of MMPs and TIMPs [24]. Examining the role HAMSCs play in these changes is vital to pin point their function in asthmatic ECM remodeling. ECM proteins from HASMC *in vitro,* have been found to be altered in response to allergic stimulae [24]. In addition, examination of asthmatic patients have identified that there is increased ECM, collagen and hyaluronan around ASM cells [29, 30]. ASMCs from asthmatics also produce more collagen type I and fibronectin than those of non asthmatics [31].These findings implicate ASMCs in ECM

remodeling, which may be further elucidated by their close proximity to the basement membrane [24]. The balance between MMPs and TIMPs has been identified as a key player in ECM remodeling as MMPs are enzymes that degrade the ECM, whereas TIMPs inhibit the degradation [32]. The activation of MMPs are controlled by proteases, membrane anchored MMPs and the activity of TIMPs [32]. MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, MMP-12 and MMP-14 have all been shown to be released from ASMCs [32].HASMC have been recognized as a source of MMPs and tissue remodeling in asthmatic has been found to be attributed to the induction of MMP-1, MMP-2, and MMP-9, implicating their importance in asthmatic ECM remodeling [33-35]. It has been found that TIMP-1 can inhibit MMP-1, MMP-2, MMP-3 and MMP-9, while TIMP-2 is specific for MMP-2 [32]. TIMP-1, TIMP-2 and TIMP-3 are secreted by ASMCs, however TIMP-1 has been found to be produced by asthmatic ASMCs however not TIMP-2 or TIMP-3 [24, 36]. ASMCs have also been associated in ECM remodeling as numerous ECM proteins have been found between muscle bundles, ASMCs can migrate into the luminal direction of the airway where they can communicate with epithelial cells, and asthmatic ASMCs can proliferate twice as fast as those from non asthmatic patients [24, 37].

Along with MMPs and TIMPs, growth factors have also been linked with ECM remodeling in HASMC. TGF-β, VEGF, and connective tissue growth factor (CTGF) are profibrotic stimulae and have all been shown to be secreted from HASMC [19, 24]. TGF-β stimulates ASMCs to release ECM proteins such as collagen type I and fribronectin.

Previous studies have identified TGF-β in asthmatic bronchoalveolar lavage (BAL) fluid and increased concentrations in asthmatic airways which has been correlated with a thicker basement membrane [38, 39]. CTGF is a downstream mediator of TGF-β and has been shown to be released by HASMC to induce production of fibronectin and collagen type I, with a greater response in correlation with TGF-β stimulation [40]. VEGF, an important growth factor in angiogenesis, can be produced by ASMCs in response to TGF-β and CTGF stimulation in ASMC [41]. Furthermore, in the presence of MMP-2, the ECM in ASMCs is reduced as it inhibits the interaction between VEGF and CTGF [41]. Although VEGF has been shown to increase ECM proteins in other cell types, the question of whether VEGF can cause fibronectin and collagen type I induction in HASMC remains to be completely understood. PDGF has also been shown to impact ECM remodeling by promoting proliferation of ASMCs [42]

1.5 Pro-inflammatory Proteins and Human Airway Smooth Muscle Cells

1.5.1. Gp130 Cytokines

The IL-6 subfamily of cytokines (also known as the gp130 cytokines) are grouped together as they share similarities between biological structure and functions [41, 43, 44]. Glycoprotein 130 (gp130), a signal transducing sub unit, is a component of the gp130 cytokine receptors [45] . Members of this family of cytokines include IL-6, IL-11,IL-31, IL-27, LIF, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and novel neurotrophin-1/B cell stimulating factor-3 (NNT-1/BSF-3) [43, 46-48]. Numerous biological activities can be initiated via these receptors such as cell differentiation,

apoptosis, inflammation, cell survival and hematopoeisis [45, 46]. The gp130 cytokines signal through receptor complexes that include the critical gp130 signaling molecule, with the exception of IL-31 which engages the OSMRBB/gp130 like protein complex [19]. In the human system, oncostatin M and LIF share many similarities in structure, function and genetics as they are both able to signal through the LIF receptor that is composed of LIFRβ and gp130 receptor chains [49]. However, OSM also engages the OSMRBB/gp130 complex, specific for OSM.

The role gp130 cytokines have in the pathogenesis of asthma is not completely understood, however they have been found to be produced in the airways of asthmatic patients in response to inflammatory stimuli and have been shown to exhibit pro-fibrotic characteristics [43]. HASMC have been stimulated with numerous gp130 cytokines to examine the impact they have on cytokine and chemokine production in these cells, however OSM seems to induce the greatest cytokine and chemokine production in these cells [19, 50].

1.5.2 Oncostatin M

Oncostatin M is a multi-functional cytokine belonging to the IL-6 subfamily of cytokines [46]. Biological activities of OSM include inflammatory responses, hematopoeisis, tissue remodeling, and growth modulation [46]. OSM can signal through two receptors, the OSMRβ (OSM/gp130 heterodimer) and LIFRβ (LIF/gp130 heterodimer, as above) receptors, both of which activate the JAK/STAT and MAPK (specifically ERK and JNK) pathways [44].

In regards to asthma, it has been shown by Simpson et al, that sputum from patients suffering from asthma contains OSM, and that OSM is expressed in airway [44] neutrophils and macrophages [51]. Specifically for ASM, the OSMRβ has been shown to be highly expressed on HASMC [43]. Furthermore, studies have also implicated OSM in the development of ECM remodeling in asthma as it has been found to be produced by infiltrating cells during an asthmatic attack (monocytes, macrophages, activated T cells and neutrophils) and contributes to the production of MMPs, TIMPs, collagen, and anti-proteases [43, 52, 53]. In human lung fibroblasts (*in vitro*), OSM has been found to induce collagen production, further implicating its role in ECM remodeling [54]. OSM, but not other gp130 cytokine members, has been shown to induce the production of VEGF, MCP-1 and IL-6 in HAMSCs [19]. OSM has also been implicated in the phosphorylation of ERK, JNK, and STAT3 in HASMC, where as other members of the gp130 cytokines have been less effective [19]. Stimulation of HASMC in culture with Th2 cytokines IL-4 and IL-13 resulted in dose dependent production of eotaxin, VEGF and MCP-1, and this response was amplified with increasing concentrations of OSM [50].

1.5.3 Interferons

Interferons (IFN) are proteins that posses anti-viral activity in response to foreign stimulae [55]. IFNs can be classified into three groups, type I IFNs, type II IFNs and IFN- like cytokines. Viral stimulae recognized by TLRs result in the expression of type I IFNs, which in humans consist of IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, when the

stimulae is specifically double stranded RNA ,IFN-α, IFN-β and IFN- ω are most commonly induced [28, 55, 56]. Although asthma has been characterized with mainly a Th2 cytokine expression, Th1 expression by the chemokine IP-10 (CXCL10) has also been identified in ASMCs stimulated with TLR3-ligand, POLY:IC [57]. IP-10 is a type I IFN inducible gene that can recruit Th1 cells to the lung and mast cells to ASM bundles [58]. Furthermore, stimulation of bronchial SMCs with Chlamydia pneumoniae produced low levels of IFN-β, signifying its relevance in bacterial respiratory infection [59]. IFN-β has also been implicated in the production of RANTES in HASMC by increasing the regulation of TNF-α [60]. Minimal studies have been performed examining the impact TLR-ligand stimulation can have on the production of type I IFNs in ASMCs, doing so would be required to further understand their importance in the pathologic exacerbations of asthma.

1.6 Toll-like Receptors in Human Airway Smooth Muscle Cells

Immune responses, such as inflammation, occur upon recognition of foreign stimulae. TLRs are responsible for notifying the innate immune system that such stimulae have come in contact with tissues. TLRs are a class of pattern recognition receptors (PRRs), that use pathogen-associated molecular patterns (PAMPs) to recognize microorganisms both outside of the cell and in intracellular endosomes and lysosomes [61, 62]. There are 10 known TLRs in humans, TLR1 to TLR10, which can be categorized into two groups depending on their localization and type of ligand [63]. TLR1, TLR2, TLR4, TLR5, and TLR6 are localized on the plasma membrane and

recognize membrane components of microbes [64].Typical ligands include: TLR1 ligand is triacyl lipoprotein found on bacteria; TLR2 ligand is lipoprotein found on bacteria, viruses, parasites; TLR4 ligand is endotoxin such as lipopolysaccharide (LPS) from bacteria, viruses; TLR5 ligand is flagellin from bacteria, and TLR6 ligand is diacyl lipoprotein from viruses and bacteria [61]. The second group of TLRs consists of TLR3, TLR8, TLR9 and TLR10, they recognize microbial nucleic acids and are localized in intracellular vesicles, endosomes and lysosomes [64]. The ligand for TLR3 is double stranded RNA from viruses; TLR8 ligand is single stranded RNA from viruses, bacteria; TLR9 ligand is the CpG motif from DNA of viruses, bacteria, protozoa, and the ligand for TLR10 remains unknown [61]. In addition, more recent data show that some mammalian TLRs can be engaged by endogenous ligands [65].

TLR3 and TLR4 signaling in HASMC in response to their ligands will be of main focus in this project. These two TLRs signal differently as mentioned previously, TLR3 is found in endosomes while TLR4 is located on the plasma membrane. Furthermore, TLR4 forms complexes with co-factors before initiating downstream signaling [66]. CD14, a co-molecule in TLR4 signaling in response to LPS, is a glycosylphosphatidylinositol- linked protein with leucine rich reapeats [67]. Along with the LPS binding protein, CD14 (soluble or membrane bound) can act on the TLR4-MD2 complex to initiate downstream signaling [67]. Numerous studies have implicated the role of both soluble and membrane CD14 in allergic asthma, however none have examined the impact of CD14 expression in HASMC.

Although few studies have questioned the association between TLRs and HASMC, TLR stimulation in asthmatics has been implicated in the exacerbation of asthma. A recent study by Kuo et al, have identified that TLR3 agonist, Poly I:C, as well as rhinovirus can induce deposition of both fibronectin and collagen in asthmatic HASMC suggesting that viruses could contribute to the severity of exacerbations through ECM remodeling [68]. It has also been shown that upon stimulation with TLR2, TLR3, and TLR4 agonists, ASMCs produce IL-8, eotaxin, IL-6, IP-10 (an IFN inducible gene) and ICAM-1 [69].Furthermore, cytokines such as TNF-α, IL-1β and IFN-γ, have the potential to up-regulate the expression of TLRs in ASMCs , resulting in increased concentrations of cytokines, chemokines and growth factors released from these cells [20]. Investigations on the expression of functional TLR2 and TLR4 in HASMC has unveiled that although these TLRs can be up-regulated with pro-inflammatory cytokines, there is minimal expression of functional TLR2 and TLR4 within these cells [57, 70]. Recent findings have established that TLR3 expression is greater than other TLRs in HASMC, signifying the rationale for focusing on TLR3 expression in these cells [18]. Further examination of the impact TLR-ligands have on ECM remodeling will aid in the search for effective treatments for the pathology of asthma.

1.7 Toll-like Receptor Signaling Pathways

TLR signaling pathways result in the up-regulation of genes that are important in the innate immune response. There are numerous signaling pathways TLRs can induce upon recognition by specific PAMPs.

1.7.1 My-D88 dependent and independent pathways

TLRs are a single-pass transmembrane protein with a toll IL-1 receptor (TIR) motif in their cytoplasmic domain [71, 72]. In the MyD88 dependent pathway, contact with a ligand causes TLRs to recruit the adaptor protein MyD88 that also contains a TIR domain which binds to the TIR domain of the TLR [72]. At the other end of MyD88 is a death domain (DD) that can bind to other molecules containing a DD. Serine/Threonine protein kinase, IL-1 receptor associated kinase (IRAK)-1 and 4, are recruited and activated by MyD88 upon hyperphosphorylation of IRAK-1 [73]. At this point, IRAK-1 is autophosphorylated and tumor necrosis factor receptor associated factor 6 (TRAF6) binds to the IRAK complex and recruits a mitogen activated protein kinase kinase kinase (MAPKKK) called TGF-β activated kinase (TAK1) [74]. TAK1 allows the activation of two pathways, the nuclear factor kappa light chain enhancer of activated B cells (NFκB) pathway and the MAPK pathway [74] . The MyD88 independent pathway allows for the production of type I IFNs. As opposed to MyD88 binding to the TIR of the TLR, the adaptor protein TIR domain containing adapter inducing IFN β (TRIF) is bound [71]. TRIF can also bind to TRAF6 stimulating the NFκB pathway or it can bind to tank binding kinase (TBK)-1 and IkKε which result in the activation of transcription factors known as IFN regulatory factors (IRFs) [75]. Once IRFs, IRF-3 and IRF-7, are phosphorylated they are translocated to the nucleus as dimers and are then capable of type I interferon gene expression [4] . Most TLRs contain a proline residue that is essential for MyD88 signaling and the production of inflammatory cytokines, however TLR3 has been found to lack this residue and in place has an alanine residue which

results in larger amounts of type I IFN being produced, suggesting it signals through the MyD88 independent pathway [74].

1.7.2 NFκB Signaling Pathway

NFκB is a transcription factor that is essential for immune responses as it is involved in the expression of cytokines, chemokines, adhesion molecules and growth factors [76]. Previous findings have implicated its role in asthma as increased markers for NFκB activity have been observed in the airways of asthmatic patients [77]. Upon TAK1 recruitment to the IRAK complex, TAK1 recruits the Ikβ kinase (IKK) complex which is made up of IKK-α, IKK-β and IKK-γ (NEMO), resulting in the phosphorylation and degradation of Ikβ, allowing NFκB to translocate from the cytolplasm into the nucleus [61]. IKK-β has been found to be more active than IKK-α in phosphorylating IkB and has also been recognized as essential in NFκB activation in ASMCs [78]. NFκB is most commonly made up of dimers of members of the DNA bindings Rel family of proteins, P50 and P65, however P52, c-Rel and Rel-B may also be found in specific cells [16]. Numerous cytokines, chemokines, and growth factors produced by ASMCs have been correlated with the NFκB signaling cascade. IL-8, TGF-β, ICAM-1 and VCAM have all been shown to be expressed via this pathway [16]. In addition, LPS elicits activation of the MAPK signaling pathway, ERK1/2 and p38, which subsequently activate the NFκB pathway for IL-6 production in ASMCs [79]. Furthermore, IKK-β inhibition through knockouts has demonstrated its function in the expression of ICAM-1, IL-6, IL-8, RANTES and MCP-1 in ASMCs [78].

1.7.3 MAPK Signaling Pathway

The mitogen activated protein kinases (MAPK) signal transduction signaling pathway consists of the MAPK cascade. The MAPK cascade is made up of MAPK (ERK1/2, JNK1/2, P38), MAPK kinase (MEK1/2, MKK3/4/6/7), and MAPK kinase kinase (Raf-1, MEKK 1-4) [80]. As an example, in the MAPK/ERK pathway, upon recognition of growth factors by receptors tyrosine kinase receptors, GTP-binding protein RAS is activated by guanine-nucleotide exchange factors by allowing GTP to bind to the protein [81]. As a result, a MAPKKK (RAF-1) is recruited and phosphorylates a MAPKK (MEK) which then phosphorylates a MAPK (ERK), activation of the ERK allows it to translocate to the nucleus and phosphorylate a transcription factor (ELK) which binds at the serum response element (SRE) of c-Fos for gene expression [81]. Another example is the JNK pathway; JNK binds to the transcription factor c-Jun in the cytoplasm to cause phosphorylation and translocation into the nucleus where it then binds to c-Fos to form AP-1 which is responsible for the cellular response of environmental stimulae [82]. The p38 pathway can result in the activation of transcription factors ELK, CREB, and CHOP [81]. There are three subfamilies of kinases that have been implicated in ASMCs proliferation and inflammatory responses, extracellular signal-regulated kinase (ERK), p38, and c-Jun N terminal kinase (JNK) all of which can be activated by cytokines as well other molecules [79, 81].

ERK activation, through phosphorylation, has been identified to be stimulated by OSM, PDGF, EGF, and LIF in HASMC, which have led to the expression of MMP-9,

eotaxin, RANTES, IL-8, VEGF, and IL-17 [18-20, 83-85]. P38 has been correlated with IFN-β production and with IL-6, IL-8 and eotaxin secretion in response to IL-17, as well as increasing IL-6 and RANTES secretion in TNF-α stimulated cells [20]. JNK has been demonstrated in the production of RANTES and IL-8 release from HASMC after stimulation with IL-1β and TNF-α [86]. As mentioned previously, LPS elicits activation of the MAPK signaling pathway, ERK1/2 and p38, which subsequently activated the NFκB pathway for IL-6 production in ASMCs [79]. Further examination of the MAPK signaling pathways involved in TLR and OSM stimulation is required to advance the understanding of these mechanisms.

1.7.4 JAK-STAT signaling Pathway

The Janus tyrosine kinases (JAKs) are a group of non-receptor tyrosine kinases that activate the signal transducer and activator of transcription (STAT) pathway in response to various inflammatory molecules [87]. JAKs are positioned at the cytoplasmic tail of cytokine receptors that results in tyrosine phosphorylation of the receptor and thus the recruitment of STATs (STAT-1 through STAT-6), leading to the production of homo or heterodimers that translocate into the nucleus for gene expression [87, 88]. The gp130 family of cytokines has been shown to signal through the activation of JAK 1, JAK2 and TYK2 and by recruiting STAT3 [38, 43, 49]. In accordance, results from previous studies have indicated that the effects of OSM to induce VEGF and eotaxin expression are mediated by STAT3 [19, 50]. In response to IL-4, HASMC secreted eotaxin in a STAT6 dependent pathway, however previous findings in the lab have shown that increased

collagen production by AdmOSM in mice was STAT6 independent [27, 89]. In addition, PDGF induction has been shown to activate JAK2, STAT1 and STAT3 in asthma [87]. Type I IFNs also signal through the JAK-STAT pathway mediated by the activation of STAT1, STAT2, STAT3, TYK2 and JAK1, where IFN –stimulated response element (ISRE) is responsible for their production [55]. Very few studies have examined the role TLR ligands have on the activation of the JAK-STAT pathway in HASMC, and none have determined the function and mechanisms of OSM in this response.

1.8. Thesis Proposal

1.8.1. Hypothesis

OSM markedly increases lung airway smooth muscle cell responses to external stimulae, such as products of bacteria, viruses or TLR-ligands that activate toll-like receptors. This exacerbates inflammation and extracellular matrix remodeling which contributes to pathology in asthmatic patients**.**

1.8.2. Specific Hypothesis for this Project

In HASMC *in vitro*, OSM augments the production of cytokines, chemokines, and ECM modulators in response to TLR-ligands. This occurs through up-regulation of TLR expression and amplified signaling cascades.

In order to test the above hypothesis, two main aims have been of central focus.

1.8.3. Rationale: Production of Pro-inflammatory and ECM Proteins

Various cytokines and chemokines serve as stimulants for signaling pathways that modulate ECM remodeling and therefore require further attention to better understand inflammatory signaling in HASMC. Previous findings have identified that HASMC express TLR1 through TLR10 [69] at the mRNA level, although this has not been confirmed at the protein level. In addition, upon stimulation with TNF-α , IL-1 β, and IFN- γ there is an increased expression of IL-8, a modulator of chemotaxis for neutrophils; eotaxin, a chemoattractant for eosinophils, and an up-regulation of TLR2 and TLR3 expression [69]. These findings demonstrate that HASMC can produce pro-inflammatory chemokines upon stimulation of the TLRs they express. Furthermore, stimulation with inflammatory mediators, such as TNF- α, IL-1β and IFN- γ up regulate TLR expression which has been associated with an increased production of IL-8 [69]. However, the effects of OSM and other gp130 cytokines in this context are unknown.

Recent findings in the lab have demonstrated that OSM can increase MCP-1 and IL-6 production in HASMC stimulated with TLR3 and TLR4-ligands (unpublished data), suggesting that OSM can also modulate cytokine and chemokine production in response to TLR agonists. OSM has also been recognized as a modulator of VEGF production in HASMC, demonstrating its ability to potentiate ECM remodeling. Examining the cytokines and chemokines that are up-regulated by OSM after TLR stimulae is important to determine potential roles of HASMC in infections that exacerbate asthmatic pathology.

Using three objectives, this aim will examine the ability of TLR-ligands and OSM in HASMC to alter inflammatory cytokines and chemokines, type I IFNs, and ECM modulators *in vitro*, at the protein and mRNA level.

A: Identifying cytokines and chemokines produced in response to TLR-ligands and OSM

B: Determining RNA expression of cytokines and chemokines by TLR-ligands and OSM

C: Examining protein and RNA levels of ECM modulators by TLR-ligands and OSM

1.8.4. Rationale: Receptor Regulation and Signaling Cascades

Identifying the mechanisms that modulate OSM and TLR responses in inflammation and ECM remodeling is essential to pin point effective modulation. The synergy between OSM and TLR-ligands may involve receptor modulation or post-transcriptional modifications of target genes. As mentioned earlier, stimulation with inflammatory mediators, such as TNF- α, IL-1β and IFN- γ have been shown to up regulate TLR2 and TLR3 expression [69]. In addition, previous findings in the lab using PCR and gel analysis suggested that in HASMC, TLR3 expression is slightly increased with OSM stimulation, TLR4 is unaffected while CD14 may be up regulated upon the addition of OSM. These data, as well as others that have shown up regulation of TLRs in response to inflammatory cytokines, suggest that OSM induced inflammatory responses in HASMC may be in part due to the regulation of TLRs [75] .

Synergy between OSM and TLR-ligands in HASMC may also involve alteration of signaling cascades. Various signaling pathways have been recognized in the mediation of

cytokine, chemokine, growth factors and enzyme production in HASMC including MyD88, NFκB, MAPK (specifically ERK1/2, p38 and JNK) and JAK-STAT .In regards to OSM, the gp130 family of cytokines have been shown to signal through the activation of JAK 1, JAK2 and Tyk2 and by recruiting STAT3 [38, 43, 49]. In HASMC stimulated with OSM, STAT3 activation has been implicated in VEGF and eotaxin production, while ERK and JNK have also been shown to mediate eotaxin induction [19, 50]. Type I IFNs also signal through the JAK-STAT pathway, mediated by the activation of STAT1, STAT2, STAT3, TYK2 and JAK1 where IFN –stimulated response element (ISRE) is responsible for their production, however further investigation is necessary to determine how OSM stimulation in response to TLR-ligands could impact this mechanism in HASMC [55]. Examining the signaling pathways that TLR-ligands induce in these cells and how OSM alters this response will aid in understanding the mechanisms by which TLR-ligands and OSM combine to regulate cell phenotypes.

Two objectives will be used to examine TLR receptor regulation and amplified signaling cascades in HASMC upon OSM and TLR-ligand stimulation.

A: Examining receptor modulation in response to OSM and TLR-ligands in HASMC

B: Identifying signaling pathways induced by OSM and TLR-ligands in HASMC

-CHAPTER 2-

MATERIALS AND METHODS

2.1 Cell Culture

In collaboration with Dr. P. Nair (McMaster University, Ontario, Canada) we obtained cultures of human airway smooth muscle cells derived from airways of several different patients. Resected lung portions were obtained at St. Joseph's Healthcare, Hamilton from patients undergoing surgery for lung cancer. Patient characteristics can be found in **[Table 1]** in the appendix. Smooth muscle was isolated from healthy un-affected areas of the bronchi. Patient consent was obtained and tissue resection was approved by the hospital Research Ethics Board. HASMC were obtained from a different patient on each day, March 11 (HASMC-M), February 23 (HASMC-F), June 24 (HASMC-J) and April 5 (HASMC-A). These were cultured in RPMI medium supplemented with 1% penicillin/streptomycin, 1% L-glutamine, 0.1% fungizone, and 10% FBS, have a distinctive morphology in culture, and we used them for analysis at no more than 10 passages *in vitro*.

2.1.1 Gp130 Cytokine Stimulations

Upon confluence in 75cm² cell culture flasks, HASMC were lifted from flasks with 0.5% trypsin-EDTA (stock diluted 10X in PBS) and were seeded into 96 well plates at 10^4 cells per well. Upon 75-80% confluence, HASMC were serum deprived for 3

hours (replaced 10% FBS RPMI medium with 2% FBS RPMI medium) followed by stimulation with various concentrations of Gp130 cytokines *in vitro*. Serum deprivation was performed to reduce basal levels of cytokine and chemokine detection in supernatants. Recombinant human Gp130 ctyokines were purchased from R&D Systems (Minneapolis, MN) and included recombinant human OSM (R&D Systems Cat.# 295-OM) , recombinant human IL-6 (R&D Systems, Cat.# 206-IL-010), recombinant human IL-11 (R&D Systems Cat.# 218-IL), recombinant human IL-31 (R&D Systems Cat.# 2824-IL) and recombinant human LIF (R&D Systems Cat.# 249-LR-050). These cytokines were diluted to optimal concentrations with 2% FBS RPMI medium. Diluted cytokines were then added to HASMC in 96 well plates (100µl per well) and were incubated at 37˚C for 18 hours. After the time course, supernatants were removed from the cells and placed at -20˚C to be used for protein analysis with ELISA and MSD assays.

2.1.2. TLR-ligand Challenges

Upon confluence in 75cm² cell culture flasks, HASMC were lifted from flasks with 0.5% trypsin-EDTA (stock diluted 10X in PBS) and were seeded into 96 well plates at 10^4 cells per well. Upon 75-80% confluence, HASMC were serum deprived for 3 hours (replaced 10% FBS RPMI medium with 2% FBS RPMI medium) followed by stimulation with various concentrations of TLR-ligands *in vitro*. Serum deprivation was performed to reduce basal levels of cytokine and chemokine detection in supernatants. TLR-ligands and soluble CD14 were obtained from the lab of Dr. A. Ashkar (McMaster University, Ontario, Canada). TLR3-lignads (POLY IC) included preparations from

Sigma-Aldrich (a long and a short) and a preparation from General Electric. TLR4-ligand used was lipopolysaccharide. Diluted TLR-ligands (in 2% FBS RPMI) were then added to HASMC in 96 well plates (100µl per well) and were incubated at 37˚C for 18 hours. After the time course, supernatants were removed from the cells and placed at -20˚C to be used for protein analysis with ELISA and MSD assays.

2.1.3. Neutralizing Antibody Experiments

Upon confluence in 75cm² cell culture flasks, HASMC were lifted from flasks with 0.5% trypsin-EDTA (stock diluted 10X in PBS) and were seeded into 96 well plates at 10^4 cells per well. Upon 75-80% confluence, HASMC were serum deprived for 3 hours as mentioned previously. PBMCs were isolated from a healthy subject and stimulated with LPS for 24 hours. PBMC conditioned media (CM) was added to HASMC at a 1:800 dilution in 2% FBS RPMI for 18 hours in the absence or presence of anti-OSM, anti-OSMRB, or mouse IgG. Anti-OSM was purchased from R&D Systems (Minneapolis, MN) and was incubated with PBMC CM for 2 hours in eppendorf tubes at 37˚C at 1.3 and 3.8µg/ml prior to HASMC stimulation. Anti-OSMRB was received from Amgen Canada (Mississauga, ON) and HASMC were primed with 1.3 and 7.6ng/ml of antibody for 1 hour at 37˚C prior to HASMC stimulation with PBMC CM. Cells neutralized with anti-OSMRB and PBMC received a second dose of antibody at the same concentration in combination with PBMC CM. HASMC were also challenged with mouse IgG at the same concentrations as the anti-OSM and anti-OSMRB as a control.

HASMC were stimulated for 18 hours; the supernatants were removed and stored at -20˚C until use with MSD and ELISA.

2.2. Protein Analysis

2.2.1. Enzyme-linked Immunosorbent Assays (ELISAs)

Supernatants from HASMC cultures previously stimulated with Gp130 cytokines and TLR-ligands were thawed and used to determine supernatant concentrations of IL-6, MCP-1, CD14, IFN-α, IFN-β, and VEGF. ELISA DuoSets were purchased from R&D Systems (Minneapolis, MN) and included human IL-6 DuoSet (R&D Systems Cat.# DY206), human CCL2/MCP-1 DuoSet (R&D Systems Cat.# DY279), human VEGF Duoset (R&D Systems Cat.# DY293B) , human CD14 DuoSet (R&D Systems Cat.# DY383), human OSM DuoSet (R&D Systems Cat.# DY295), VeriKine human IFN-α (R&D Systems Cat.# 41410 and VeriKine human IFN-β (R&D Systems Cat.# 41100). Sensitivities of ELISAs varied between kits and were as follows: human IL-6 (≤ 9.4 pg/ml), human MCP-1 (≤ 15.6 pg/ml), human VEGF (≤ 31.25 pg/ml), human CD14 (≤ 62.5 pg/ml), human OSM (≤ 31.25 pg/ml), human IFN-α (≤ 12.5 pg/ml) and human IFN-β (≤ 25 pg/ml). ELISA assays were performed according to the manufacturer's instructions.

2.2.2. MSD Mesoscale Analysis

Supernatants from HASMC cultures previously stimulated with Gp130 cytokines and TLR-ligands were thawed and used to determine supernatant concentrations using MSD Multi-Spot assays (MesoScale Discovery, Gaithersburg, MD). Multi-Spot assays

used included: human chemokine 9-plex (Cat.# K15001C-1) to quantify CC and CXC chemokines (MCP-1, MCP-4, MIP-1β, eotaxin-1, eotaxin-3, TARC, MDC, IP-10 and IL-8), human chemokine 7-plex (Cat.# K15031C-1) to quantify CC and CXC chemokines (MCP-1, MCP-4, eotaxin-1, TARC, MIP-1β, IP-10, IL-8), human MMP-3 (Cat.# K15034C-1) and human MMP-2 (Cat.# K15033C-1) to quantify MMP-1, MMP-2, MMP-3, MMP-9 and MMP-10, human growth factor panel II (Cat.# K15029C-1) to quantify ECM modulators (bFGF, PIGF, sFIT-1, and VEGF) and human Th1/Th2 10-plex (Cat.# K15010C-1) to quantify cytokines (IFN-γ, IL-10, IL-12p70, IL-13, IL-2, IL-5, IL-4, IL-8, TNF-α, IL-1β). Multi-Spot assays were performed according to the manufacturer's instructions and plates were read with MSD SECTOR® Imager 2400.

2.2.3. Flow Cytometry

Upon confluence in 75cm² cell culture flasks, HASMC were lifted from flasks with 0.5% trypsin-EDTA (stock diluted 10X in PBS) and were seeded into 75cm² cell culture dishes. HASMC were serum deprived for 3 hours (replaced 10% FBS RPMI medium with 2% FBS RPMI medium) followed by stimulation with 2µg/ml or 10µg/ml of OSM. Diluted OSM was made with 2% FBS RPMI medium and added to cells in dishes (5ml) for 2 hours, 6 hours, or 24 hours. After the time courses, supernatants were removed and 1mM EDTA PBS was added to HASMC for 30 minutes in the incubator (37˚C). After 30 minutes, cells were removed from dishes by pipetting cells off and cells were collected in FACS tubes. Cells were then spun down at 1200 rpm for 5 mins, liquid was splashed out and cells were re-suspended in 200µl PBS to be transferred to a 96 well

plate for staining. After spinning down cells (as mentioned above) Fc block [1:200 dilution with FACS buffer (0.3% BSA in PBS)][51, 90] was added to cells for 30 minutes (25µl) at 4˚C. After the 30 minutes, 150µl of FACS buffer was added to wells and cells were then spun down (as mentioned above) followed by the addition of 25µl of human TLR4 (Anti-Human CD284 Alexa Fluor®488, eBioscience, San Diego, CA, Cat.# 53-9917) and human CD14 antibodies (Anti-human CD14 APC, eBioscience, San Diego, CA, Cat.# 17-0149) at 1:25 dilution in FACS buffer for 30 minutes at 4˚C. After the TLR4 and CD14 staining, 150µl of FACS buffer was added to wells and cells were spun down (as mentioned above) followed by the addition of 150µl of fixative cytofix/cytoperm fixation/permeabilization kit (BD Bioscience, San Diego, CA, Cat.# 554715) for 30 minutes at 4˚C. After fixation cells were spun down (as mentioned above) and human TLR3 antibody (Anti-human CD283 PE, eBioscience, San Diego, CA, Cat.# 12-9039) was added (25µl at a 1:50 dilution in Perm wash from BD kit mentioned above) for 30 minutes at 4˚C. After TLR3 staining, 150µl of FACS buffer was added to wells and cells were spun down (as mentioned above) followed by the addition of 200µl of FACS buffer. Cells (in FACS buffer) were then transferred to FACS tubes to be read with BD LSR II flow cytometer (BD Bioscience, San Diego, CA). Data was analyzed using FlowJo Software (Tree Star, Ashland, OR). Gates for antibodies were set according to istoype controls/FMOs mentioned below.

Multi-color flow cytometer experiments require fluorescence minus one (FMOs) in order to set appropriate gates for each antibody. These were prepared by making an

FMO for each antibody which consisted of the specific antibody an FMO was required for and isotype controls for the other antibodies in the multi-color stain. Isotype controls used in the FMOs were as follows: TLR4 isotype control (Mouse IgG2a K isotype control Alexa Fluor®488, eBioscience, San Diego, CA, Cat.# 53-4724), TLR 3 isotype control (Mouse IgG1 K istotype control PE, eBioscience, San Diego, CA, Cat.# 12-4714), and CD14 isotype control (Mouse IgG1 K isotype control APC, eBioscience, San Diego, CA, Cat.# 17-4714). Lastly, multi-color experiments also require compensation beads to perform optimal fluorescence compensation. BD CompBead Plus kit (BD Bioscience, San Diego, CA, Cat.# 560497) was used to prepare the compensation by following the manufacturer's instructions.

2.3 RNA Analysis

2.3.1. RNA Isolation

Upon 75-80% confluence in 75cm² cell culture flasks, HASMC were serum deprived for 3 hours (replaced 10% FBS RPMI medium with 2% FBS RPMI medium) followed by stimulation with OSM and/or challenges with TLR-ligands. For the OSM time course experiment, 2ng/ml OSM was added to flasks for 6 hours or 24 hours. For OSM and TLR-ligand experiment, 2ng/ml OSM, and 2µg/ml Poly I:C 20ng/ml LPS in the absence or presence of 2ng/ml OSM for 6 hours. OSM and TLR-ligand concentrations were diluted with 2% FBS RPMI medium and 5ml was added to each designated flask. After the time courses, supernatants were removed from cells and RNA was isolated using Ambion Purelink RNA mini kit (Life Technologies, Carlsbad, CA,

Cat.# 12183018A) by following manufacturer's instructions. Genomic DNA was removed using Ambion DNA-free kit (Ambion, Austin, TX, Cat.# AM1906) by following the manufacturer's instructions. RNA concentrations were obtained using NanoVue spectrophotometer. Reverse transcription of RNA was performed using Invitrogen Superscript II Reverse Transcriptase kit (Life Technologies, Carlsbad, CA, Cat.# 18064014) and was carried out according to the manufacturer's instructions.

2.3.2. qRT-PCR

mRNA expression was examined for human IL-6, MCP-1, eotaxin-1, eotaxin-3, IP-10, VEGF, smooth muscle actin, elastin, collagen1A1, TLR4, TLR3, CD14 and OSMRBβ using real-time quantitative PCR (following the TaqMan protocol). Pre-developed gene-expression assays were used from Applied Biosystems (Foster City, CA) and were as follows: IL-6 (Hs00985639_m1), MCP-1 (Hs00234140_m1), eotaxin-1 (Hs00237013_m1), eotaxin-3 (Hs00171146_m1), IP-10 (Hs00171042_m1), VEGF (Hs00900055_m1), smooth muscle actin (Hs00909449_m1), collagen 1A1 (Hs00164004_m1), elastin (Hs00355783_m1), TLR4 (Hs01060206_m1), TLR3 (Hs01551078_m1), CD14 (Hs00169122_g1), and OSMRBβ (Hs00384276_m1). Endogenous control pre-developed assay for β-Actin (Applied Biosystems, Foster City, CA, Cat.# 4333762F) was used as a housekeeping gene and all mRNA data was analyzed relative to β-Actin threshold cycle values. TaqMan Universal Master Mix manufactured by Roche (Branchburg, NJ) was bought from Applied Biosystems (Foster City, CA, Cat.# 4304437) and used along with nuclease free water and the pre-developed TaqMan probes

as the plate reaction mixture. Once prepared, the plate was read and analyzed with the ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) using a ΔΔCT study which was operated by Sequence Detector version 2.1 software. Data represents one experiment, therefore error bars are for variations in responses detected in replicates. Statistics were performed to determine specific qualitative trends in HASMC mRNA expression.

2.4. Signaling Analysis

2.4.1. Cell Lysates

Upon confluence in 75cm² cell culture flasks, HASMC were lifted from flasks with 0.5% trypsin-EDTA (stock diluted 10X in PBS) and were seeded into 6 well plates. Upon 75-80% confluence , cells were serum deprived for 3 hours (replaced 10% FBS RPMI medium with 2% FBS RPMI medium) followed by stimulation with 2ng/ml OSM, or 2µg/ml Poly I:C/20ng/ml LPS in the absence and/or presence of 2ng/ml OSM for 62 minutes and 2 hours. After the time courses, the 6 well plates containing the cells were placed on ice, the supernatants were removed, the cells were washed with PBS and cell lysates were prepared with Radioimmunoprecipitation (RIPA) lysis buffer (1X PBS, 1% Igepal CA-630, 0.5% Sodium Deoxycholate, and 0.1% Sodium Dodecyl Sulfate (SDS). Immediately before adding lysis buffer to cells, inhibitors [0.5% Sodium Orthovanadate (Na3VO4), 0.5% Phenylmethylsulfonyl Fluoride (PMSF), 3% Aprotinin and 0.1% 1.0M Dithiothreitol (DTT)] were added to the buffer. 200µl of RIPA lysis buffer with inhibitors was added to each well for 2 minutes. After 2 minutes the cells were scrapped

off the plate using cell scrappers. The RIPA lysis buffer (with the cells) was collected into eppendorf tubes and incubated on ice for 1 hour. Following the incubation, the lysates were sheared using a needle and syringe and centrifuged at 12,000 rpm for 10 minutes at 4˚C. The supernatants were collected and stored at -80˚C until use for Western Blotting.

2.4.2. Western Blots

Bradford assays were performed to determine concentrations of protein in HASMC lysates. 1:32 dilution of cell lysates in water were prepared and compared to a BSA standard (0-14ug standard) after the addition of Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, Cat.# 500-0006). 15ug/ul protein per sample was added to 10% SDS-PAGE gels (along with prestained PageRuler Protein ladder (Thermo Scientific, Waltham, MA, Cat.# 26616) for electrophoresis using the Mini-PROTEAN® 3 electrophoresis cell (Bio-Rad, Hercules, CA). Gels were then transferred onto pure 0.2µm nitrocellulose membranes (PALL Life Sciences, Ann Arbor, MI, Cat.# 71445F) using Mini-Trans-Blot® cell systems (Bio-Rad, Hercules, CA). After the transfer was complete membranes were blocked in 5% fat-free powdered milk made with Tris-Buffered Saline 0.15% Tween-20 (TBS-T) for 1 hour on a rocker at room temperature.

Primary antibodies were added to membranes overnight (1:1000 dilution) on rocker at 4˚C. Monoclonal rabbit IgG Phosphorylated Tyr705 STAT3 antibody (Cell Signaling Technology, New England Biolabs, Canada, Cat.# 9145), polyclonal rabbit IgG STAT3 antibody (Cell Signaling Technology, New England Biolabs, Canada, Cat.#

9132), Polyclonal rabbit IgG Phosphorylated Tyr694 STAT5 antibody (Cell Signaling Technology, New England Biolabds, Canada, Cat.# 9351), Polyclonal rabbit IgG STAT5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, Ca.# sc-835), Polyclonal rabbit IgG Phosphorylated Tyr641 STAT6 antibody (Cell Signaling Technology, New England Biolabs, Canada, Cat.# 9361), Polyclonal rabbit IgG STAT6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, Ca.# sc-981), Monoclonal rabbit IgG Phosphorylated NFκB Ser536 p65 antibody (Cell Signaling Technology, New England Biolabs, Canada, Cat.# 3033) and Polyclonal rabbit IgG NFκB p65 antibody (Cell Signaling Technology, New England Biolabs, Canada, Ca.# 3034). Primary antibodies were diluted in 5% milk with TBS-T (STAT5 and STAT6) or 5% BSA with TBS-T (STAT3, Phospho STAT3, Phospho STAT5, Phospho-STAT6, Phospho NFκB p65, and NFκB p65). Membranes were washed with TBS-T and goat anti-rabbit IgG-HRP conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, Ca.# sc-2301) was added for 1 hour at room temperature on rocker (1:2000 dilution in 5% milk in TBS-T). Following washes with TBS-T and TBS, Pierce® ECL 2 Western Blotting Substrate (Thermo Scientific, Waltham, MA, Ca.# 80196) was then added to membranes according to manufacturer's instructions to visualize protein on membranes.

2.5. Statistics

Data was analyzed using GraphPad Prism 5(GraphPad Software, La Jolla, CA). When a Two-way ANOVA with a Bonferroni post test was used, significance was 95% CI *=$P<0.05$, **=$P< 0.001$, and ***= $P<0.0001$. When a One-way ANOVA with a

Bonferroni post test was used, significance was 95% CI #=P<0.05, ##=P< 0.001, and ###= P<0.0001. All error bars represent the mean standard error (SEM).

-CHAPTER 3-

RESULTS

3.1. Cytokine and Chemokine Production in Response to TLR-ligands and OSM

3.1.1. OSM as an Inducer of Pro-inflammatory Proteins

Initial experiments focused on confirming previous observations with multiple patient cell lines to confirm responses in our system. HASMC-M, HASMC-J and HASMC-F were cultured as discussed in materials and methods and stimulated with OSM to test if OSM can induce the production of pro-inflammatory proteins. HASMC-M were cultured and plated into 96 well plates at 10^4 cells per well. Upon 75-80% confluence HASMC-M were serum deprived with 2% FBS for 3 hours followed by stimulation with increasing concentrations of OSM (0ng/ml, 0.5ng/ml, 2ng/ml, 5ng/ml and 10ng/ml) for 18 hours. After the 18 hour time point supernatants were removed from the cells and frozen at -20˚C. ELISA Duosets for MCP-1 and IL-6 were then performed to examine concentrations (pg/ml) in supernatants **[Figure 1 A-B]**. Previous results in the laboratory from a different patient cell line have identified OSM as an inducer of MCP-1 and IL-6 on HASMC (unpublished data). The data from the HASMC-M further demonstrates this since OSM induced a significant dose dependent induction of MCP-1 and IL-6 levels. HASMC-M supernatants contained up to 6000pg/ml of MCP-1 at the

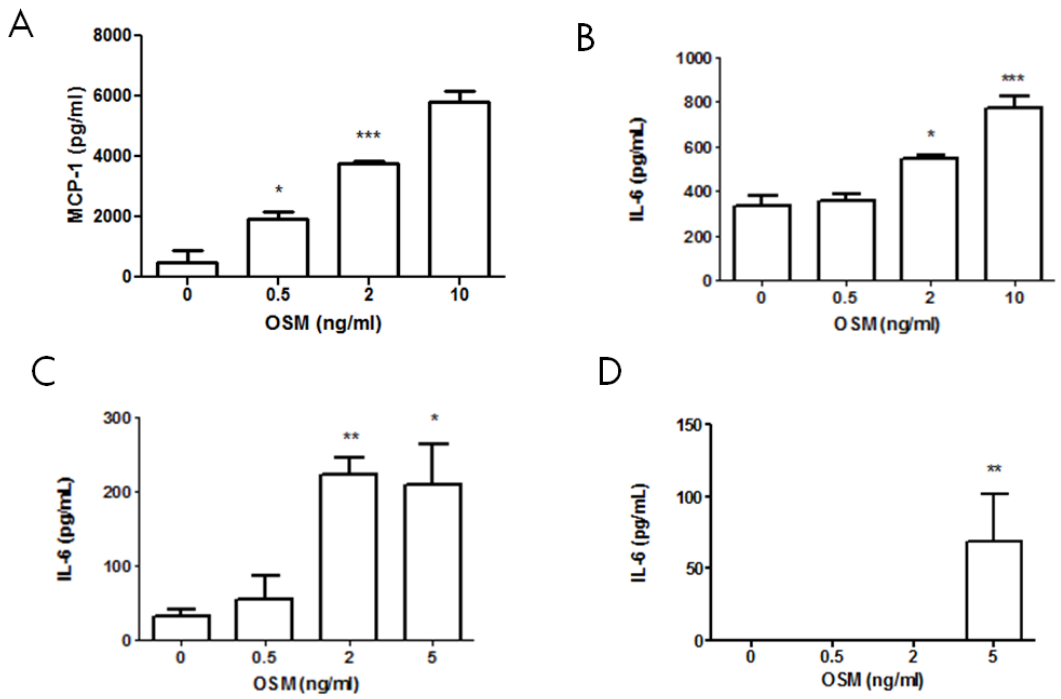
highest concentration of OSM (10ng/ml) stimulus, where as IL-6 concentrations in supernatants were lower (~800 pg/ml at highest OSM stimulus).

To confirm that this dose dependent response can be reproduced in more than one cell line, HASMC-J and HASMC-F were stimulated in the same fashion as HASMC-M followed by an IL-6 ELISA of cell culture supernatants **[Figure 1 C-D]**. Although the cell lines secreted different amounts of IL-6 at the highest concentration of OSM, a dose dependent induction of IL-6 was seen similar to HASMC-M. HASMC-M stimulated with 2ng/ml OSM responded with 600pg/ml IL-6, HASMC-J with ~200pg/ml IL-6 (3-fold less), while stimulation of HASMC-F induced low levels of IL-6 (although ~50pg/ml was present at 5ng/ml OSM). Thus it can be concluded that the three cell lines respond to OSM stimulation in the same qualitative fashion, but at varying degrees of sensitivity.

FIGURE 1. OSM induces MCP-1 and IL-6 protein expression in HASMC.

HASMC-M were stimulated with increasing concentrations of OSM for 18 hours. ELISAs were performed to examine **(A)** MCP-1 and **(B)** IL-6 concentrations in supernatants. **(C)**HASMC-J and **(D)** HASMC-F were stimulated with increasing concentrations of OSM One-way ANOVA with a Bonferroni post test was used, significance with 95% CI #P<0.0001 in comparison to control levels (OSM at 0ng/ml). Error bars represent SEM.

FIGURE 1.


A
MCP-1 (pg/ml)
8000
6000
4000
2000
0
*
***
0
0.5
2
10
OSM (ng/ml)
B
IL-6 (pg/mL)
1000
800
600
400
200
0
*
***
0
0.5
2
10
OSM (ng/ml)
C
IL-6 (pg/mL)
300
200
100
0
**
*
0
0.5
2
5
OSM (ng/ml)
D
IL-6 (pg/mL)
150
100
50
0
**
0
0.5
2
5
OSM (ng/ml)

3.1.2. Pro-inflammatory mRNA Expression in Response to OSM

In order to determine if protein responses correlated with mRNA levels in HASMC, qRT-PCR was used to asses MCP-1, IL-6, eotaxin-1, eotaxin-3 and IP-10 mRNA levels. HASMC-A and HASMC-J were stimulated with 2ng/ml OSM for 6 hours or 24 hours. After each time point, RNA was isolated, treated with DNase, followed by PCR and qRT-PCR.. TaqMan probes for the proteins mentioned above were used along with β-Actin as an endogenous control. **[Figure 2]** demonstrates that elevated mRNA levels are consistent with elevated protein levels **[Figure 1].** Both MCP-1 and IL-6 mRNA levels were significantly increased at 6 hours and 24 hours in HASMC-A. In HASMC-J, MCP-1 and IL-6 mRNA levels were significantly increased at 6 hours however mRNA levels return back to normal at 24 hours. This data could suggest that HASMC-J respond earlier than HASMC-A in regards to MCP-1 and IL-6 expression. Eotaxin-1 and eotaxin-3 mRNA levels were also significantly up-regulated upon OSM stimulation at 6 hours and 24 hours in both cell lines (with the exception of eotaxin-3 in HASMC-A at 6 hours). Interestingly, IP-10 expression was significantly increased at 6 hours and returned back to control levels by 24 hours in both cell lines.

FIGURE 2. OSM increases cytokine/chemokine mRNA expression in HASMC. HASMC-A and HASMC-J were stimulated with 2ng/ml OSM for 6H and 24H. RNA levels were examined using qRT-PCR using TaqMan probes for **(A)** MCP-1 and IL-6, **(B)** eotaxin-1, eotaxin-3 and IP-10. Data was corrected to β-Actin endogenous levels as control. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. Error bars represent SEM within the qRT-PCR experiment since the data are an n=1.

FIGURE 2.A.

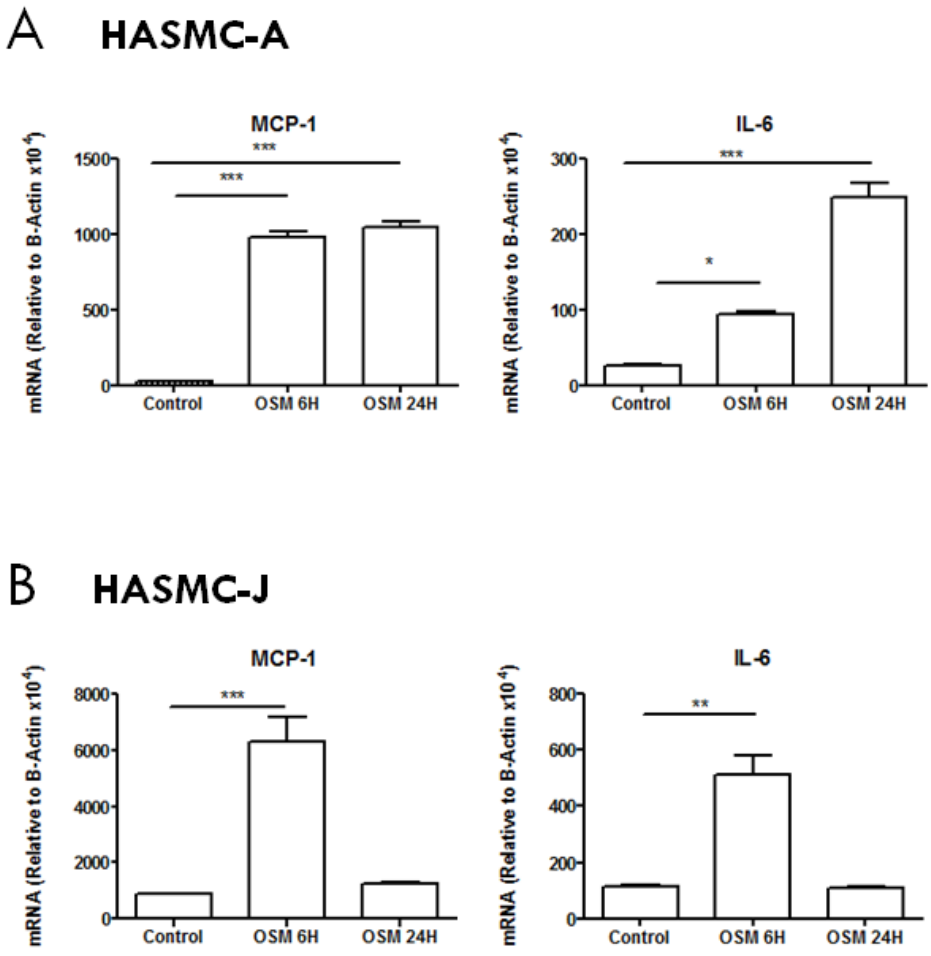
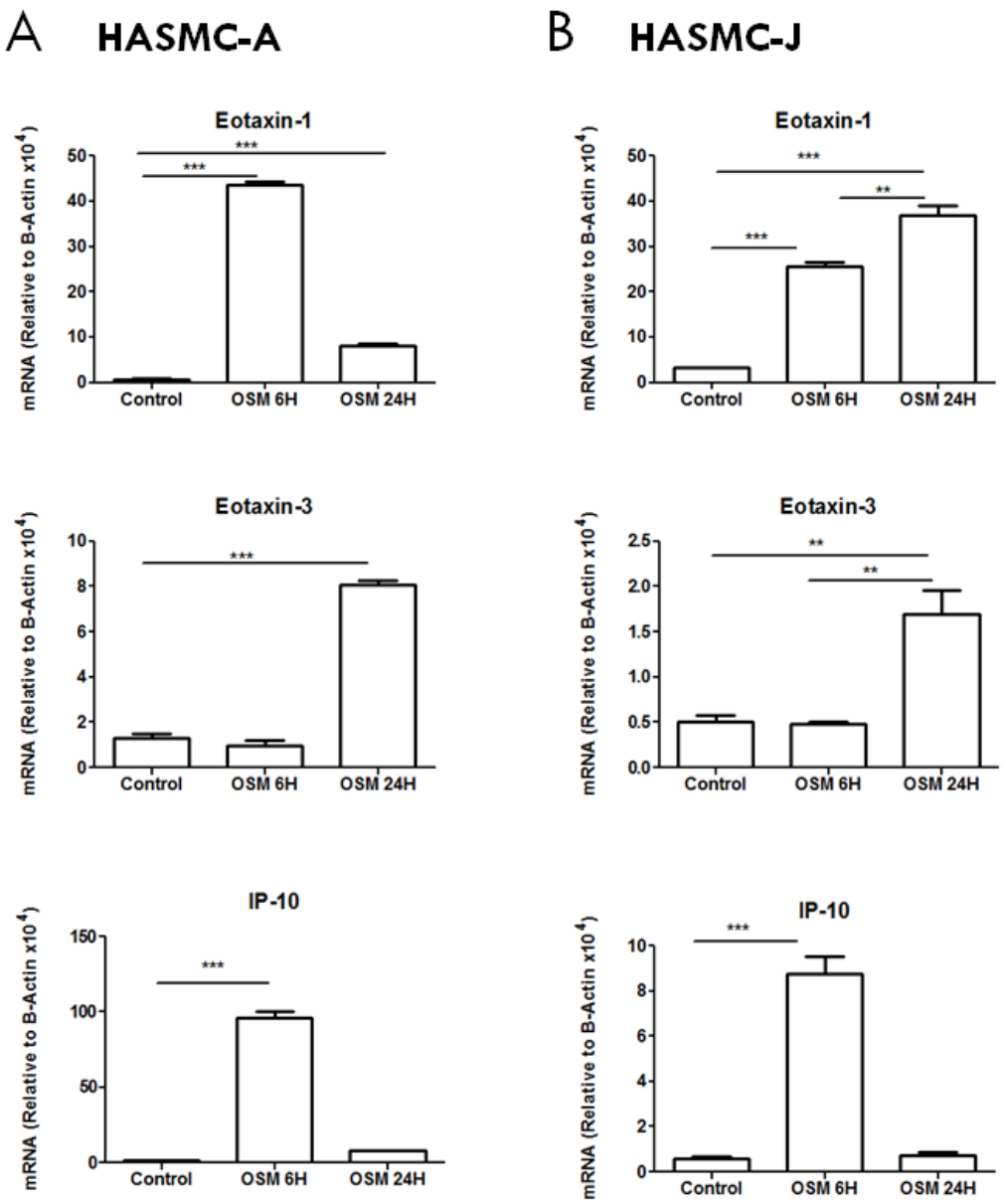

A
HASMC-A
MCP-1
IL-6
mRNA (Relative to B-Actin x10⁻⁴)
Control
OSM 6H
OSM 24H
B
HASMC-J
MCP-1
IL-6
mRNA (Relative to B-Actin x10⁻⁴)
Control
OSM 6H
OSM 24H

FIGURE 2.B


A HASMC-A
B HASMC-J
Eotaxin-1
Eotaxin-3
IP-10
mRNA (Relative to B-Actin x10-4)
Control
OSM 6H
OSM 24H
***
**

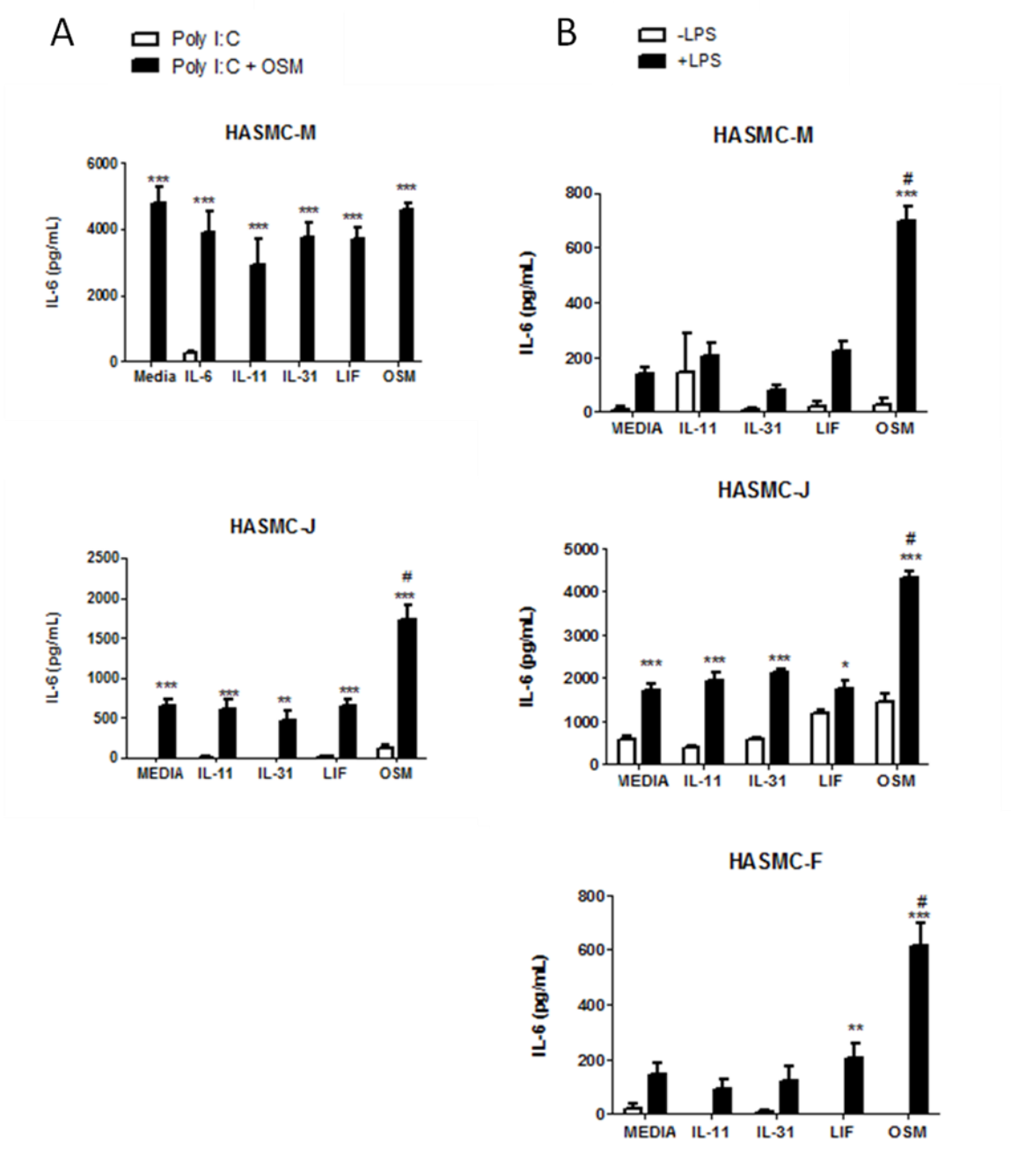
3.1.3. Pro-inflammatory Responses by TLR-ligands and Gp130 cytokines

HASMC-M, HASMC-J and HASMC-F were cultured and plated into 96 well plates at 10^4 cells per well. Upon 75-80% confluence HASMC were serum deprived with 2% FBS for 3 hours followed by stimulation wi th 0.5ng/ml of various gp130 cytokines (IL-11, IL-31, LIF and OSM) in the absence or presence of 2µg/ml TLR3-ligand (Poly I:C) or 20ng/ml TLR4-ligand (LPS) for 18 hours. After the 18 hour time point supernatants were removed from the cells and frozen at -20˚C. ELISA Duoset for IL-6 was performed to examine concentrations (pg/ml) in supernatants **[Figure 3]**. Both HASMC-M and HASMC-J stimulated with gp130 cytokines in the absence of Poly I:C produced exceptionally low levels of IL-6, however IL-6 production was significantly increased with the addition of Poly I:C. Furthermore, OSM stimulation on HASMC-J resulted in significantly more IL-6 levels than any of the other gp130 cytokines tested. This trend was not seen however in HASMC-M. In regards to the LPS challenged cells, HASMC-M, HASMC-J and HASMC-F demonstrated similar results to the Poly I:C challenged cells where, in the absence of LPS, IL-6 levels were low and increased upon the addition of LPS. All three cell lines displayed a similar trend but to varying degrees of sensitivity. Lastly, OSM in the presence of LPS stimulated significantly more IL-6 levels than the other gp130 cytokines in all three cell lines. These data suggest that in HASMC, co-stimulation with TLR agonists and OSM is a greater pro-inflammatory inducer than other gp130 cytokines. This is evident by the enhancement of IL-6 levels by OSM in comparison to the other gp130 cytokines tested (IL-11, IL-31, LIF and OSM).

FIGURE 3. OSM enhances responses to TLR-ligands in HASMC.

(A) HASMC-M and HASMC-J were stimulated with 0.5ng/ml gp130 cytokines in the presence or absence of 2µg/ml Poly I:C. ELISA performed for IL-6 with supernatants from 18 hours. **(B)** Three patient cell lines (HASMC-J, M, F) were stimulated with 0.5ng/ml gp130 cytokines in the presence or absence of 20ng/ml LPS. ELISA performed for IL-6 with supernatants from 18 hours. Two-way ANOVA with a Bonferroni post test was used to determine significance between gp130 cytokine stimulation and the presence of TLR agonists in co-stimulations, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI #P<0.0001. Error bars represent SEM.

FIGURE 3.


A
Poly I:C
Poly I:C + OSM
HASMC-M
IL-6 (pg/mL)
Media
IL-6
IL-11
IL-31
LIF
OSM
HASMC-J
B
-LPS
+LPS
HASMC-M
MEDIA
IL-11
IL-31
LIF
OSM
HASMC-J
HASMC-F

3.1.4. Pro-inflammatory Responses in HASMC Subsequent to TLR3-ligand Stimulus

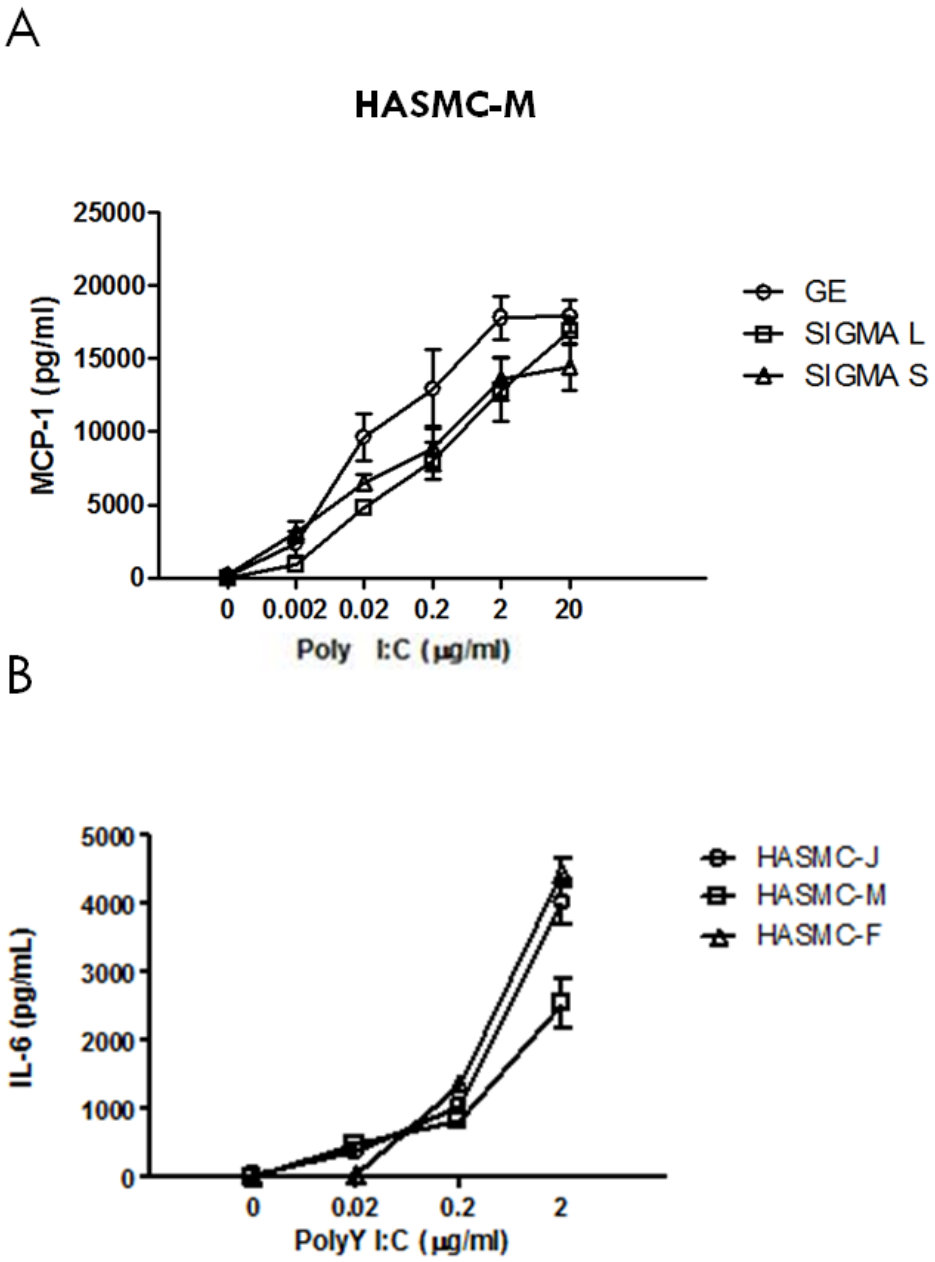
Innate immune responses occur upon recognition of foreign stimulae by innate immune cells through TLRs. However, non-immune cells including smooth muscle and stromal cells can also respond to TLR ligands. To determine the impact of TLR stimulae on HASMC, HASMC-M were challenged with increasing concentrations (0.002µg/ml, 0.02µg/ml, 0.2µg/ml, 2µg/ml, 20µg/ml) of TLR3-ligand (Poly I:C). In an initial experiment to establish the most appropriate TLR3-ligand for HASMC, three lengths of Poly I:C were used, a short (Sigma S ~0.5kbp), medium (Sigma L ~1.5kbp) and long (GE ~8kbp). **[Figure 4 A]** shows a dose dependent induction of MCP-1 in response to Poly I:C, where the GE form of Poly I:C induced the greatest amount of MCP-1 in comparison to Sigma S and Sigma L forms. Approximately 17000pg/ml of MCP-1 was detected in supernatants at the highest concentration of both GE and Sigma L, while Sigma S was only able to induce around 15000pg/ml. These data provide the rationale for using the GE form as the TLR3-ligand in subsequent experiments with HASMC. In addition, HASMC release high amounts of MCP-1 which could stimulate monocyte chemotaxis *in vivo*.

As shown previously, HASMC stimulated with OSM confirmed that although different patient cell lines respond with similar tends, there are differences in their sensitivity. Therefore, examining the variation in IL-6 secretion in response to TLR3-ligand in HASMC-M, HASMC-J and HASMC-F will determine if these varying degrees of responsiveness are also seen with TLR3 challenge. HASMC-M, HASMC-J and HASMC-F were challenged with Poly I:C at 0µg/ml, 0.02µg/ml, 0.2µg/ml and 2µg/ml.

[Figure 4 B] demonstrates that both HASMC-J and HASMC-F expressed ~4000pg/ml IL-6 in response to 2ng/ml GE where as the HASMC-M expressed ~2500pg/ml, a 1.6 fold change. Taken together, these data demonstrate that similarly to OSM stimulation, HASMC respond with a comparable trend to TLR3-liand however with varying degrees of sensitivity.

FIGURE 4. **TLR3-ligand stimulates MCP-1 and IL-6 production in HASMC**. **(A)** HASMC-M were stimulated with increasing concentrations of different preparations of Poly I:C for 18 hours, a long and short from manufacturer Sigma (Sigma L and Sigma S) and the longest form from manufacturer GE. ELISA was performed to examine MCP-1 concentrations in supernatants. **(B)** Three patient cell lines (HASMC-J, M, F) were stimulated with TLR3-ligand, Poly I:C. ELISA performed to examine IL-6 concentrations in supernatants. Error bars represent SEM.

FIGURE 4.


A
HASMC-M
MCP-1 (pg/ml)
25000
20000
15000
10000
5000
0
0
0.002
0.02
0.2
2
20
Poly I:C (µg/ml)
GE
SIGMA L
SIGMA S
B
IL-6 (pg/mL)
5000
4000
3000
2000
1000
0
0
0.02
0.2
2
PolyY I:C (µg/ml)
HASMC-J
HASMC-M
HASMC-F

3.1.5. Pro-inflammatory Responses in HASMC Subsequent to TLR4-ligand Stimulus

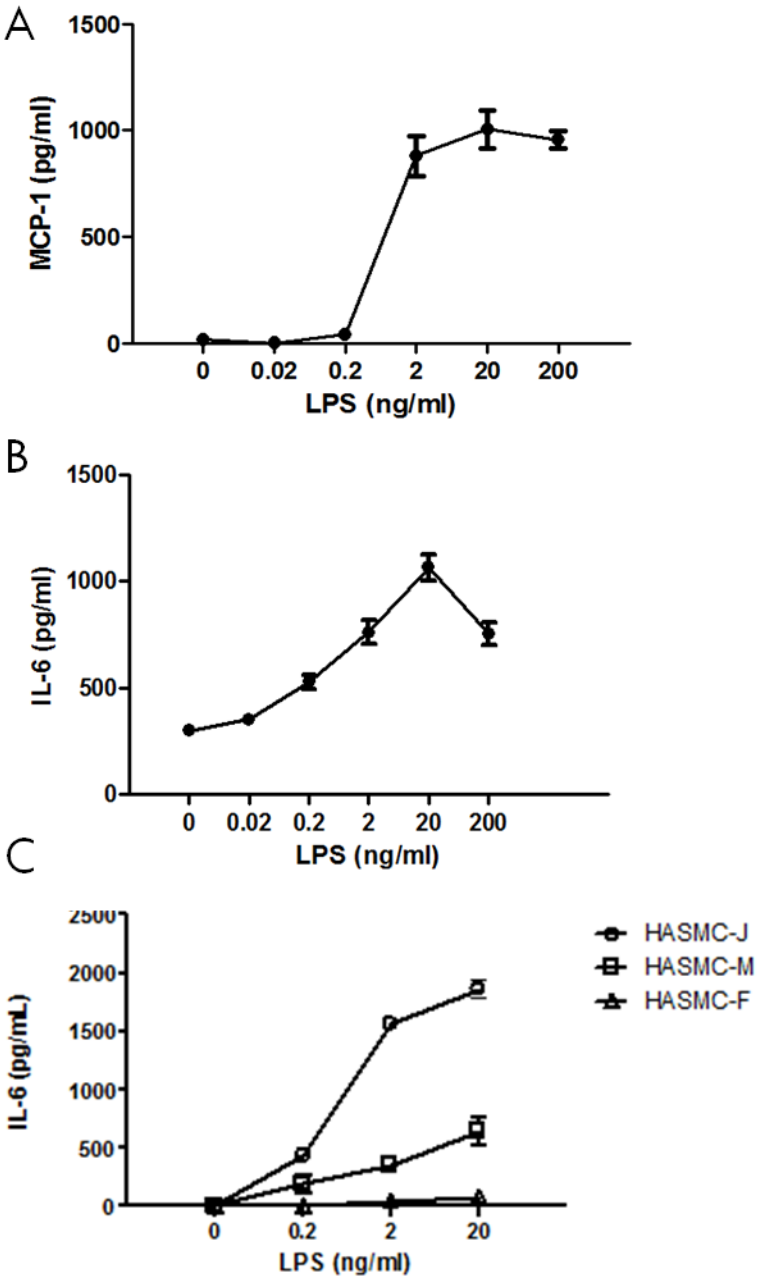
Similarly to TLR3-ligand challenge, HASMC-M were stimulated *in vitro* with increasing concentrations (0.02ng/ml, 0.2ng/ml, 2ng/ml, 20ng/ml, 200ng/ml) of TLR4-ligand (LPS) to determine the potential role of bacterial agonists in regulating HASMC. Stimulation of HASMC **[Figure 5 A-B]** elevated both MCP-1 and IL-6 levels in supernatants (800pg/ml) upon addition with LPS (20ng/ml). At 200ng/ml LPS stimulus, IL-6 concentrations were decreased suggesting the possibility of LPS toxicity at this concentration. Furthermore, MCP-1 concentrations at the highest IL-6 inducing TLR4-ligand stimulus were ~1000pg/ml, 17 fold lower than MCP-1 levels in the presence of TLR3-ligand (17000pg/ml) **[Figure 4 A]**. As for IL-6 protein levels, the response to LPS seemed to peak at 20ng/ml LPS, indicating that about 1000pg/ml of IL-6 is the maximal response by HAMSCs when challenged with LPS, whereas levels were above 4000pg/ml with TLR3-ligand **[Figure 4 B]**. TLR3-ligand also induced higher levels of MCP-1 in comparison to TLR4-ligand (TLR3-ligand induced ~17000pg/ml of MCP-1 whereas maximal TLR4-ligand stimulation resulted in could only initiate about 1000pg/ml). These data confirmed previous observations by others in the laboratory using a different HASMC line (data not shown) where MCP-1 and IL-6 production by HASMC is greater upon Poly I:C challenge in comparison to LPS challenge.

To determine if different HASMC respond to TLR4-ligand with varying degrees of sensitivity as shown with OSM and TLR3-ligand, HASMC-M, HASMC-J and

HASMC-F were stimulated with increasing concentrations of LPS (0ng/ml, 0.2ng/ml, 2ng/ml and 20ng/ml). **[Figure 5 C]** further elucidates this trend since upon stimulation with 20ng/ml LPS HASMCJ supernatants contained 1900pg/ml of IL-6, HASMC-M ~600pg/ml, and ~75pg/ml in HASMC-F. Of interesting note, cell lines that responded lower to Poly I:C did not necessarily respond lower to LPS. Furthermore, the data indicated there was greater variation between cell lines in response to LPS challenge in comparison to Poly I:C challenge.

FIGURE 5. TLR4-ligand stimulates MCP-1 and IL-6 production in HASMC. HASMC-M were stimulated with increasing concentrations of TLR4-ligand (LPS) at the indicated concentrations for 18 hours. ELISA was performed to examine **(A)** IL-6 and **(B)** MCP-1 concentrations in supernatants. **(C)** Three patient cell lines (HASMC-J, M, F) were stimulated with TLR4-ligand (LPS) at the indicated concentrations for 18 hours. ELISAs were performed to examine IL-6 concentrations in 18 hour supernatants. Error bars represent SEM.

FIGURE 5.


A
1500
1000
500
0
MCP-1 (pg/ml)
0
0.02
0.2
2
20
200
LPS (ng/ml)
B
1500
1000
500
0
IL-6 (pg/ml)
0
0.02
0.2
2
20
200
LPS (ng/ml)
C
2500
2000
1500
1000
500
0
IL-6 (pg/mL)
0
0.2
2
20
LPS (ng/ml)
HASMC-J
HASMC-M
HASMC-F

3.1.6. TLR3 and TLR4-ligand Challenge in the Presence of OSM

In **Figures 4 and 5,** it has been shown that HASMC can secrete IL-6 and MCP-1 in response to TLR3 and TLR4-ligand challenge. The following experiments were conducted to determine if a significant increase in pro-inflammatory proteins can be induced by HASMC challenged with Poly I:C or LPS in the presence of OSM. In order to determine at which concentrations of Poly I:C or LPS that co-stimulation with OSM may amplify cytokine/chemokine secretion, the HASMC lines were stimulated with increasing concentrations of Poly I:C (0µg/ml, 0.02µg/ml, 0.2µg/ml and 2µg/ml) or increasing concentrations of LPS (0ng/ml, 0.2ng/ml, 2ng/ml and 20ng/ml) in the presence or absence of 0.5ng/ml OSM. **[Figure 6]** illustrates IL-6 ELISA data from 18 hour supernatants of Poly I:C challenged HASMC. Significant increases in IL-6 production can be seen in all three cell lines at 2µg/ml Poly I:C. However HASMC-J and HASMC-M cultured supernatants showed significant increases in IL-6 with 10-fold less Poly I:C than that from HASMC-F. Since 0µg/ml Poly I:C represents 0.5ng/ml OSM alone it is possible to determine if the increase in IL-6 secretion at various concentrations is additive or potentially synergistic with the addition of OSM. HASMC-M and HASMC-J IL-6 levels could be synergistic with as little as 0.2ug/ml Poly I:C, while HASMC-F appear to have additive responses. Lastly, all three cells lines, although responding somewhat differently, secrete similar amounts of IL-6 at the highest concentration of Poly I:C.

In regards to LPS challenge **[Figure 7]**, IL-6 levels in supernatants of all three cells lines was potentiated by 0.5ng/ml OSM at 2ng/ml of LPS. In contrast to the findings

with the Poly I:C challenged cells, all three cells lines produce varying amounts of IL-6 at the highest concentration of LPS (20ng/ml). HASMC-J responded with the greatest induction of IL-6 (~3000pg/ml), followed by HASMC-M (~1000pg/ml) and HASMC-F with the least (~500pg/ml). Since IL-6 levels induced by the combination of OSM and LPS were greater than that of either stimulus alone, the results indicate synergistic responses occurred.

FIGURE 6. OSM augments TLR3-ligand production of IL-6 in HASMC. Patient cell lines (HASMC-J, M, F) were stimulated with increasing concentrations of Poly I:C in the absence or presence of 0.5ng/ml OSM. ELISAs were performed to examine IL-6 concentrations in supernatants after 18 hours. Two-way ANOVA with a Bonferroni post test was used to determine significance between Poly I:C alone and Poly I;C and OSM co-stimulations, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. Error bars represent SEM.

FIGURE 7. OSM augments TLR4-ligand production of IL-6 in HASMC. Patient cell lines (HASMC-J, M, F) were stimulated with increasing concentrations of LPS in the absence or presence of 0.5ng/ml OSM. ELISAs were performed to examine IL-6 concentrations in supernatants after 18 hours. Two-way ANOVA with a Bonferroni post test was used to determine significance between LPS alone and LPS and OSM co-stimualtions, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. Error bars represent SEM.

FIGURE 6.

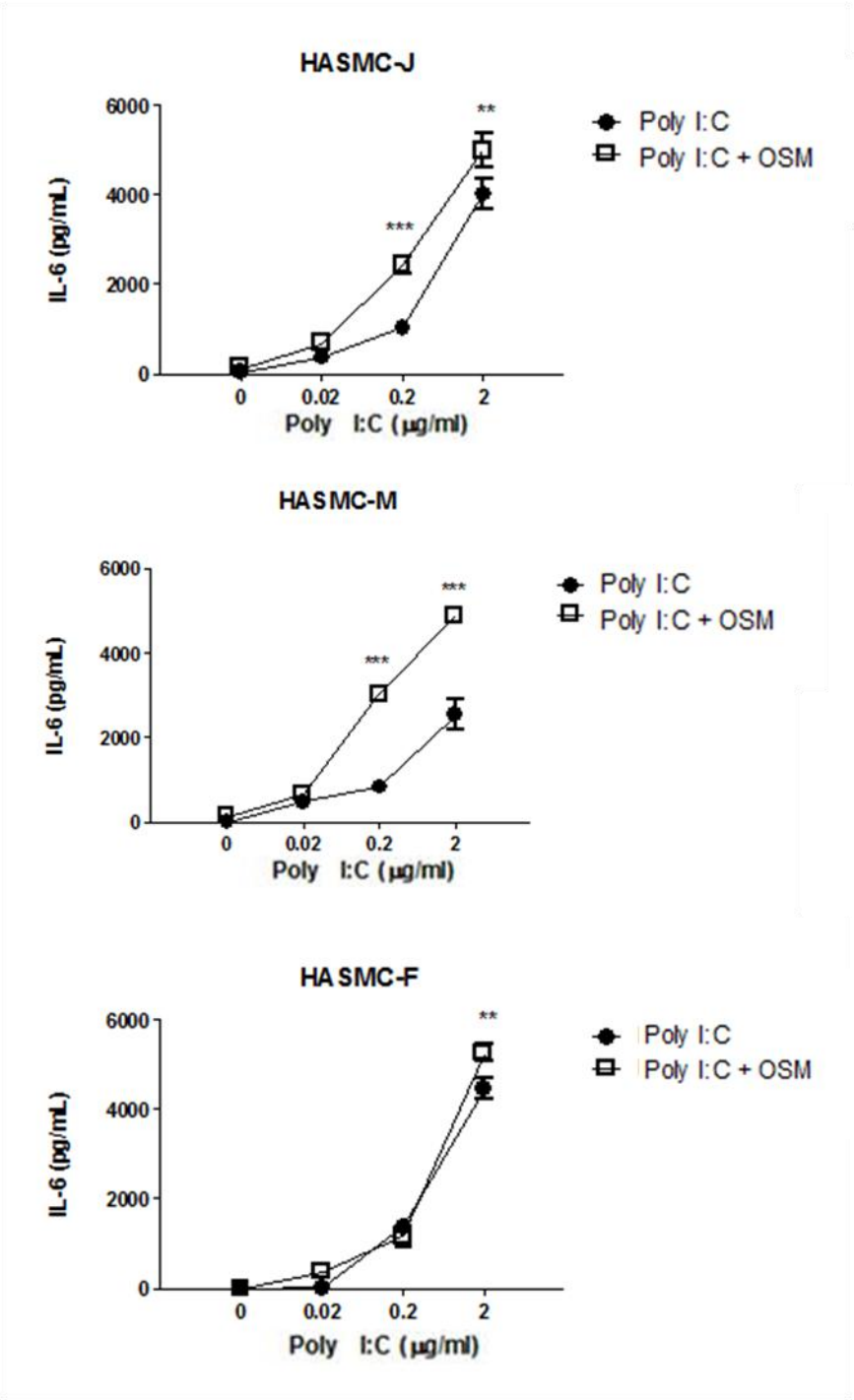
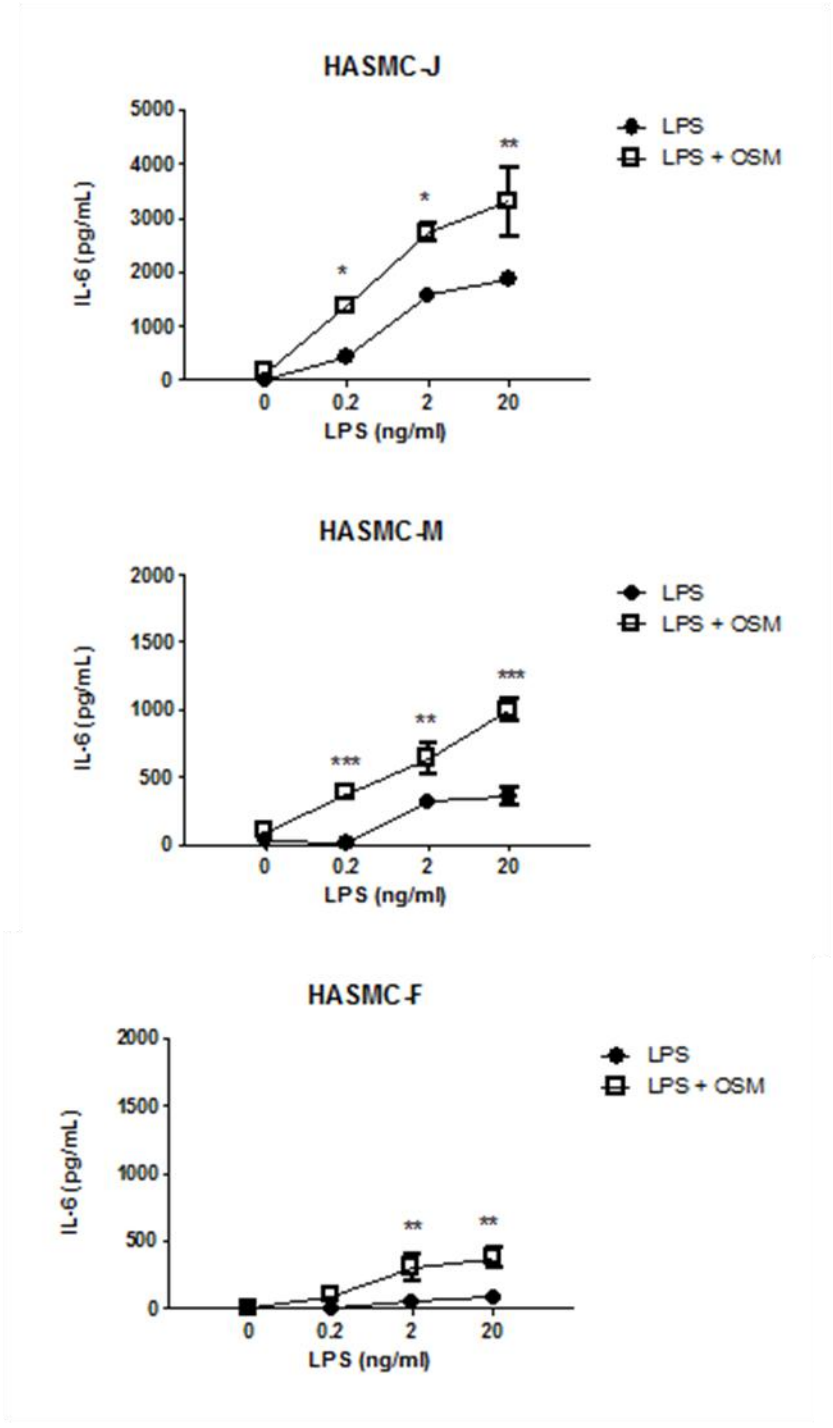

HASMC-J
IL-6 (pg/mL)
6000
4000
2000
0
0
0.02
0.2
2
Poly I:C (µg/ml)
**
***
Poly I:C
Poly I:C + OSM
HASMC-M
IL-6 (pg/mL)
6000
4000
2000
0
0
0.02
0.2
2
Poly I:C (µg/ml)
***
***
Poly I:C
Poly I:C + OSM
HASMC-F
IL-6 (pg/mL)
6000
4000
2000
0
0
0.02
0.2
2
Poly I:C (µg/ml)
**
Poly I:C
Poly I:C + OSM

FIGURE 7.


HASMC-J
IL-6 (pg/mL)
5000
4000
3000
2000
1000
0
0
0.2
2
20
LPS (ng/ml)
LPS
LPS + OSM
*
*
**
HASMC-M
IL-6 (pg/mL)
2000
1500
1000
500
0
LPS (ng/ml)
***
**
***
HASMC-F
IL-6 (pg/mL)
2000
1500
1000
500
0
LPS (ng/ml)
**
**

The above experiments examined IL-6 production by HASMC with TLR3 and TLR4-ligand and OSM co-challenge. Determining levels of other cytokines and chemokines that are produced subsequent to TLR3, TLR4 and OSM stimulation would aid in further elucidating the immunomodulatory functions of HASMC *in vitro*. HASMC-M and HASMC-J received the aforementioned concentrations of OSM, and both Poly I:C or LPS in the absence or presence of 0.5ng/ml OSM. HASMC-A were not stimulated with increasing concentrations of OSM, and were challenged with lower concentrations of Poly I:C (0ng/ml, 10ng/ml, 50ng/ml, 100ng/ml) and LPS (0ng/ml, 0.1ng/ml, 0.5ng/ml, 1ng/ml). MSD analysis of supernatants was performed to determine various protein concentrations. MSD human chemokine 9-plex/7-plex was used to quantify CC and CXC chemokines (MCP-1, MCP-4, eotaxin-1, eotaxin-3, TARC, IP-10 and IL-8). Figures are organized according to analyte, MCP-1 **[Figure 8]**, MCP-4 **[Figure 9]**, Eotaxin-1 **[Figure 10]**, Eotaxin-3 **[Figure 11]**, TARC **[Figure 12]**, IL-8 **[Figure 13]** and IP-10**[Figure 14]**.

MCP-1**[Figure 8]**

MCP-1 levels were significantly increased in response to OSM in both HASMC-M and HASMC-J lines. Poly I:C challenge increased MCP-1 levels in HASMC-M, HASMC-J and HASMC-A lines. LPS challenge increased MCP-1 levels in all three cell lines, however at lower concentrations in HASMC-M and HASMC-J lines. Poly I:C and OSM co-stimulation significantly increased MCP-1 detection in HASMC-M, HASMC-J and HASMC-A in comparison to Poly I:C alone, however at varying concentrations

between cell lines. MCP-1 production in response to LPS and OSM co-stimulation was also significantly higher in comparison to response to LPS alone at varying concentrations in HASMC-M, HASMC-J and HASMC-A lines.

MCP-4**[Figure 9]**

Contrary to MCP-1, MCP-4 concentrations were not altered by OSM in either HASMC-M or HASMC-J lines. Poly I:C challenge increased MCP-4 production in all three cells lines, however HASMC-J was the only cell line with increased MCP-4 production in response to LPS. HASMC-M and HASMC-A challenged with Poly I:C in the presence of OSM significantly increased MCP-4 secretion. An increase was seen in HASMC-J however was not statistically significant. Lastly, MCP-4 levels were not significantly altered upon co-stimulation in HASMC-M, HASMC-J or HASMC-A lines.

Eotaxin-1**[Figure 10]**

HASMC-M stimulated with OSM produced a significant increase in eotaxin-1 levels however this trend was not seen with HASMC-J. Eotaxin-1 levels were increased with Poly I:C challenge while LPS challenge elicited low levels of eotaxin-1 secretion in HASMC-M, HASMC-J and HASMC- lines. Poly I:C and OSM co-stimulation did not significantly alter eotaxin-1 production in HASMC-M, however there was a significant decrease in eotaxin-1 levels at 2µg/ml Poly I:C and OSM co-stimulation in comparison to Poly I:C alone in HASMC-J. On the other hand, Poly I:C challenge with OSM induced a significant increase in eotaxin-1 concentrations at 100ng/ml in comparison to Poly I:C

alone in HASMC-A. Lastly, LPS and OSM co-stimulation did not alter eotaxin-1 production in HASMC-M, HASMC-J or HASMC-F lines.

Eotaxin-3**[Figure 11]**

Stimulation with OSM of both HASMC-M and HASMC-J did not alter eotaxin-3 levels detected. HASMC-M and HASMC-A both exhibited increased production of eotaxin-3 in response to Poly I:C and LPS. However, Poly I:C at 2µg/ml and OSM co-stimulation in HASMC-M induced a significant increase in eotaxin-3 levels. As seen with eotaxin-1, HASMC-J challenged with Poly I:C at 2µg/ml and OSM co-stimulation resulted in a significant decrease in eotaxin-3 in comparison to 2µg/ml Poly I:C alone. Similarly to eotaxin-1, LPS challenge in the presence of OSM did not significantly alter eotaxin-3 concentrations.

TARC **[Figure 12]**

OSM induced a significant increase in TARC levels expressed by HASMC-M however not in HASMC-J. Poly I:C stimulation induced increased production of TARC in all three cell lines. With the exception of HASMC-J, LPS stimulation did not greatly alter TARC production in the cell lines. Both Poly I:C and LPS co-stimulations with OSM did not significantly alter TARC production in HASMC-M or HASMC-J, however Poly I:C challenge at 50ng/ml or 100ng/ml in the presence of OSM did result in further increase in TARC levels by HASMC-A. HASMC-A, HASMC-M and HAMSCs-J lines TARC production was not augmented upon LPS and OSM co-stimulation.

IL-8 **[Figure 13]**

In both HASMC-M and HASMC-J, OSM did not induce a significant increase in IL-8 levels. Poly I:C elicited IL-8 production in HASMC-M, HASMC-J and HASMC-A lines, however LPS only increased IL-8 in HASMC-J and HASMC-A. Co-stimulation with Poly I:C and OSM in HASMC-M resulted in a significant increase in IL-8 levels at 2µg/ml, however in HASMC-J and HASMC-A lines a significant decrease was observed at 2µg/ml Poly I:C. In regards to LPS and OSM co-stimulation, HASMC-M and HASMC-J lines did not induce a significant change in IL-8 detection, however HASMC-A IL-8 production was significantly decreased with the presence of 1ng/ml LPS.

IP-10 **[Figure 14]**

OSM stimulation at 2ng/ml significantly increased IP-10 concentrations (although very low) in comparison to control in HASMC-M, however this trend was not seen with HASMC-J. Both Poly I:C and LPS challenges resulted in increased IP-10 production. Co-stimulation with Poly I:C and OSM significantly increased IP-10 detection in HASMC-M at 2µg/ml Poly I:C, however a significant increase was not seen in HASMC-J and HASMC-A. LPS co-challenge with OSM did not alter IP-10 levels in comparison to LPS alone in all three cell lines with the exception of HASMC-A where 0.5ng/ml LPS induced a significant increase in IP-10 which decreased by the following concentration.

Taken together, these findings demonstrate that eotaxin-1, eotaxin-3, MCP-1, MCP-4, TARC, IL-8 and IP-10 could all be chemokines in the pro-inflammatory

response in HASMC upon TLR3 and TLR4 challenge, therefore potentially contributing to asthmatic pathology. TLR3-ligand with OSM, significantly altered the detection of eotaxin-1, eotaxin-3, MCP-1, MCP-4 , TARC, IL-8 and IP-10; however TLR4-ligand only significantly altered MCP-1 and IL-8 levels. Similarly to IL-6 production shown previously, Poly I:C. In regards to OSM stimulation alone, significant dose dependent increases were seen with eotaxin-1, MCP-1, TARC and IP-10 in some but not all lines.

FIGURES 8-14. MCP-1/4, Eotaxin-1/3, TARC, IL-8 and IP-10 production in response to OSM and TLR3 and TLR4-ligands. (A) HASMC-M, **(B)** HASMC-J and **(C)** HASMC-A were stimulated with increasing concentrations of OSM, increasing concentrations of Poly I:C in the absence or presence of 0.5ng/ml OSM, or increasing concentrations of LPS in the absence or presence of 0.5ng/ml OSM. Data are organized according to analyte, MCP-1 **[Figure 8]**, MCP-4 **[Figure 9]**, Eotaxin-1 **[Figure 10]**, Eotaxin-3 **[Figure 11]**, TARC **[Figure 12]**, IL-8 **[Figure 13]**, and IP-10 **[Figure 14]**. MSD analysis was performed to examine concentrations of various chemokines in supernatants at 18 hours. White bars represent Poly I:C or LPS alone and black bars represent the addition of OSM to the ligands. Two-way ANOVA with a Bonferroni post test was used to determine significance between TLR agonist and TLR agonist and OSM co-stimulations, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. One-way ANOVA with a Bonferroni post test was used to determine significance of OSM stimulation in comparison to control (0ng/ml OSM), significance with 95% CI #=P<0.05, ##=P< 0.001, and ###= P<0.0001. Error bars represent SEM.

FIGURE 8.

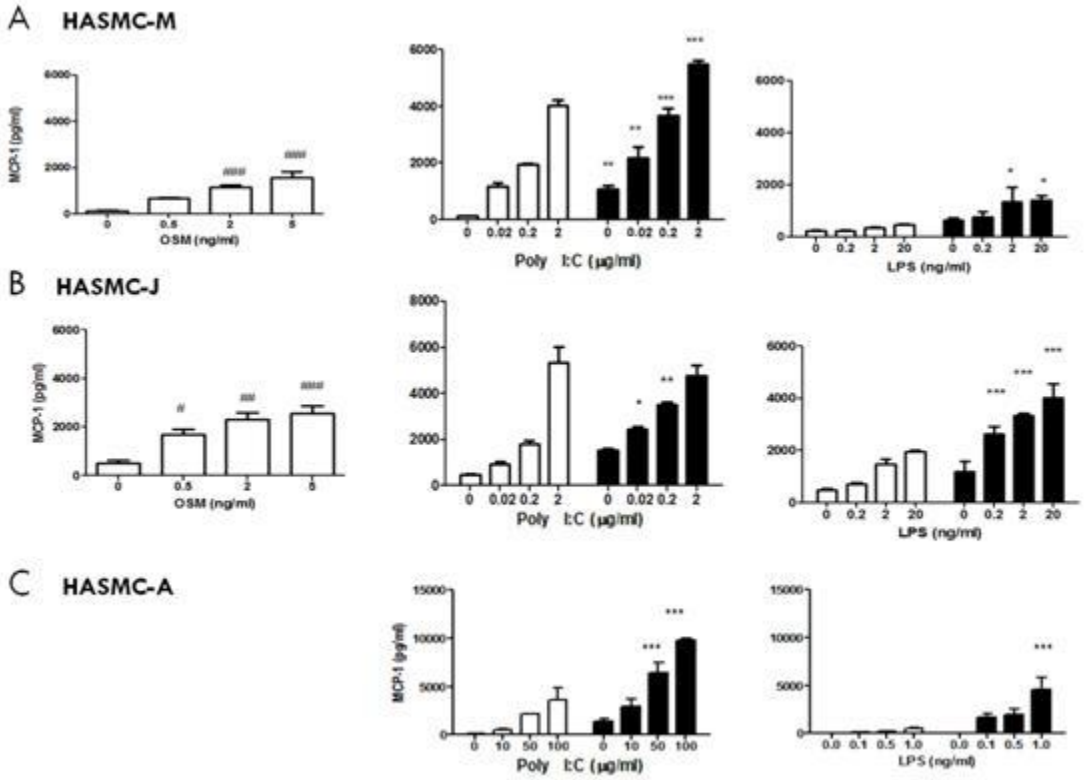

A HASMC-M
MCP-1 (pg/ml)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
B HASMC-J
MCP-1 (pg/ml)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
C HASMC-A
MCP-1 (pg/ml)
Poly I:C (µg/ml)
LPS (ng/ml)

FIGURE 9.

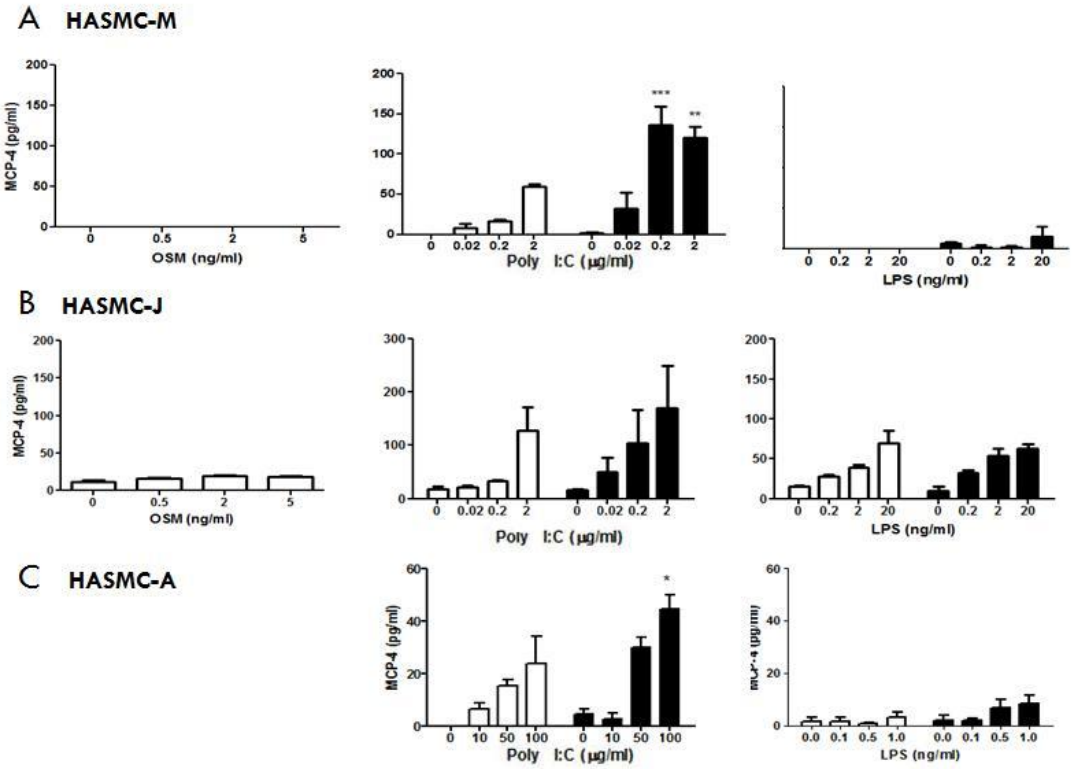
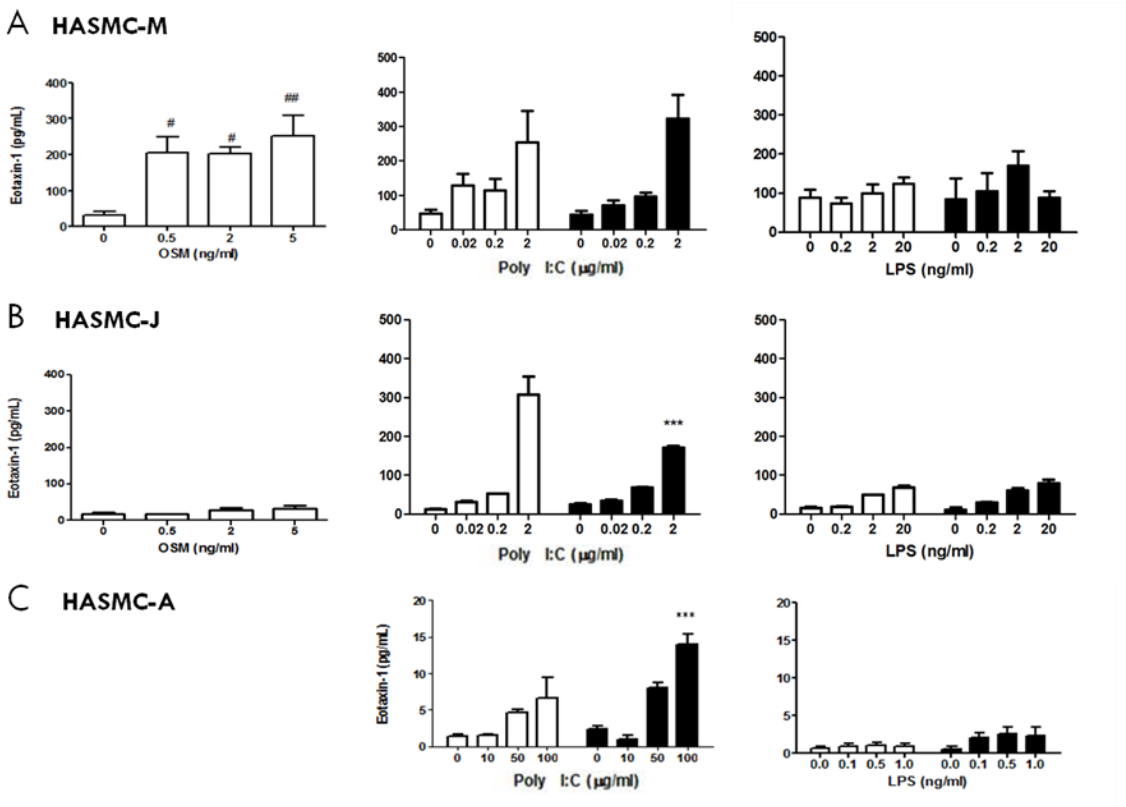

A HASMC-M
MCP-4 (pg/ml)
OSM (ng/ml)
Poly I:C (μg/ml)
LPS (ng/ml)
B HASMC-J
C HASMC-A

FIGURE 10.


A HASMC-M
Eotaxin-1 (pg/mL)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
B HASMC-J
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OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
C HASMC-A
Eotaxin-1 (pg/mL)
Poly I:C (µg/ml)
LPS (ng/ml)

FIGURE 11.

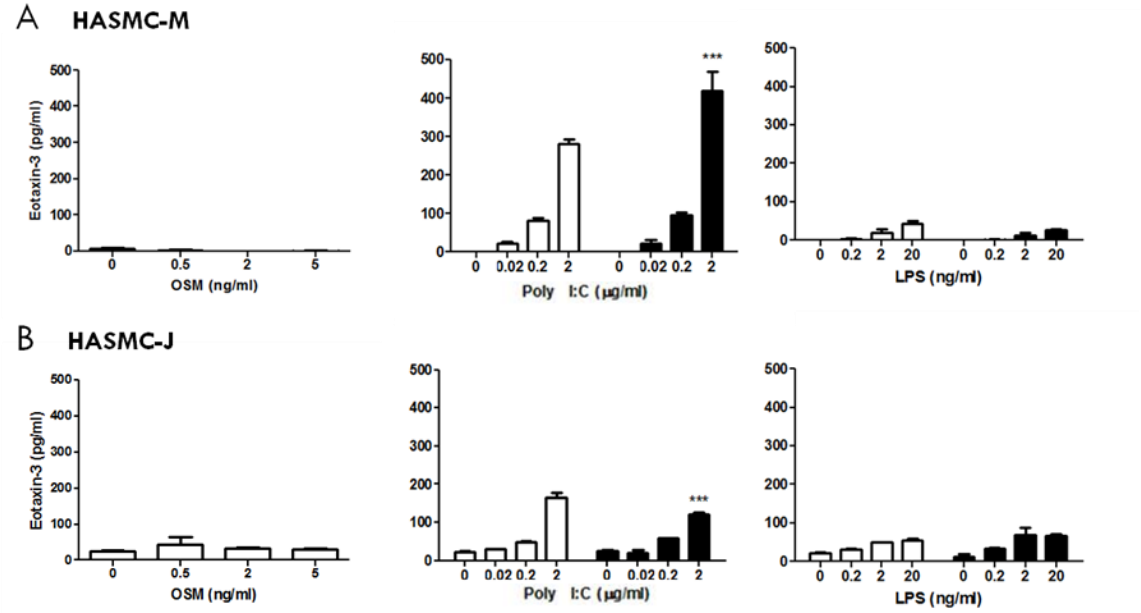

A
HASMC-M
Eotaxin-3 (pg/ml)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
***
B
HASMC-J
Eotaxin-3 (pg/ml)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
***

FIGURE 12.

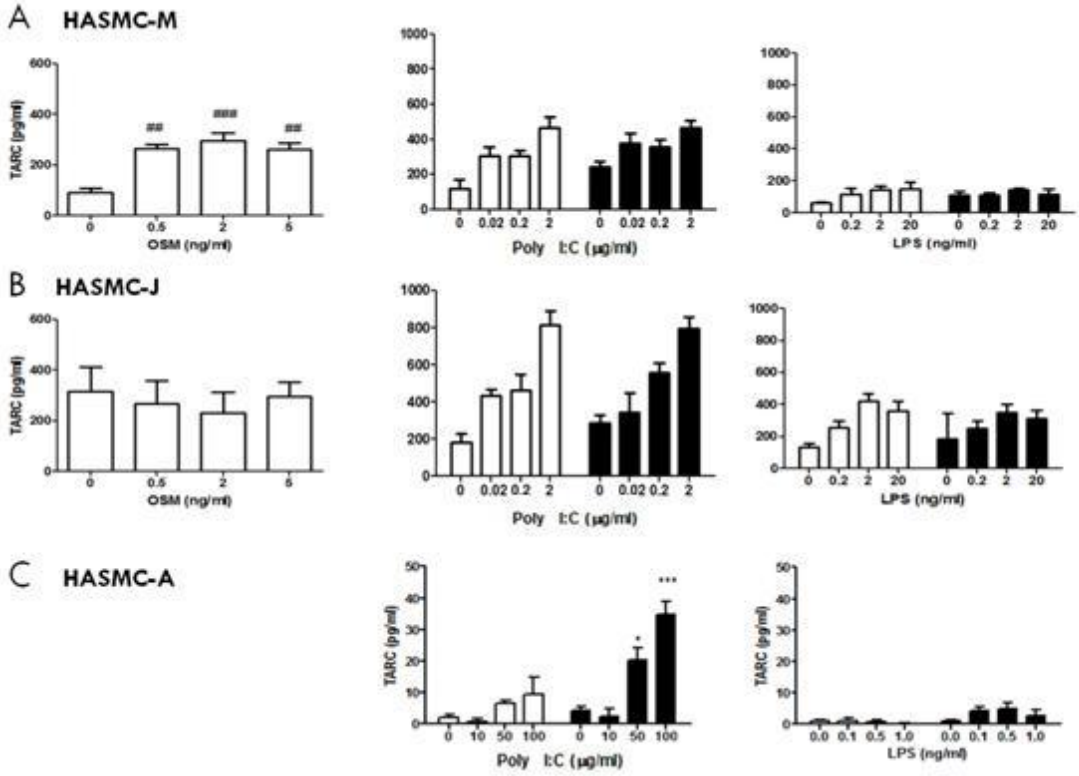

A HASMC-M
TARC (pg/ml)
OSM (ng/ml)
Poly I:C (μg/ml)
LPS (ng/ml)
B HASMC-J
TARC (pg/ml)
OSM (ng/ml)
Poly I:C (μg/ml)
LPS (ng/ml)
C HASMC-A
TARC (pg/ml)
Poly I:C (μg/ml)
TARC (pg/ml)
LPS (ng/ml)

FIGURE 13.

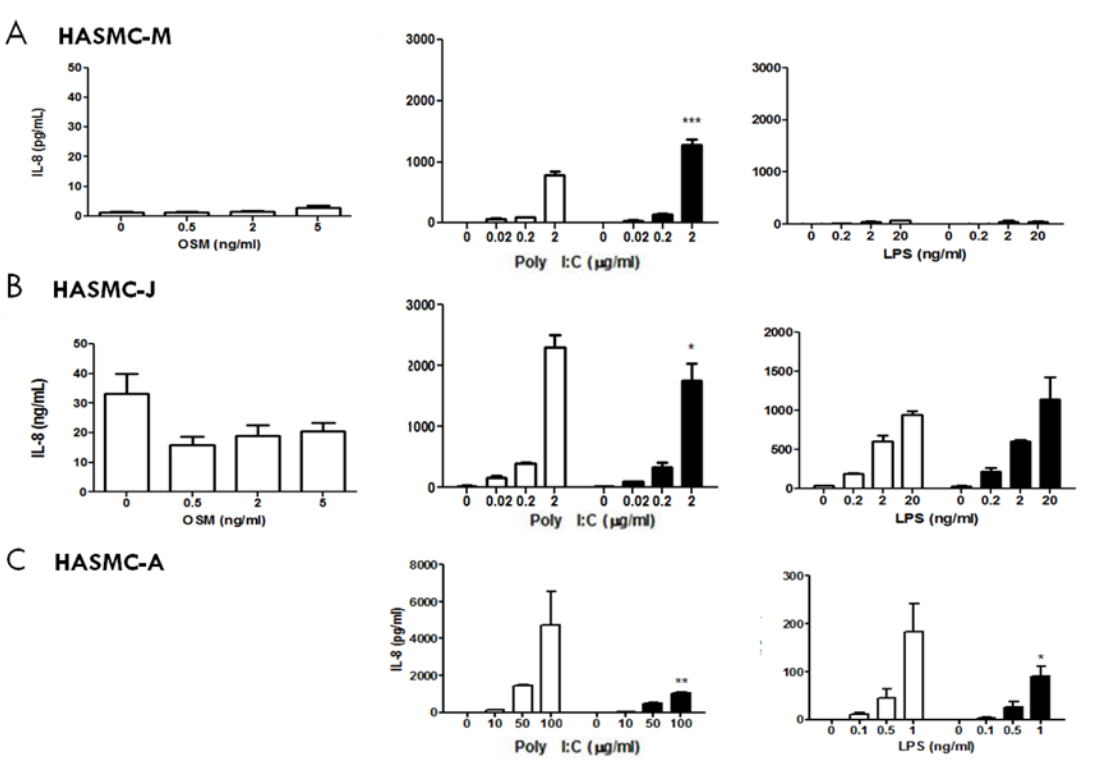
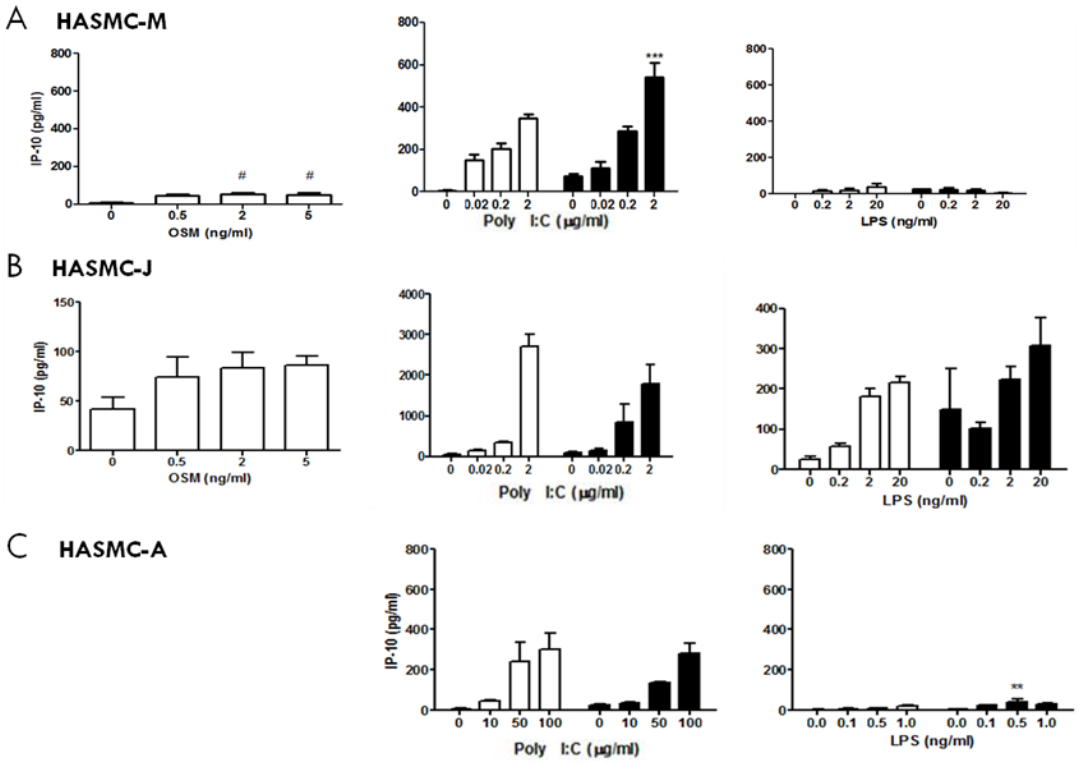

A
HASMC-M
IL-8 (pg/mL)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
B
HASMC-J
IL-8 (ng/mL)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
C
HASMC-A
IL-8 (pg/ml)
Poly I:C (µg/ml)
LPS (ng/ml)

FIGURE 14.

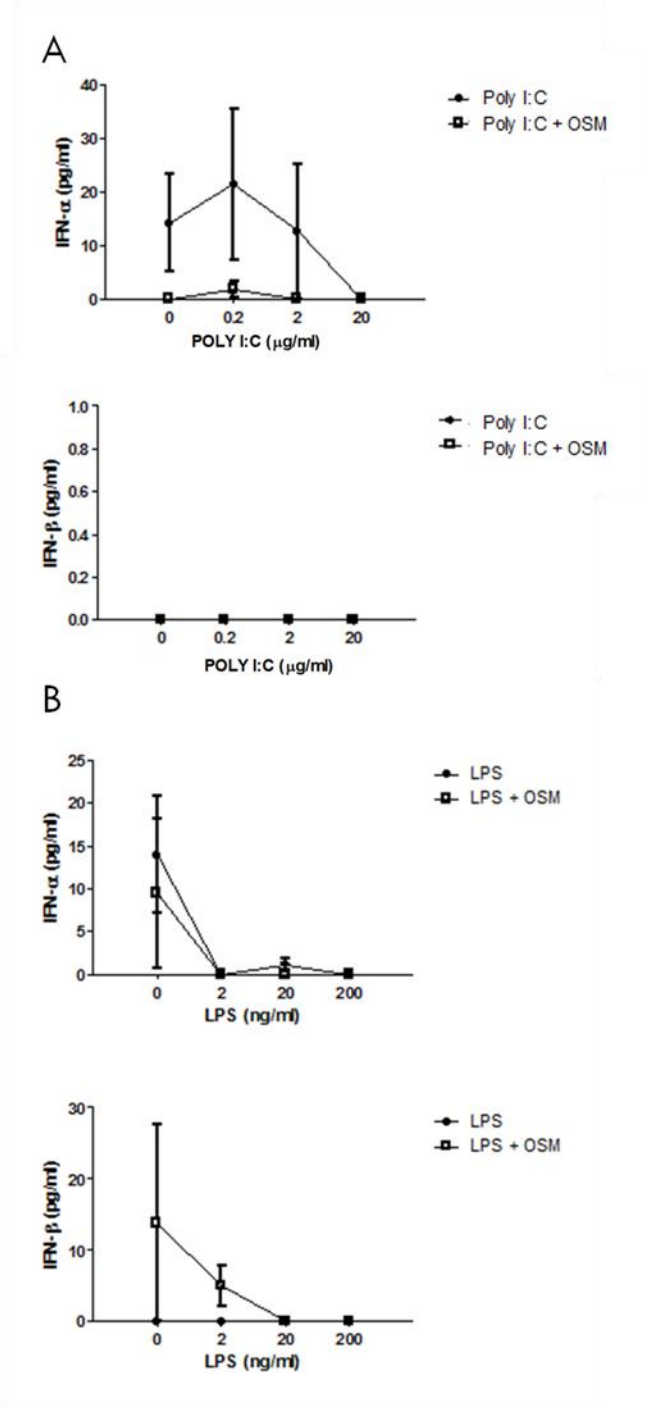

A HASMC-M
IP-10 (pg/ml)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
B HASMC-J
IP-10 (pg/ml)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)
C HASMC-A
IP-10 (pg/ml)
Poly I:C (µg/ml)
LPS (ng/ml)

3.1.7. Interferon Production in Response to TLR-ligands

Interferons can be produced in response to pathogens in numerous cell lines. In order to determine if HASMC can secrete interferon upon TLR3 and TLR4-ligand challenge, HASMC-M were stimulated with increasing concentrations of Poly I:C and LPS. IFN-α and IFN-β ELISAs were conducted to analyze concentrations in supernatants **[Figure 15]**. These findings illustrate that this cell line cannot induce IFN-α or IFN-β at concentrations detectable by the ELISA (limit of detection for these assays were above concentrations detected) upon Poly I:C and LPS challenge. Furthermore, there was no change in levels detected in supernatants of cells stimulated with OSM in the presence of Poly I:C or LPS.

FIGURE 15. TLR3 and TLR4-ligands do not induce IFN-α or IFN-β in HASMC. HASMC-M were stimulated with increasing concentrations of **(A)** Poly I:C or **(B)** LPS in the absence or presence of OSM. ELISAs were performed to examine IFN-α and IFN-β concentrations in 18 hour supernatants. Limit of detection was 12 pg/ml for IFN-α and 50pg/ml IFN-β. Error bars represent SEM.

FIGURE 15.

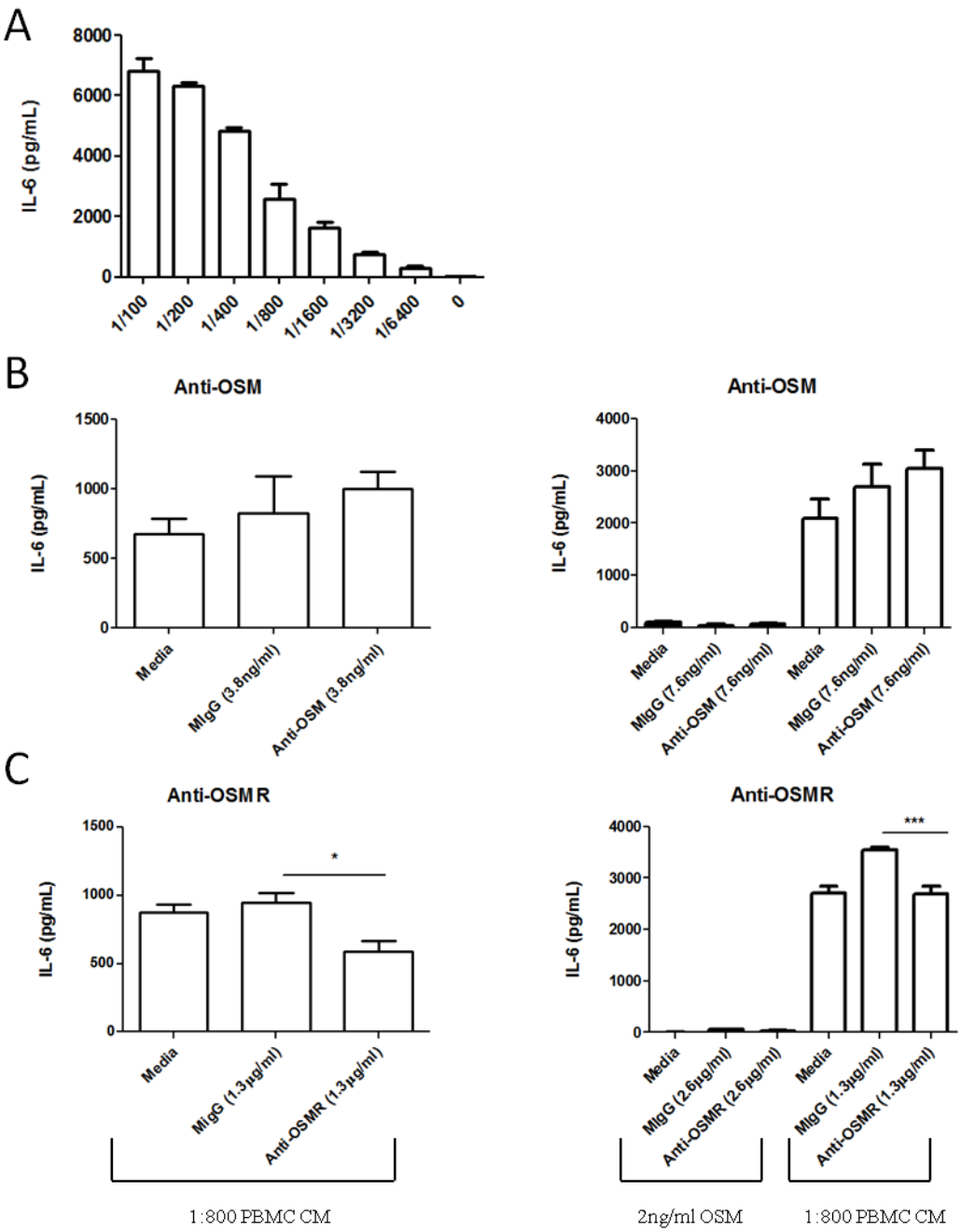

A
IFN-α (pg/ml)
40
30
20
10
0
0
0.2
2
20
POLY I:C (μg/ml)
Poly I:C
Poly I:C + OSM
IFN-β (pg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.2
2
20
POLY I:C (μg/ml)
Poly I:C
Poly I:C + OSM
B
IFN-α (pg/ml)
25
20
15
10
5
0
0
2
20
200
LPS (ng/ml)
LPS
LPS + OSM
IFN-β (pg/ml)
30
20
10
0
0
2
20
200
LPS (ng/ml)
LPS
LPS + OSM

3.1.8. Modulation by Anti- OSM and Anti-OSMRB

Antibodies to the OSM ligand and OSMRB were used to determine to what extent the pro-inflammatory responses seen in HASMC can be modulated if OSM is inhibited. Peripheral blood mononuclear cells were obtained from healthy subjects and stimulated with LPS for 24 hours and 48 hours. Supernatants (PBMC conditioned media) were then removed and assessed (using MSD TH1/TH2-7 plex/OSM ELISA) for cytokine levels where numerous cytokines were present, including 2240pg/ml OSM (Data not shown). PBMC conditioned media was then diluted and used to challenge HASMC-J for 6 hours and 24 hours alongside 2ng/ml OSM stimulation as a control, at which point the HASMC supernatants were analyzed for IL-6 concentrations using an IL-6 ELISA, **Figure 16 A** shows the IL-6 concentrations of HASMC supernatants upon stimulation with a titration of LPS treated PBMC conditioned media. This experiment was used to determine the appropriate sub-optimal concentration (1:800) of PBMCs required for HASMC challenge to assess effects of the antibodies. **[Figure 16 B]** demonstrates a significant reduction in IL-6 concentrations in cells treated with anti-OSMRB (1.3µg/ml) in comparison to a mouse IgG control (two different experiments shown in left and right panels), where as **[Figure 16 C]** demonstrates that anti-OSM (3.8 and 7.6ng/ml) was unable to modulate IL-6 production in comparison to a mouse IgG control (two different experiments shown in left and right panels).

FIGURE 16. Anti-OSMRB decreases IL-6 concentrations in HASMC supernatants. Supernatants of peripheral blood mononuclear cells (PBMCs) treated with LPS for 24 hours and 2ng/ml OSM were used to stimulate HASMC. **(A)** PBMC supernatants were diluted in RPMI medium and placed on HASMC followed by IL-6 ELISA. Supernatants of PBMCs treated with LPS for 24 hours (conditioned medium at 1:800) and 2ng/ml OSM were used to stimulate HASMC with the addition of **(B)** anti-OSMRB and **(C)** anti-OSM or control non-specific IgG antibody. Black bars represent OSM stimulated cells and white bars represent PBMC conditioned medium (1:800) stimulated cells. IL-6 ELISA was performed on HASMC supernatants after 18 hours. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. Error bars represent SEM.

FIGURE 16.


A
IL-6 (pg/mL)
8000
6000
4000
2000
0
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
0
B
Anti-OSM
IL-6 (pg/mL)
1500
1000
500
0
Media
MIgG (3.8ng/ml)
Anti-OSM (3.8ng/ml)
Anti-OSM
IL-6 (pg/mL)
4000
3000
2000
1000
0
Media
MIgG (7.6ng/ml)
Anti-OSM (7.6ng/ml)
Media
MIgG (7.6ng/ml)
Anti-OSM (7.6ng/ml)
C
Anti-OSMR
IL-6 (pg/mL)
1500
1000
500
0
*
Media
MIgG (1.3µg/ml)
Anti-OSMR (1.3µg/ml)
1:800 PBMC CM
Anti-OSMR
IL-6 (pg/mL)
4000
3000
2000
1000
0
***
Media
MIgG (2.6µg/ml)
Anti-OSMR (2.6µg/ml)
Media
MIgG (1.3µg/ml)
Anti-OSMR (1.3µg/ml)
2ng/ml OSM
1:800 PBMC CM

3.2. Cytokine and Chemokine mRNA Alterations in Response to TLR-ligands and OSM

3. 2.1. Pro-inflammatory mRNA Expression in Response to TLR-ligands and OSM

HASMC stimulated with OSM, Poly I:C or LPS in the absence or presence of OSM were used to determine if TLR-ligand challenge responses seen at the protein level were consistent with mRNA expression. HASMC-M and HASMC-J lines were stimulated with 2ng/ml OSM, 2µg/ml Poly I:C in the absence or presence of 2ng/ml OSM, or 20ng/ml LPS in the absence or presence of 2ng/ml OSM at 6 hours. At various times, RNA was isolated, treated with DNase, followed by PCR and assessed with qRT-PCR. HASMC-M and HASMC-J mRNA data are shown in **[Figure 17]**. MCP-1 and IL-6 mRNA expression upon OSM stimulation in HASMC-M was not significantly increased in comparison to control. On the other hand, consistent with the protein data, MCP-1 mRNA expression was significantly increased in HASMC-M that were challenged with Poly I:C and OSM in comparison to Poly I:C alone challenged cells, however this trend was not seen with the LPS stimulated cells. Also consistent with the protein data, both MCP-1 and IL-6 mRNA induced by Poly I:C challenge was significantly greater than the mRNA expressed in the LPS challenged cells. Contrary to the mRNA in the OSM stimulated HASMC-M, HASMC-J OSM stimulation significantly increased MCP-1 and IL-6 mRNA expression, however none of the other stimulations (Poly I:C, LPS and Poly I:C and LPS co-stimulations with OSM) altered MCP-1 or IL-6 mRNA levels. In regards to eotaxin-1 and eotaxin-3 mRNA expression, HASMC-M and

HASMC-J responded differently to the stimulations. HASMC-M showed a significant increase in eotaxin-1 mRNA levels in response to LPS. In addition co-stimulation with OSM with TLR3 or TLR4-ligands augmented eotaxin-1 and eotaxin-3 mRNA expression in comparison to the ligands alone. This trend was not seen in HASMC-J, where only Poly I:C induced significant eotaxin-1 and eotaxin-3 mRNA expression. Alterations in IP-10 expression were however consistent between cell lines, where Poly I:C stimulus significantly increased expression. In addition, HASMC-M challenged with Poly I:C and OSM co-stimulation demonstrated a significant increase in IP-10 in comparison to ligand alone, however this trend was not seen with HASMC-J. In general, all mRNA expression data was consistent with the previously shown protein data.

FIGURE 17. Expression of cytokine/chemokine mRNA is increased by TLR3-ligand and OSM. HASMC-M and HASMC-J were stimulated with 2ng/ml OSM, 2µg/ml Poly I:C in the absence or presence of 2ng/ml OSM, or 20ng/ml LPS in the absence or presence of 2ng/ml OSM. RNA expression was examined using qRT-PCR using probes for **(A)** MCP-1 and IL-6, **(B)** eotaxin-1, eotaxin-3 and IP-10. Data was corrected to β-Actin endogenous levels as control. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=$P<0.05$, **=$P< 0.001$, and ***= $P<0.0001$. Error bars represent SEM within the qRT-PCR experiment since the data are an n=1.

FIGURE 17.A

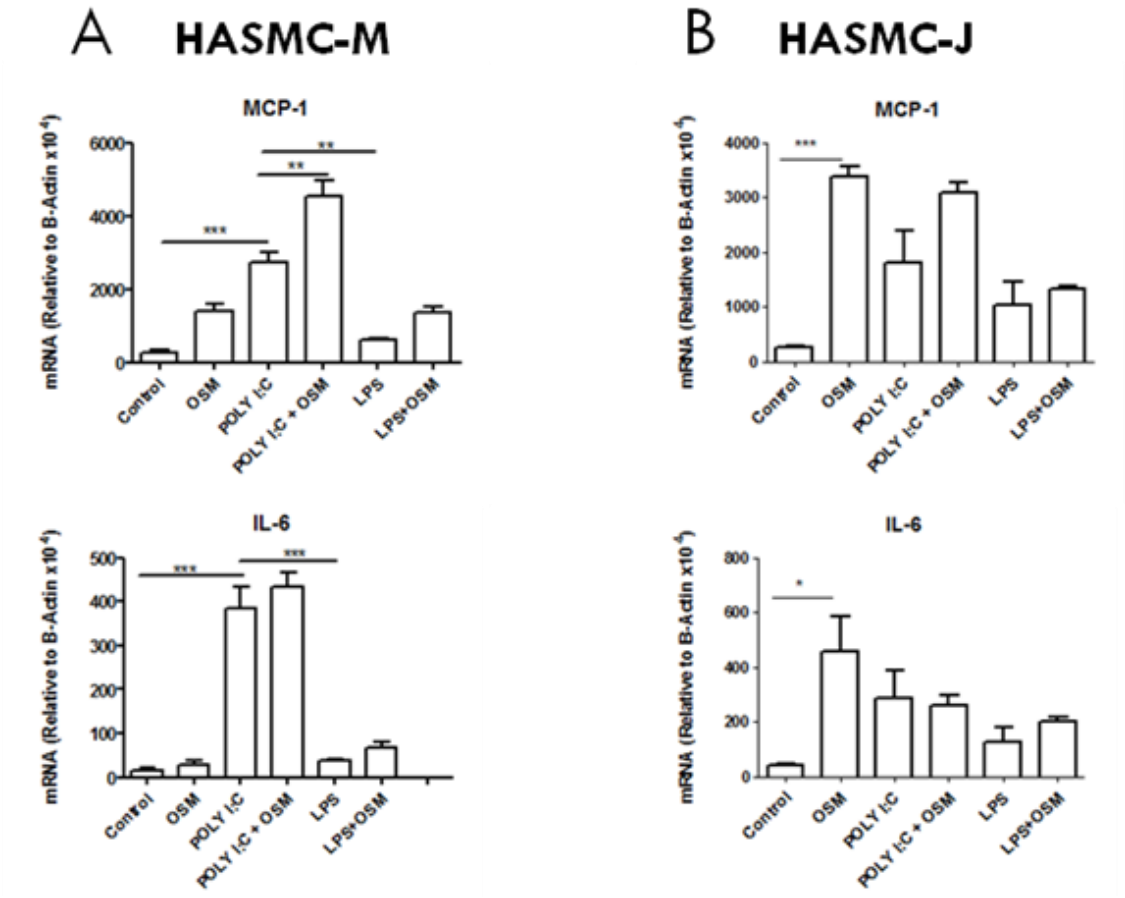
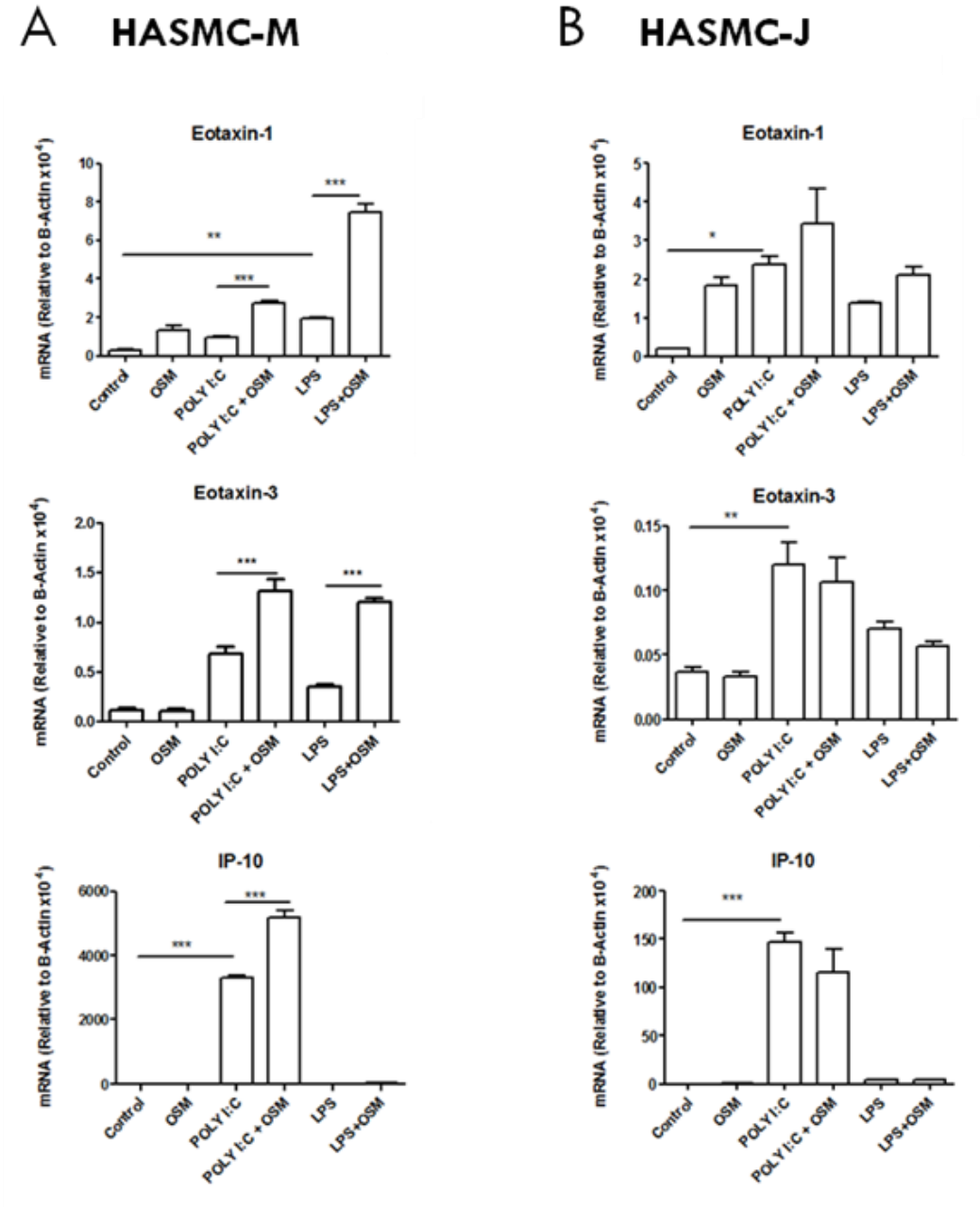

A HASMC-M
MCP-1
mRNA (Relative to B-Actin x10⁻⁴)
6000
4000
2000
0
***
**
**
Control
OSM
POLY I:C
POLY I:C + OSM
LPS
LPS+OSM
IL-6
mRNA (Relative to B-Actin x10⁻⁴)
500
400
300
200
100
0
***
***
Control
OSM
POLY I:C
POLY I:C + OSM
LPS
LPS+OSM
B HASMC-J
MCP-1
mRNA (Relative to B-Actin x10⁻⁴)
4000
3000
2000
1000
0
***
Control
OSM
POLY I:C
POLY I:C + OSM
LPS
LPS+OSM
IL-6
mRNA (Relative to B-Actin x10⁻⁴)
800
600
400
200
0
*
Control
OSM
POLY I:C
POLY I:C + OSM
LPS
LPS+OSM

FIGURE 17.B


A HASMC-M
B HASMC-J
Eotaxin-1
Eotaxin-3
IP-10
mRNA (Relative to B-Actin x10⁻⁴)
Control
OSM
POLY I:C
POLY I:C + OSM
LPS
LPS+OSM

3.3. Modulation of ECM modulators in response to TLR-ligands and OSM

3.3.1. VEGF Production in Response to OSM and TLR-ligands

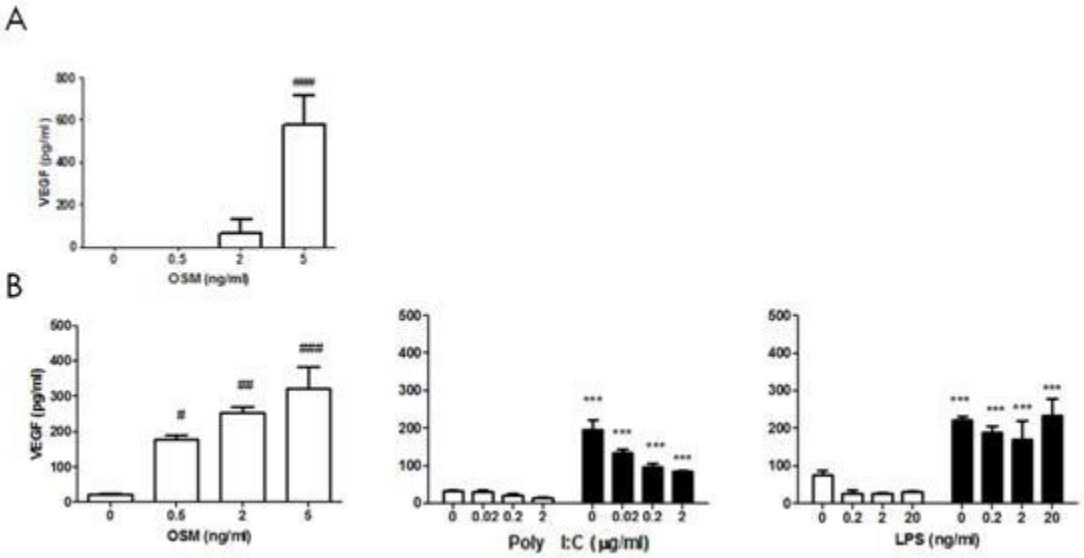
Growth factors such as VEGF can modulate ECM remodeling; thus alterations in growth factors, such as VEGF may contribute to the pathogenesis of asthma. Determining if the production of VEGF expression is modulated by OSM and TLR3 and TLR4-ligands in HASMC would aid in determining potential roles of HASMC in ECM remodeling. As performed previously, the HASMC-J line was stimulated with increasing concentrations of OSM (0ng/ml, 0.5ng/ml, 2ng/ml, 5ng/ml and 10ng/ml) for 18 hours. After the 18 hour time point, supernatants were removed from the cells and assessed using a VEGF ELISA to examine concentrations (pg/ml) in supernatants **[Figure 18 A]**. This finding demonstrates that in HASMC-J, 5ng/ml of OSM stimulation significantly induces VEGF production.

To determine if this trend could be seen in another cell line, as well as to examine the effect of TLR3 and TLR4-ligand stimulation on VEGF production, HASMC-M were stimulated with increasing concentrations of OSM (0ng/ml, 0.5ng/ml, 2ng/ml, 5ng/ml and 10ng/ml) as well as increasing concentrations of Poly I:C (0µg/ml, 0.02µg/ml, 0.2µg/ml and 2µg/ml) or increasing concentrations of LPS (0ng/ml, 0.2ng/ml, 2ng/ml and 20ng/ml) in the presence or absence of 0.5ng/ml OSM. The supernatants were analyzed with the MSD human growth factor panel I to examine various growth factors (bFGF,VEGF,sFIT-1,PIGF) **[Figure 18 B]**. Similarly to HASMC-J, HASMC-M stimulated with OSM significantly increased VEGF production. On the other hand, Poly I:C or LPS stimulation

did not increase VEGF concentrations. Interestingly, as the concentration of Poly I:C increased in co-stimualtion with OSM, VEGF levels decreased.. This could suggest that although OSM induces VEGF production, increases in TLR3-ligand inhibits VEGF. No significant changes were identified in levels of bFGF, sFIT-1 or PIGF protein detected.

FIGURE 18. OSM induces VEGF production in HASMC. (A) HASMC-J were stimulated with increasing concentrations of OSM for 18 hours. ELISA was performed to examine VEGF concentrations. **(B)** HASMC-M were stimulated with increasing concentrations of OSM, increasing concentrations of Poly I:C in the absence or presence of 0.5ng/ml OSM, or increasing concentrations of LPS in the absence or presence of 0.5ng/ml OSM. MSD (Human Growth Factor Panel I) analysis was performed to examine concentrations of various growth factors in supernatants at 18 hours. White bars represent Poly I:C or LPS alone and black bars represent the addition of OSM to the TLR-ligands. Two-way ANOVA with a Bonferroni post test was used to determine significance between TLR agonist and TLR agonist and OSM co-stimulations, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. One-way ANOVA with a Bonferroni post test was used to determine significance between OSM stimulation in comparison to control (0ng/ml OSM), significance with 95% CI #=P<0.05, ##=P< 0.001, and ###= P<0.0001. Error bars represent SEM.

FIGURE 18.

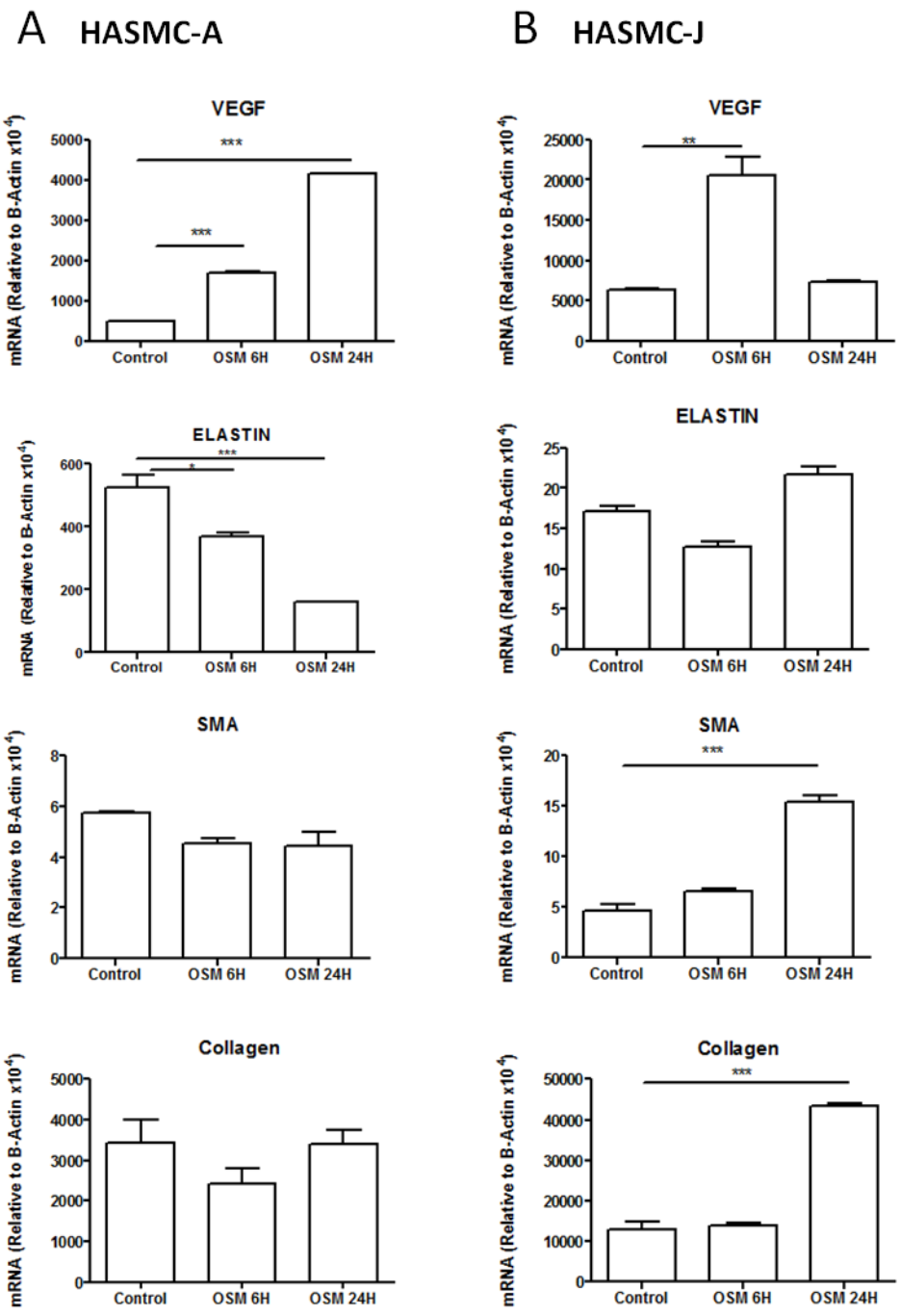


3.3.2. Alterations in mRNA expression of ECM modulators in response to OSM

mRNA levels for VEGF and ECM proteins (elastin, smooth muscle actin, and collagen 1A1) were also examined. HASMC-J and HASMC-A lines were stimulated with 2ng/ml OSM for 6 hours and 24 hours. After each time point, RNA was isolated, treated with DNase, followed by PCR and assessed by qRT-PCR **[Figure 19]**. Both HASMC-J and HASMC-A lines showed a significant increase in mRNA expression of VEGF upon stimulation with OSM, however levels further increased at 24 hours in HASMC-A while levels in HASMC-J returned to the same level as control. As mentioned previously, the differences in mRNA responses between HASMC-J and HASMC-A suggest that although a similar trend is seen, differences in kinetics were evident. In regards to elastin, HASMC-A demonstrated a significant decrease in mRNA expression after OSM stimulation, while HASMC-J mRNA levels were unaffected. Collagen and smooth muscle actin were both significantly increased in HASMC-J however not in HASMC-A.

FIGURE 19. OSM alters mRNA expression of VEGF, elastin, SMA and collagen. **(A)** HASMC-A and **(B)** HASMC-J were stimulated with 2ng/ml OSM, 2µg/ml Poly I:C in the absence or presence of 2ng/ml OSM, or 20ng/ml LPS in the absence or presence of 2ng/ml OSM. RNA expression was examined using qRT-PCR using TaqMan probes for VEGF, elastin, SMA and collagen. Data was corrected to β-Actin endogenous levels as control. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. . Error bars represent SEM within the qRT-PCR experiment since the data are an n=1.

FIGURE 19.

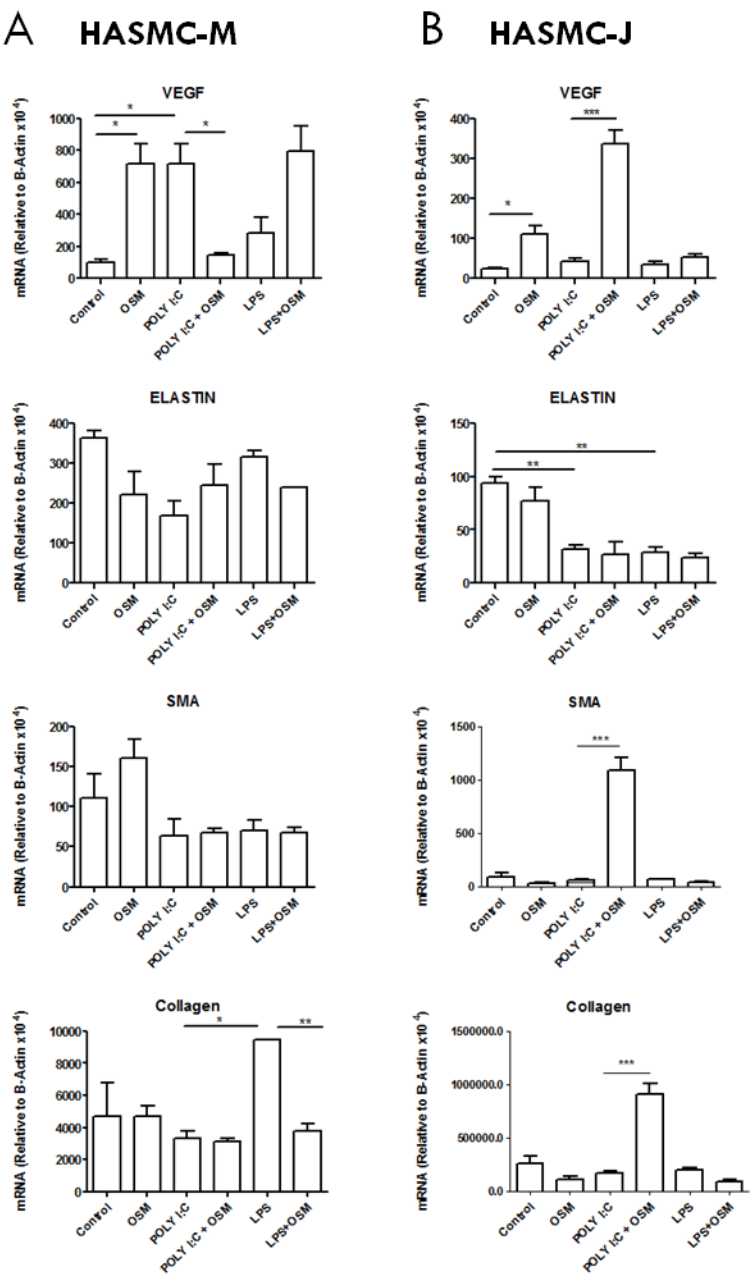

A HASMC-A
B HASMC-J
VEGF
ELASTIN
SMA
Collagen
mRNA (Relative to B-Actin x10⁻⁴)
Control
OSM 6H
OSM 24H
***
**
*

3.3.3. mRNA Expression of ECM Modulators in Response to OSM and TLR-ligands

To identify the mRNA levels of VEGF, elastin, collagen and smooth muscle actin in response to TLR3 and TLR4-ligands in the absence or presence of OSM., HASMC-M and HASMC-J lines were stimulated with 2ng/ml OSM, 2µg/ml Poly I:C in the absence or presence of 2ng/ml OSM, or 20ng/ml LPS in the absence or presence of 2ng/ml OSM at 6 hours followed by the protocol for qRT-PCR **[Figure 20]**. The mRNA expression for VEGF, smooth muscle actin and collagen in HASMC-J stimulated with Poly I:C and OSM was significantly increased in comparison to Poly I:C alone. In addition, elastin expression was significantly decreased with Poly I:C and LPS stimulation in comparison to control. Alterations in mRNA expression were seen at a lesser extent in HASMC-M. VEGF mRNA levels were significantly increased with Poly I:C alone and contrary to HASMC-J, significantly decreased with the addition of OSM. Expression of both elastin and smooth muscle actin mRNA was unchanged in HASMC-M with all stimulations; however LPS stimulated cells induce a significant increase in collagen expression, which was decreased with the addition of OSM. As previously mentioned, these findings suggest that individual HASMC lines could respond differently in regards to elastin, smooth muscle actin and collagen mRNA expression.

FIGURE 20. VEGF, elastin, SMA and collagen mRNA expression are modulated by TLR-ligands and OSM Stimulation (A) HASMC-M and **(B)** HASMC-J were stimulated with 2ng/ml OSM, 2µg/ml Poly I:C in the absence or presence of 2ng/ml OSM, or 20ng/ml LPS in the absence or presence of 2ng/ml OSM. RNA expression was examined using qRT-PCR using TaqMan probes for VEGF, Elastin, SMA, and Collagen. Data was corrected to β-Actin endogenous levels as control. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. . Error bars represent SEM within the qRT-PCR experiment since the data are an n=1.

FIGURE 20.

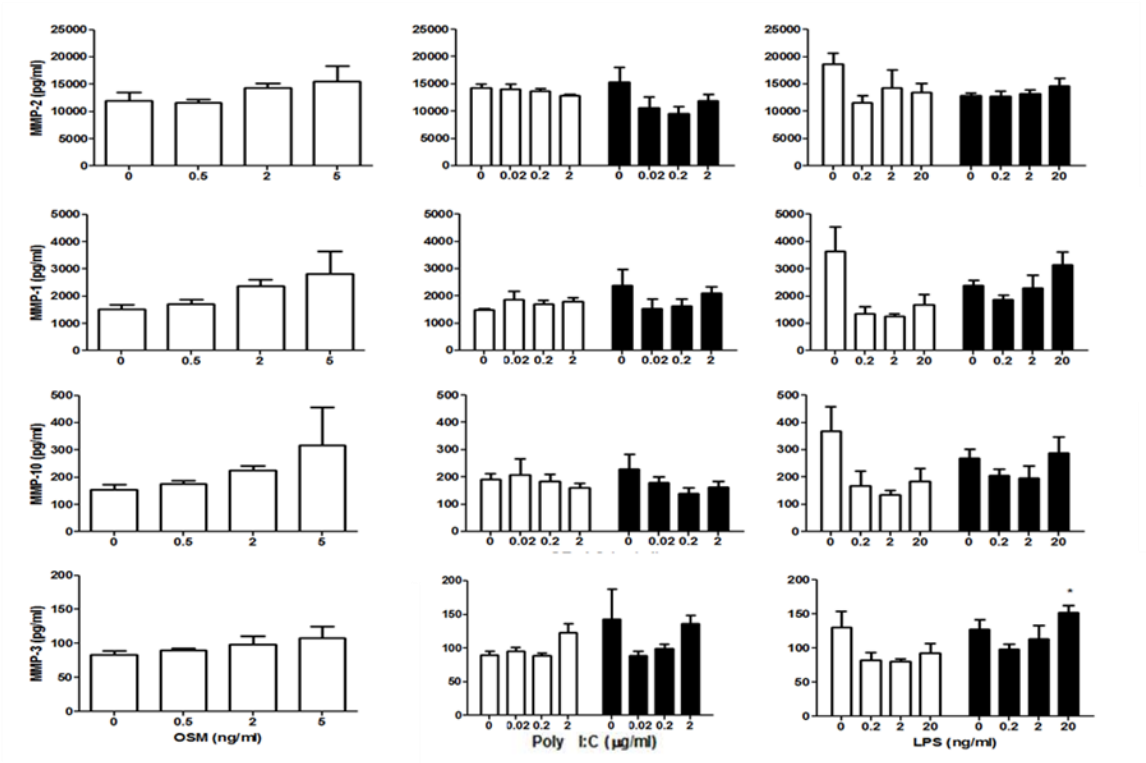

A HASMC-M
B HASMC-J
VEGF
ELASTIN
SMA
Collagen
mRNA (Relative to B-Actin x10⁻⁴)
Control
OSM
POLY I:C
POLY I:C + OSM
LPS
LPS+OSM

3.3.4. Modulation of MMPs in Response to OSM and TLR-ligands

In addition to cytokines, chemokines, growth factors and ECM proteins, HASMC have been shown to express matrix MMPs and TIMPs which are important in maintaining the balance between ECM degradation and remodeling. The HASMC-M line was stimulated with increasing concentrations of OSM (0ng/ml, 0.5ng/ml, 2ng/ml, 5ng/ml and 10ng/ml) as well as increasing concentrations of Poly I:C (0µg/ml, 0.02µg/ml, 0.2µg/ml and 2µg/ml) or increasing concentrations of LPS (0ng/ml, 0.2ng/ml, 2ng/ml and 20ng/ml) in the presence or absence of 0.5ng/ml OSM. The supernatants were analyzed for concentrations of various MMPs using MSD human MMP-2 plex and human MMP-3 plex **[Figure 21**]. MMP-1, MMP- 2, MMP-3 and MMP-10 levels were all unaffected by OSM stimulation or by Poly I:C or LPS in the presence of OSM, with the exception of MMP-3 which was significantly increased with LPS and OSM co-stimulation in comparison to LPS alone.

Figure 21. MMP production is not modulated by OSM or TLR-ligands. The HASMC-M line was stimulated with increasing concentrations of OSM, increasing concentrations of Poly I:C in the absence or presence of 0.5ng/ml OSM, or increasing concentrations of LPS in the absence or presence of 0.5ng/ml OSM. MSD (Human MMP- 2-plex and MMP 3-plex) analysis was performed to examine concentrations of various MMPs in supernatants at 18 hours. White bars represent Poly I:C or LPS alone and black bars represent the addition of OSM to the TLR-ligand stimulation. Two-way ANOVA with a Bonferroni post .One-way ANOVA with a Bonferroni post test was used, significance with 95% CI #=P<0.05, ##=P< 0.001, and ###= P<0.0001. Error bars represent SEM.

FIGURE 21.


MMP-2 (pg/ml)
MMP-1 (pg/ml)
MMP-10 (pg/ml)
MMP-3 (pg/ml)
OSM (ng/ml)
Poly I:C (µg/ml)
LPS (ng/ml)

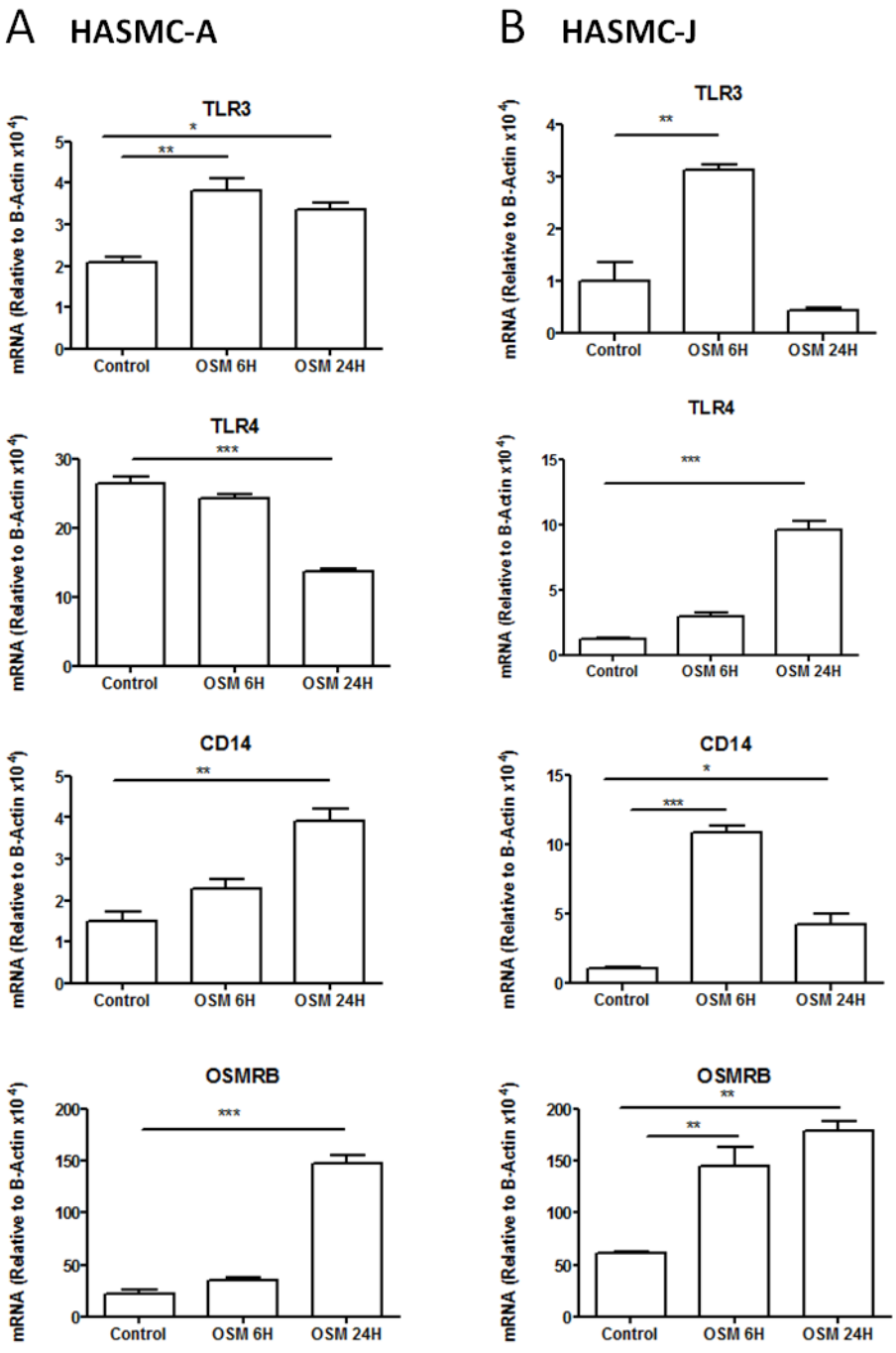
3.4. Receptor Modulation in Response to OSM and TLR-ligands

3.4.1. TLR and OSMRB mRNA Expression in Response to OSM

Identifying whether OSM can regulate receptors including TLR3, TLR4, CD14 and OSMRB in HASMC was examined by stimulating HASMC with OSM. HASMC-A and HASMC-J lines were stimulated with 2ng/ml OSM for 6 hours or 24 hours. After each time point, RNA was isolated, treated with DNase, followed by PCR and assessed by qRT-PCR. TLR3, TLR4, CD14 and OSMRB TaqMan probes were used along with β-Actin as an endogenous control **[Figure 22]**. TLR3, CD14 and OSMRB mRNA levels were all significantly increased in response to OSM in comparison to control. This increase was shown at 6 hours in HASMC-J for TLR3 and at both 6 hours and 24 hours for CD14 and OSMRB. While in HASMC-A, CD14 and OSMRB mRNA expression was not significantly increased until 24 hours. TLR3 mRNA expression was increased at both 6 hours and 24 hours. In regards to TLR4, a decrease in expression was seen with HASMC-A at 24 hours while an increase in expression was seen in HASMC-J.

FIGURE 22. TLR3, TLR4, CD14 and OSMRβ mRNA expression increases with OSM. (A) HASMC-A and **(B)** HASMC-J were stimulated with 2ng/ml OSM for 6H or 24H. RNA expression was examined using qRT-PCR using TaqMan probes for TLR3, TLR4, CD14 and OSMRBβ. Data was corrected to β-Actin endogenous levels as control. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. . Error bars represent SEM within the qRT-PCR experiment since the data are an n=1.

FIGURE 22.

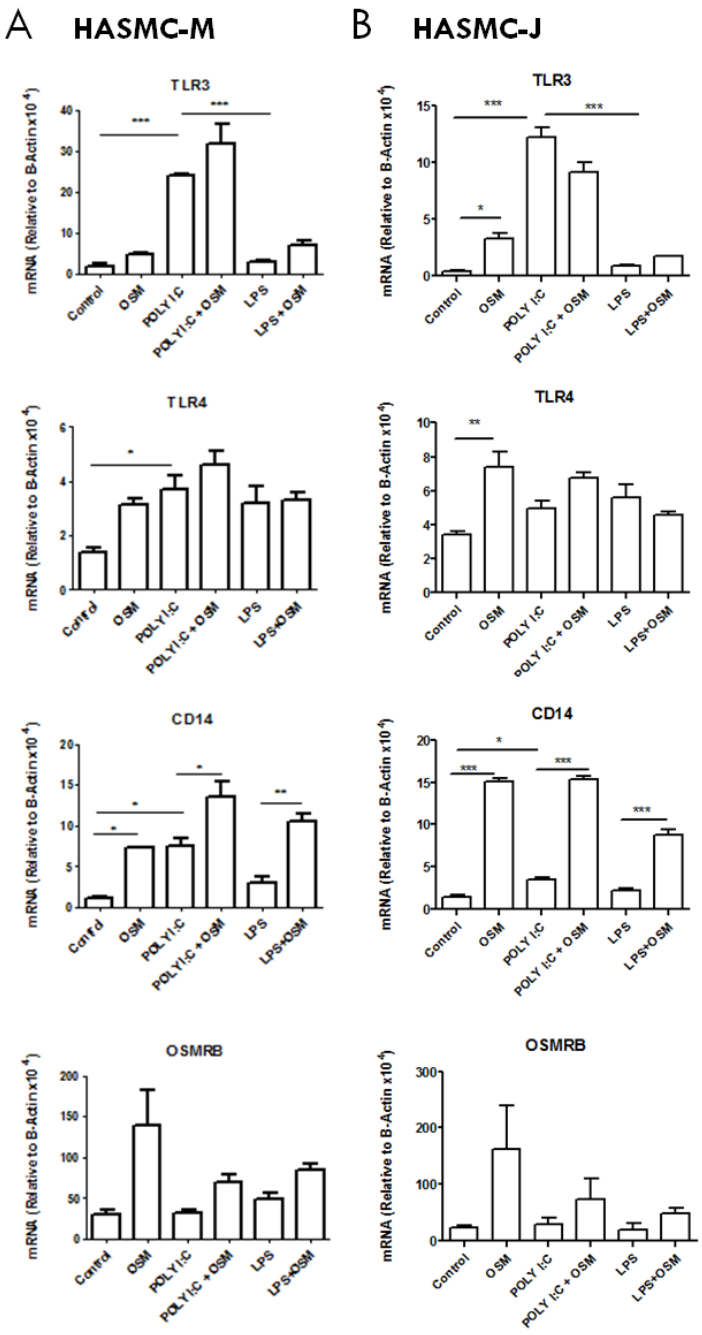

A HASMC-A
B HASMC-J
TLR3
TLR4
CD14
OSMRB
mRNA (Relative to B-Actin x10⁻⁴)
Control
OSM 6H
OSM 24H

3.4.2. TLR and OSMRB mRNA Expression in Response to TLR-ligands and OSM

The above findings implicate OSM as a regulator of TLRs and OSMRB mRNA levels in HASMC. To determine if TLR3 and TLR4-ligands can also modulate receptor expression *in vitro* and if the presence of OSM further augments the expression induced by these TLRs, HASMC were stimulated with TLR3 and TLR4-ligands in the absence or presence of OSM. HASMC-M and HASMC-J were stimulated with 2ng/ml OSM, 2µg/ml Poly I:C in the absence or presence of 2ng/ml OSM, or 20ng/ml LPS in the absence or presence of 2ng/ml OSM at 6 hours. After the time course, RNA was isolated, treated with DNase, followed by PCR and assessed by qRT-PCR with TaqMan probes for TLR3, TLR4, CD14 and OSMRB **[Figure 23]**. Poly I:C induced a significant increase in TLR3, TLR4 and CD14 expression in comparison to control in both HASMC-M and HASMC-J while OSMRB expression was not regulated by LPS or Poly I:C. Of interesting note, in both cell lines CD14 expression was significantly up-regulated by Poly I:C and OSM co-stimulation as well as with LPS and OSM co-stimulation, in comparison to stimulation by the TLR-ligands alone.

FIGURE 23.TLR3, TLR4 and CD14 mRNA is modulated by OSM and TLR-ligands. (A) HASMC-M and **(B)** HASMC-J were stimulated with 2ng/ml OSM, 2µg/ml Poly I:C in the absence or presence of 2ng/ml OSM, or 20ng/ml LPS in the absence or presence of 2ng/ml OSM. RNA expression was examined using qRT-PCR using TaqMan probes for TLR3, TLR4, CD14 and OSMRBβ. Data was corrected to β-Actin endogenous levels as control. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. . Error bars represent SEM within the qRT-PCR experiment since the data are an n=1.

FIGURE 23.


A HASMC-M
B HASMC-J
TLR3
TLR4
CD14
OSMRB
mRNA (Relative to B-Actin x10⁻⁴)
Control
OSM
POLY I:C
POLY I:C + OSM
LPS
LPS+OSM

3.4.3. Modulation of TLR Protein Expression in Response to OSM

Since preliminary evidence supported up regulation of CD14, the TLR4 and associated receptor chains became of interest. Preliminary experiments were conducted to determine if soluble CD14 could be detected in HASMC supernatants stimulated with OSM. Assessement using an ELISA for soluble CD14 revealed no presence of soluble CD14 in HASMC supernatants (data not shown), this suggested that signaling could be occurring though membrane bound CD14. An additional experiment was then performed by challenging HASMC- M with increasing concentrations of LPS (0ng/ml, 0.2ng/ml, 2ng/ml, 20ng/ml) in the absence or presence of 100ng/ml soluble CD14 (data not shown) which demonstrated that soluble CD14 in combination with LPS did not elicit detectable increased production of IL-6 in these cells. Together, these findings support that soluble CD14 is not essential for LPS challenged IL-6 responses by HASMC.

In order to further explore this question, flow cytometry for TLR3, TLR4 and CD14 was conducted using HASMC to examine changes in protein levels upon OSM stimulation. HASMC-M were stimulated with OSM for 2, 6 or 24 hours at 2ng/ml or 10ng/ml OSM. After the time points, cells were place in 1mM EDTA in PBS for 30 minutes, followed by the removal of cells from the plates. The cells then received Fc block, were stained with TLR4, CD14 and TLR3 to be analyzed with the LSRII flow cytometer. As shown in **[Figure 24]**, TLR3 and CD14 expression could be detected in these cells, however TLR4 levels were discernible since the background fluorescence was greater than the antibody fluorescence. CD14 expression was shown to be slightly

increased in the HASMC stimulated with 2ng/ml OSM at both 6 hours and 24 hours. Upon analysis, it became evident that the CD14 expression on HASMC was heterogeneous in that only a small population of these cells were CD14 positive. Based on these findings, the analysis gates were altered to compare only the changes in fluorescence in the CD14+ population. When this gate was applied, CD14 expression remained significantly increased at 24 hours and TLR3 expression was shown to also be significantly increased with 2ng/ml OSM at 24 hours.

To further examine this question, HASMC-F were stimulated with 10ng/ml OSM at 2 hours or 24 hours followed by the flow staining protocol mentioned above **[Figure 25]**. TLR3 and CD14 fluorescent antibodies were used, however not for TLR4 as it was previously shown to be non-specific. HASMC-F stimulated with OSM significantly increased TLR3 expression at both 2 hours and 24 hours (total cells and CD14+ cells). These finding suggests that TLR3 expression is present in the total HASMC-F population, in comparison to HASMC-M where TLR3 expression was induced only in the CD14+ population. In regards to CD14 expression in HASMC-F, a significant increase was identified at 2 hours in the total population however not in the CD14+ population. In addition, HASMC-J were also stimulated with OSM (2ng/ml) to examine how their TLRs were modulated in response to OSM (at 6 hours and 24 hours) **[Figure 26]**. HASMC-J stimulated with OSM demonstrated increased expression of CD14 at 6 hours in the total cell population, the expression of CD14 in the CD14+ population was

similar to that in the total cell population (data not shown). In regards to TLR3 expression in HASMC-J, specific TLR3 was not detectetable.

As previously mentioned, plots of the mean fluorescence for the CD14 antibody demonstrated a CD14+ side population implying that these cells are heterogeneous for CD14 expression. To examine the variation in CD14 expression and TLR4-ligand responses in HASMC-M, HASMC-J and HASMC-F, the percentage of CD14+ cells were gated for each experiment **[Figure 27]**. The percentage of CD14 expression was shown to be the greatest in HASMC-J (16%) and was similar between HASMC-M (7-9%) and HASMC-F (8%). In addition, the percentage of CD14+ population increased in all three cells lines between 2 and 6 hours after OSM stimulation, with HASMC-J increasing the most, followed by a decrease back to the control percentage levels by 24 hours.

FIGURE 24. OSM increases TLR3 and CD14 protein expression in HASMC-M. HASMC-M were stimulated with OSM (2ng/ml or 10ng/ml) for 2 hours, 6 hours or 24 hours. Cells were stained with fluorescent antibodies for TLR3, TLR4 and CD14 and samples were run on LSRII flow cytometry. **(A)** total cells and **(B)** gated CD14+ population. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. Error bars represent SEM.

FIGURE 25. OSM increases TLR3 and CD14 protein expression in HASMC-F. HASMC-F were stimulated with OSM (10ng/ml) for 2 hours or 24 hours. Cells were stained with fluorescent antibodies for TLR3 and CD14 and samples were run on LSRII flow cytometry. **(A)** represents total cells and **(B)** represents the gated CD14+ population. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. Error bars represent SEM.

FIGURE 26. OSM increases CD14 expression in HASMC-J. HASMC-J were stimulated with OSM (2ng/ml) for 6 hours or 24 hours. Cells were stained with fluorescent antibodies for **(A)** CD14 and **(B)** TLR3. Samples were run on LSRII flow cytometer.

FIGURE 27. Variation in CD14+ population between HASMC lines. (A) HASMC-J and HASMC-M were stimulated with OSM (2ng/ml) for 6 hours or 24 hours and **(B)** HASMC-F and HASMC-M were stimulated with OSM (10ng/ml) for 2 hours or 24 hours. Cells were stained with fluorescent antibodies for CD14 and run on LSRII flow cytometer and were gated based on an isotype control.

FIGURE 24.

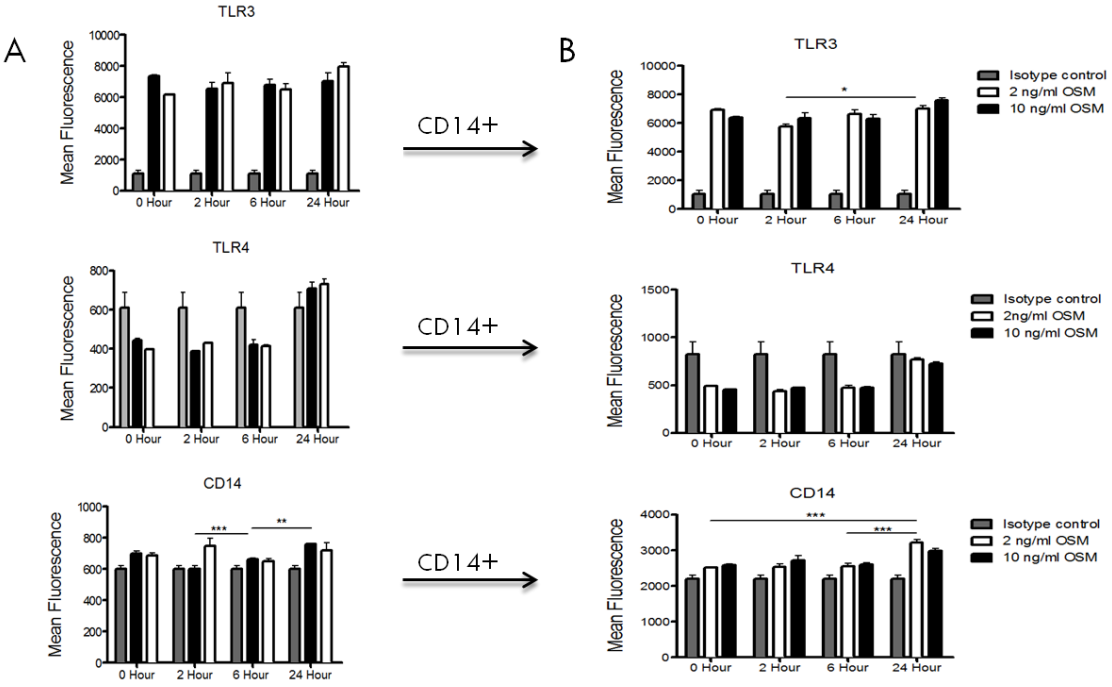

A
TLR3
Mean Fluorescence
0 Hour
2 Hour
6 Hour
24 Hour
CD14+
B
TLR3
Isotype control
2 ng/ml OSM
10 ng/ml OSM
TLR4
2ng/ml OSM
CD14
***
**

FIGURE 25.

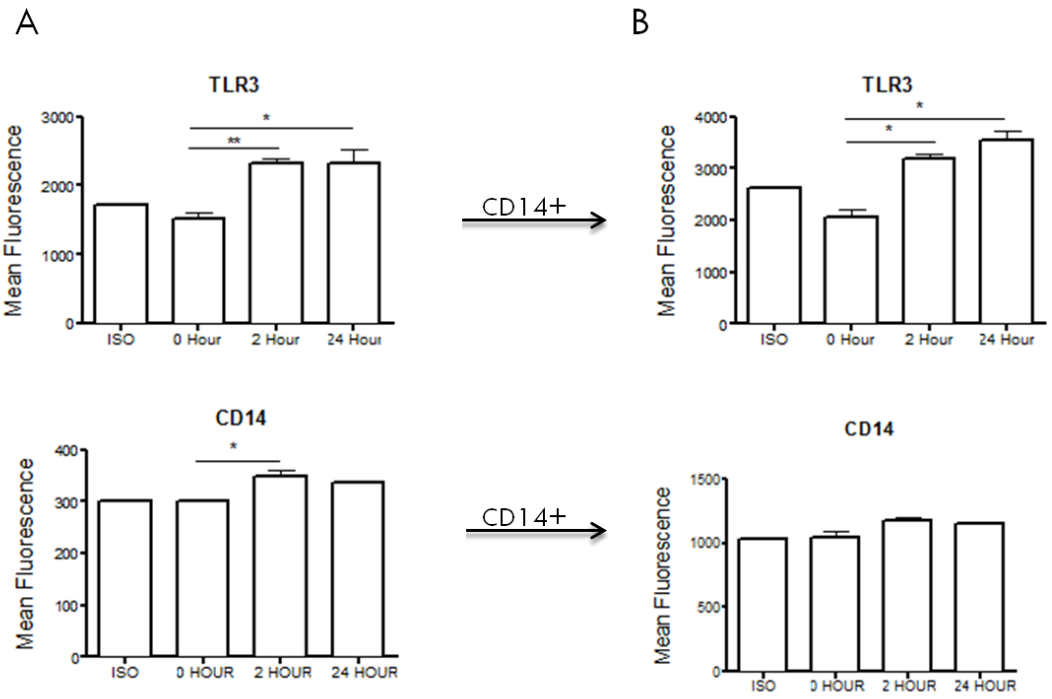

A
B
TLR3
Mean Fluorescence
3000
2000
1000
0
*
**
ISO
0 Hour
2 Hour
24 Hour
CD14+
TLR3
Mean Fluorescence
4000
3000
2000
1000
0
*
*
ISO
0 Hour
2 Hour
24 Hour
CD14
Mean Fluorescence
400
300
200
100
0
*
ISO
0 HOUR
2 HOUR
24 HOUR
CD14+
CD14
Mean Fluorescence
1500
1000
500
0
ISO
0 HOUR
2 HOUR
24 HOUR

FIGURE 26.

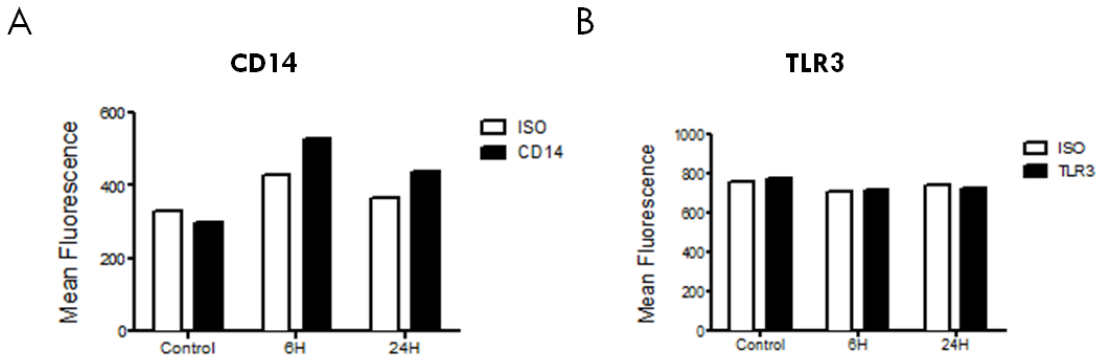
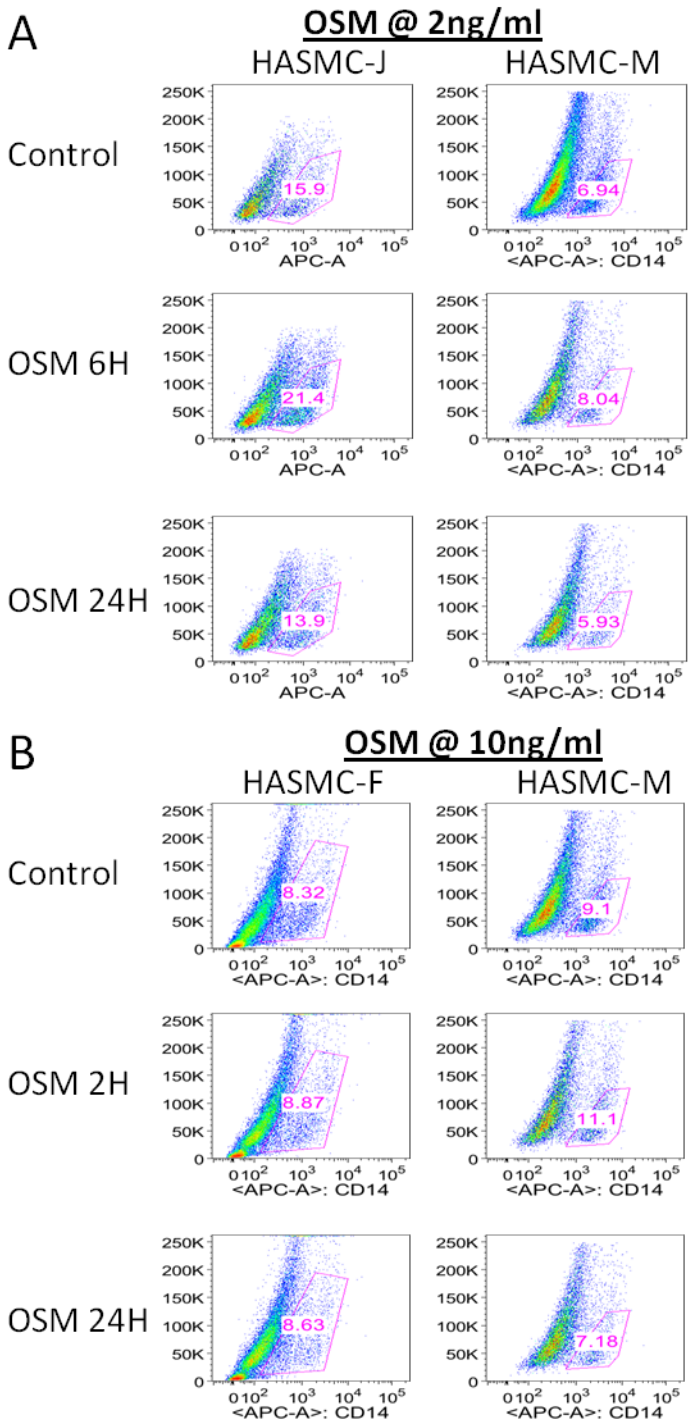

A
CD14
Mean Fluorescence
600
400
200
0
Control
6H
24H
ISO
CD14
B
TLR3
Mean Fluorescence
1000
800
600
400
200
0
Control
6H
24H
ISO
TLR3

FIGURE 27.

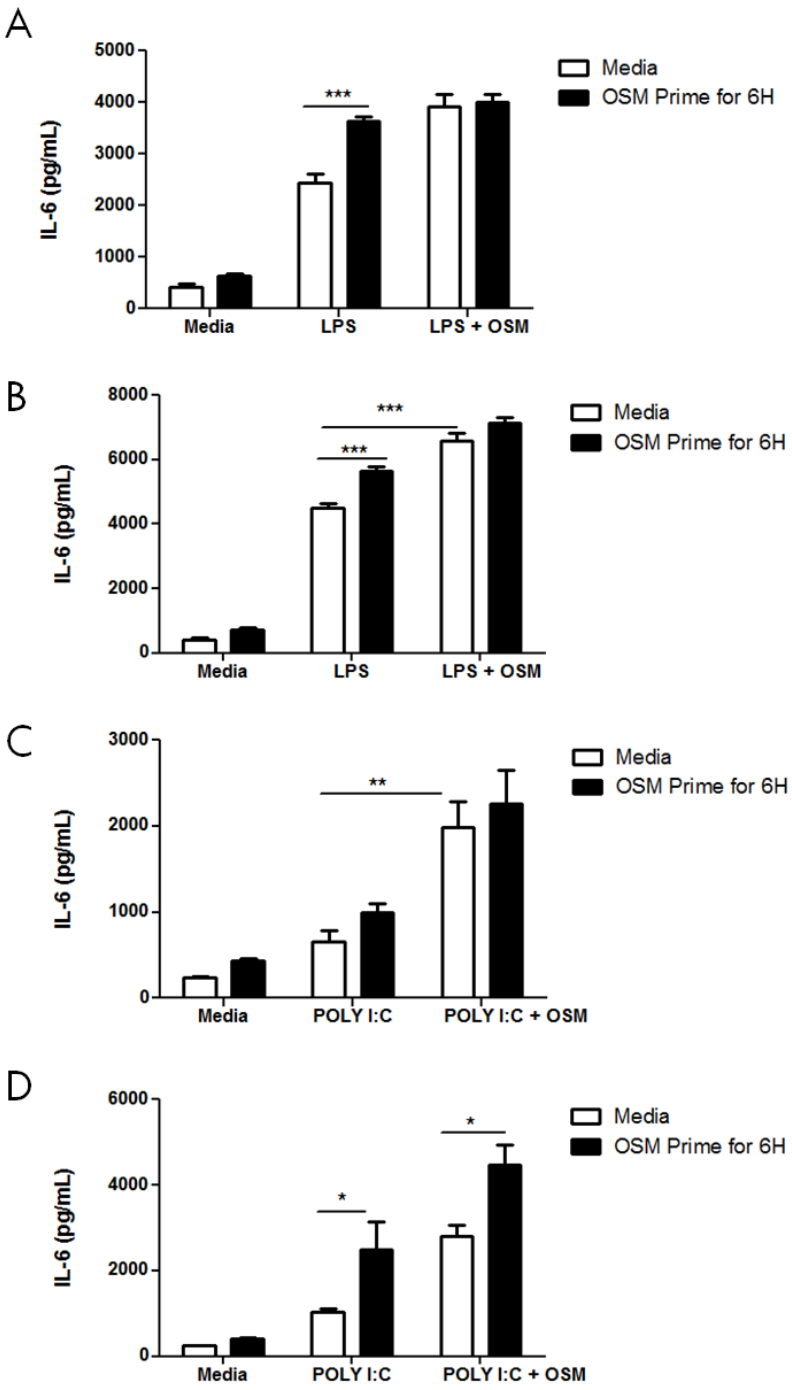

A
OSM @ 2ng/ml
HASMC-J
HASMC-M
Control
OSM 6H
OSM 24H
15.9
6.94
21.4
8.04
13.9
5.93
APC-A
<APC-A>: CD14
B
OSM @ 10ng/ml
HASMC-F
HASMC-M
Control
OSM 2H
OSM 24H
8.32
9.1
8.87
11.1
8.63
7.18
<APC-A>: CD14

3.4.4 Effectiveness of OSM Priming prior to TLR-ligand Challenge

Previous findings mentioned above have demonstrated that OSM up-regulates TLR3, TLR4 and CD14 expression. To determine if preceding up-regulation of receptors by OSM followed by TLR3 and TLR4-ligand stimulation in HASMC induces a greater pro-inflammatory response in comparison to co-stimulation, HASMC were primed with OSM for 6 hours. HASMC-J were stimulated with increasing concentrations of Poly I:C or increasing concentration of LPS in the absence of presence of 2ng/ml OSM after a 6 hour OSM prime (control prime was performed with media for 6 hours). **[Figure 28]** demonstrates that for both Poly I:C and LPS stimulated cells, OSM priming for 6 hours was sufficient to elicit the same IL-6 response seen with 18 hour OSM and TLR-ligand co-stimulation.

FIGURE 28. OSM priming elicits same response as co-stimulation with TLR-ligands. HASMC-J were stimulated ("primed') with 2ng/ml OSM or media for 6 hours followed by challenge with **(A)** LPS at 2ng/ml, **(B)** LPS at 20ng/ml, **(C)** Poly I:C at 0.2µg/ml, or **(D)** Poly I:C at 2µg/ml in the presence or absence of 0.5ng/ml OSM for 18 hours. Supernatants were assessed by IL-6 ELISA. One-way ANOVA with a Bonferroni post test was used, significance with 95% CI *=P<0.05, **=P< 0.001, and ***= P<0.0001. Error bars represent SEM.

FIGURE 28.


A
IL-6 (pg/mL)
5000
4000
3000
2000
1000
0
***
Media
OSM Prime for 6H
Media
LPS
LPS + OSM
B
IL-6 (pg/mL)
8000
6000
4000
2000
0
***
***
Media
OSM Prime for 6H
Media
LPS
LPS + OSM
C
IL-6 (pg/mL)
3000
2000
1000
0
**
Media
OSM Prime for 6H
Media
POLY I:C
POLY I:C + OSM
D
IL-6 (pg/mL)
6000
4000
2000
0
*
*
Media
OSM Prime for 6H
Media
POLY I:C
POLY I:C + OSM

3.5. Signaling Pathways Induced by TLR-ligands and OSM

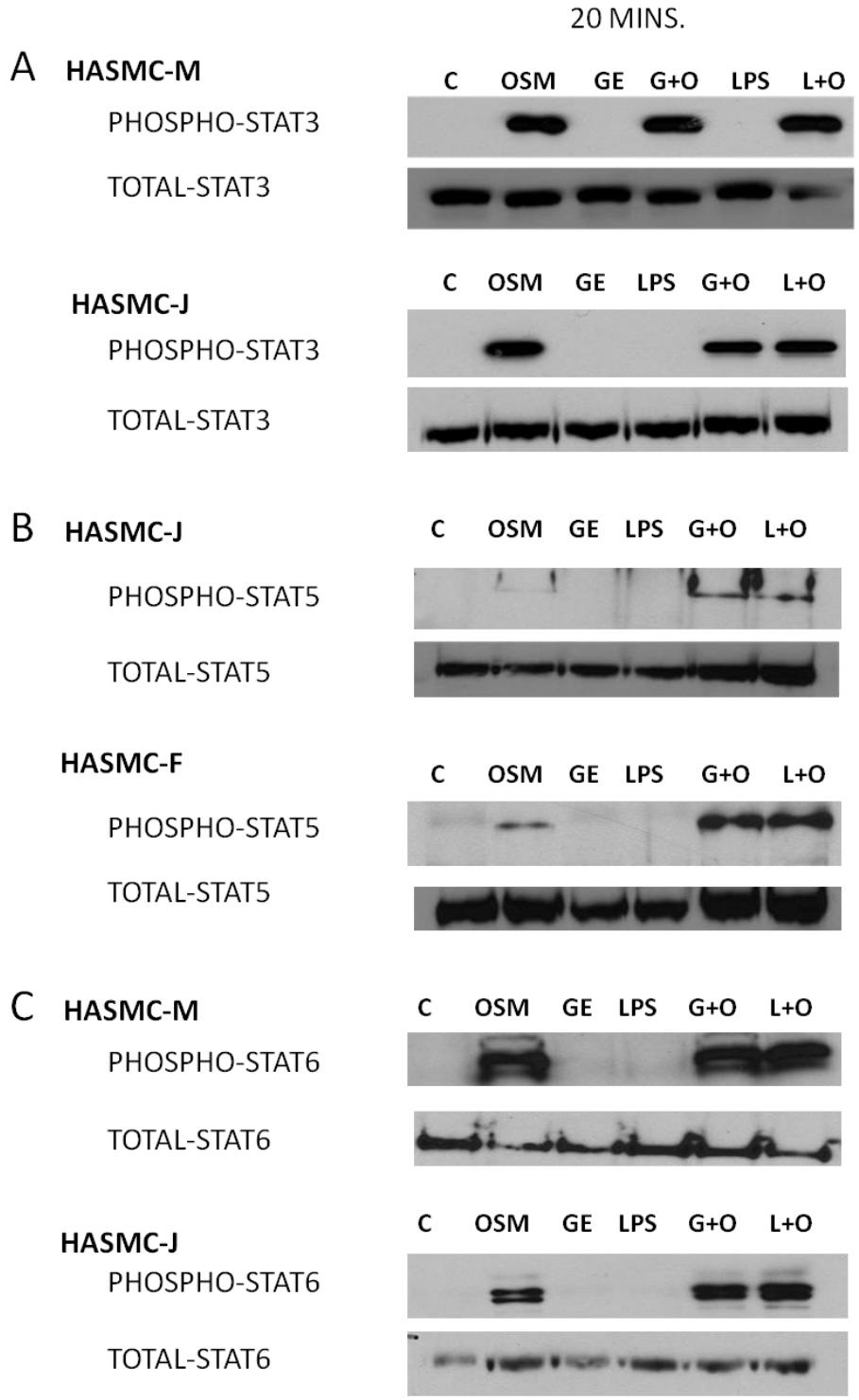
3.5.1. STAT Signaling Pathways in Response to OSM and TLR-ligands

Since it is known that OSM can induce the JAK-STAT pathway and previous findings have shown that STAT3 and STAT6 are involved in HASMC inflammatory responses; STAT3, STAT5 and STAT6 activation were examined in HASMC in response to OSM and TLR-ligands. HASMC-M, HASMC-J and HASMC-F were stimulated with 2ng/ml OSM, and 2µg/ml Poly I:C or 20ng/ml LPS in the absence or presence of 2ng/ml OSM for 20 minutes. Cell lysates were collected after the time course and used for SDS PAGE Westerns blots. Nitrocellulose membranes were probed with STAT3, STAT5 and STAT6 **[Figure 29]**. At 20 minutes, STAT3 phosphorylation was present in HASMC-M and HASMC-J stimulated with OSM, Poly I:C with OSM, and LPS with OSM, while it was not activated with Poly I:C and LPS stimulation alone. 20 minute stimulation with OSM, Poly I:C with OSM and LPS with OSM also induced STAT5 activation in HASMC-J and HASMC-F. Interestingly, the band signals for Poly I:C with OSM and LPS with OSM appear larger than OSM alone, suggesting increased STAT5 activation in these challenges in comparison to OSM alone. However, total STAT5 band signals also appear larger in these stimulations suggesting more protein was loaded in these wells. Therefore, to better understand this signaling quantitative Western blots would be helpful. Phosphorylated STAT6 was also present at 20 minutes in HASMC-J and HASMC-M with OSM, Poly I:C with OSM and LPS with OSM co-stimulations. Taken together, these data suggest that OSM activates STAT3, STAT5 and STAT6 alone and in

combination with Poly I:C and LPS. However, Poly I:C and LPS do not activate STAT3, STAT5 or STAT6 without the presence of OSM.

Figure 29. OSM activation of STAT3, STAT5 and STAT6 in HASMC. HASMC-M, HASMC-J and HASMC-F were stimulated with OSM, or Poly I:C (GE) and LPS in the absence or presence of OSM for 20 mins, Total cell lysates were prepared for SDS Page gel followed by western blots for **(A)** Total STAT3 and Phosphorylated STAT3, **(B)** Total STAT5 and phosphorylated STAT5, and **(C)** Total STAT6 and Phosphorylated STAT6.

FIGURE 29.

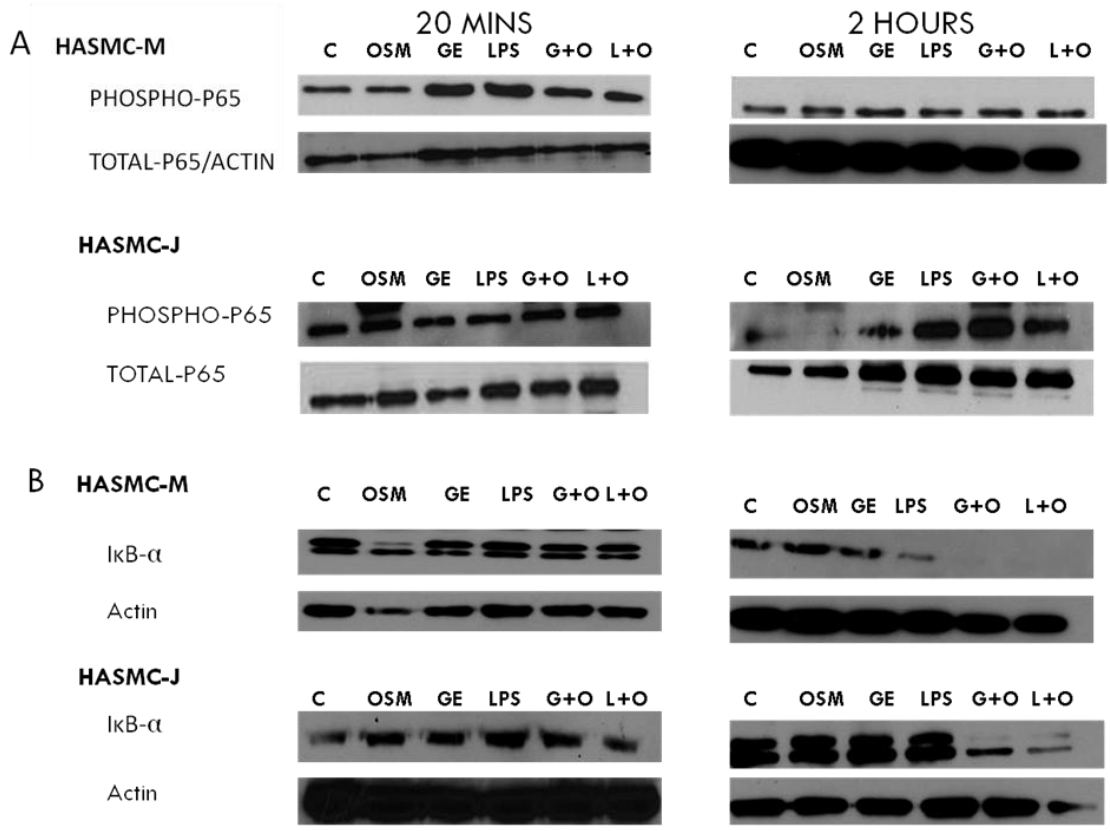

20 MINS.
A
HASMC-M
C OSM GE G+O LPS L+O
PHOSPHO-STAT3
TOTAL-STAT3
HASMC-J
C OSM GE LPS G+O L+O
PHOSPHO-STAT3
TOTAL-STAT3
B
HASMC-J
C OSM GE LPS G+O L+O
PHOSPHO-STAT5
TOTAL-STAT5
HASMC-F
C OSM GE LPS G+O L+O
PHOSPHO-STAT5
TOTAL-STAT5
C
HASMC-M
C OSM GE LPS G+O L+O
PHOSPHO-STAT6
TOTAL-STAT6
HASMC-J
C OSM GE LPS G+O L+O
PHOSPHO-STAT6
TOTAL-STAT6

3.5.2. NFκB Signaling Pathways in Response to OSM and TLR-ligands

Activation of the NFκB signaling cascade has been correlated with the production of IL-8, TGF-β, ICAM-1 and VCAM in HASMC [16]. In order to examine the activation of NFκB in response to OSM and TLR3 and TLR4-ligand stimulation, HASMC-J and HASMC-M cell lysates from cells stimulated with as seen in Figure 29. Nitrocellulose membranes were probed with phosphorylated p65, a subunit of the NFκB dimer.and IκB-α. **[Figure 30]** demonstrated[50] that phosphorylated p65 is present in all stimulation at 20 minutes (OSM, Poly I:C, LPS, Poly I:C with OSM and LPS with OSM). However at 2 hours the control and OSM stimulated cells do not demonstrate that p65 is activated, while this is not seen in HASMC-J. Contrary to NFκB activation, IκB-α degradation (suggesting activation) can be only be seen at 2 hours in cell lysates that were stimulated with LPS and Poly I:C in the presence of OSM.

Figure 30. NFκB P65/IκB-α activation in HASMC by OSM and TLR-ligands. HASMC lines were stimulated with OSM, or Poly I:C (GE) and LPS in the absence or presence of OSM for 20 mins and 2 hours. Total cell lysates were prepared for SDS Page gel followed by western blots for **(A)** Total P65 and Phosphorylated P65, and **(B)** IκB-α.

FIGURE 30.


A
HASMC-M
20 MINS
2 HOURS
C OSM GE LPS G+O L+O
PHOSPHO-P65
TOTAL-P65/ACTIN
HASMC-J
PHOSPHO-P65
TOTAL-P65
B
HASMC-M
IκB-α
Actin
HASMC-J
IκB-α
Actin

-CHAPTER 4-

DISCUSSION

4.1. Cytokine and Chemokine Production in Response to OSM

Recent research examining the implications of OSM on pro-inflammatory responses in HASMC has demonstrated that the OSMR complex (OSMRβ and gp130) is highly expressed on these cells. In addition, OSM stimulation of HASMC *in vitro* induces the production of VEGF, MCP-1 and IL-6 in cell supernatants, and other cytokines such as Th2 cytokines (IL-4 and IL-13) have also been shown to produce dose dependent increases in eotaxin-1 [19, 50]. Furthermore, these responses can be amplified by co-stimulating HASMC with OSM and Th2 cytokines [50]. Faffe et al, as well as previous results in the laboratory have identified OSM as a key gp130 cytokine in the initiation of pro-inflammatory responses by HASMC [50]. OSM stimulation, in comparison to other gp130 cytokines tested (IL-11, IL-31 and LIF) resulted in a greater detection of IL-6 in supernatants when cells were co-stimulated with LPS or Poly I:C in the presence of OSM **[Figure 3]**. In human systems, OSM can signal through both the OSMR and LIFR complexes, therefore it is possible that OSM induced IL-6 production is due to greater receptor availably [44]. In addition, OSM has been shown to induce the most abundant levels of IL-6 detection and STAT3 phosphorylation in these cells in comparison to other gp130 cytokines even though many of the gp130 cytokine receptors

have been shown to be expressed in these cells [50, 91]. Since it is known that HASMC express the receptors required for gp130 cytokine stimulation it is possible that the OSMR or LIFR complexes are more highly present and active on the cells in comparison to other family members. Taken together, these findings indicate that among the cytokines tested, OSM is more active in this model. The data mentioned above as well as previous findings in the laboratory propose that OSM could induce a variety of pro-inflammatory mediators in HASMC at both the protein and mRNA level.

In order to test this hypothesis, HASMC isolated from a number of patients were stimulated with OSM *in vitro*. Consistent with findings above, HASMC stimulated with increasing concentrations of OSM in the system also resulted in a dose dependent increase of detectable IL-6 and MCP-1. These findings were identified at both the protein **[Figure 1]** and mRNA levels **[Figure 2]**. The data demonstrated that 6 hours of OSM stimulation (at 2ng/ml OSM) can increase mRNA expression for IL-6 and MCP-1. Of relevance, this concentration of OSM is biologically significant in asthma since patients suffering from asthma have been shown to have up to 1ng/ml OSM in their sputum upon challenge [51]. Furthermore, between 50 and 800pg/ml of IL-6 could be detected in HASMC supernatants upon OSM stimulation, and recent findings have demonstrated that up to 600pg/ml of IL-6 can also be detected in sputum of challenged asthmatics [92]. Interestingly, all three cell lines tested in this project responded to OSM with varying concentration of IL-6 and MCP-1 protein and mRNA expression. This suggests that although a similar qualitative trend can be seen in HASMC stimulated with OSM,

different cell lines respond with varying degrees of sensitivity. A possible mechanism for this variation will be further discussed later in this chapter. In addition, previous findings in the laboratory demonstrate that this trend can be reproduced in a fourth HASMC line. These data suggest that in response to OSM, HASMC produce pro-inflammatory mediators that are found in asthmatic patients and contribute to the recruitment of inflammatory cells and participate in the acute phase response [19]. MCP-1 increases in HASMC by OSM also implicate these cells in the activation of mast cells and basophils in asthma since MCP-1 has been linked to the activation of monocytes, basophils, mast cells, eosinophils and lymphocytes in inflammatory disorders [93]. Furthermore, histamine generation has been found to be associated with RANTES, IL-8 and MCP-1 [94]. As a result, the up-regulation of MCP-1 in this system by OSM could therefore implicate HASMC in the acute inflammatory and allergic responses seen in asthma.

Along with IL-6 and MCP-1, an array of cytokines and chemokines has been shown to be associated with the pathogenesis of asthma including CC and CXC chemokines [95]. Examining the protein and mRNA levels of chemokines such as MCP-4, eotaxin-1, eotaxin-3, IL-8, TARC, and IP-10 was helpful in determining the role of OSM on their release by HASMC **[Table 2]**. Interestingly, preliminary experiments identified that 18s (endogenous control for qRT-PCR) mRNA expression was up-regulated by OSM in the HASMC system *in vitro*. Therefore various endogenous control genes (GAPDH, 18s, β-actin, and β-2-microglobulin) were examined to identify the most consistent control. These findings demonstrated that β-actin mRNA expression had the

least variation between samples (no more than 1 cycle) and was therefore used as an internal control. Furthermore, mRNA statistics were performed only on one experiment therefore standard error represents alterations during qRT-PCR as opposed to between experiments. mRNA expression for eotaxin-1, eotaxin-3 and IP-10 were all affected by OSM stimulation **[Figure 2]**. 6 hour OSM stimulation up-regulated eotaxin-1 and IP-10 mRNA levels however 24 hour OSM stimulation was required to up-regulate eotaxin-3 levels. As seen with MCP-1 and IL-6 mRNA, HASMC lines responded with similar trends however with varying degrees of sensitivity. MCP-1, IL-6 and eotaxin-3 expression in HASMC-A continued to increase at 24 hours however levels in HASMC-J returned back to control by 24 hours with the exception of eotaxin-3. The protein levels for some of these chemokines [eotaxin-1 (also shown by Faffe et al [50]), TARC and IP-10] were also elevated in response to increasing concentrations of OSM **[Figures 10, 12, 14]**. However significant up-regulation in supernatants was only seen in HASMC-M and not in HASMC-J. These findings further propose that HASMC lines from different patients do respond to OSM quantitatively different however with the same qualitative fashion. Furthermore, the production of TARC, eotaxin-1, eotaxin-3 and IP-10 by these cells demonstrates their potential role in eosinophilia, lymphocyte recruitment, Th2 mediated inflammation and airway hyperreactivity [96-98].

Since it has been previously shown that OSM can be expressed by airway macrophages and neutrophils and detected in the sputum of asthmatics [51], it is possible to suggest that the cellular source of OSM in asthma could be the influx of inflammatory

cells that are recruited to the airways upon an asthmatic exacerbation. During an exacerbation, these inflammatory cells could potentially migrate to smooth muscle upon release of chemoattractants from HASMC. These findings can therefore indicate that neutrophils, dendritic cells (DC) and macrophages that are recruited to smooth muscle and secrete OSM (LPS has been shown to regulate OSM production in neutrophils and DC) can augment the pro-inflammatory responses produced by HASMC [90, 99]. In addition, the OSM could potentially induce increased production of mediators often seen in asthma and that have been shown to be produced from HASMC [50]. Lastly, OSM has been shown to modulate neutrophil recruitment and could potentially engage a positive feedback mechanism in smooth muscle where the infiltration of OSM producing cells initiates the production of neutrophil chemoattractants from HASMC, inducing further neutrophil and therefore OSM recruitment [100]. In addition to asthma, OSM has also been implicated in allergic rhinitis and chronic obstructive pulmonary disease (COPD) suggesting its role in a variety of airway inflammatory diseases [101, 102]. Furthermore, HASMC have been shown to be hyperresponsive in COPD suggesting that the implications of OSM on these cells could be important in regulating this severe condition [103].

4.2. Cytokine and Chemokine Production in Response to TLR-ligands

Exacerbations of symptoms of asthma have been associated with both viral infections and bacterial infections [10, 104]. Through the engagement of PRRs, such as TLRs that are expressed on a variety of cells in the lung, these pathogens can induce

innate immune phenotypes [105]. Synthetic TLR ligands can be used as molecular mimics of a variety of pathogens depending on their composition. Previous research has demonstrated that upon stimulation with TLR2, TLR3, and TLR4 agonists, HASMC can produce IL-8, eotaxin-1, IL-6, IP-10 and ICAM-1, all of which have been implicated in asthma pathogenesis [69]. Furthermore, Kuo et al, have identified that upon infection with RV or administration of TLR3-ligand (double stranded RNA that is often produced during the replication of many viruses), asthmatic ASMCs showed increases in deposition of collagen (only seen with TLR3-ligand administration) and fibronectin [68, 106]. In regards to bacterial infections, LPS, a component of the outer membrane of gram negative bacteria, has been found to be associated with the development of atopy in humans [107]. In addition, Chlamydia pneumoniae (gram negative bacteria) has been shown to be present during asthma exacerbations [108]. For these reasons, to obtain a better understanding of the impact of TLR agonists on HASMC and determine if these cells can play a role in asthmatic exacerbations, TLR3 and TLR4 agonists were utilized to challenge HASMC *in vitro*.

Various preparations of Poly I:C were tested in HASMC cultures to determine the optimal form for challenges. A preparation from GE (8kbp) was found to induce the greatest MCP-1 levels in supernatants in comparison to two preparations from Sigma (a short and a long) **[Figure 4A]**. The mechanism of the ability of the GE form of Poly I:C to induce greater IL-6 levels in supernatants is not known, however it could be associated with ligand specificities such as number of modified nucleosides which has been shown

to affect capabilities of TLR3-ligand induced activation [109]. These findings could also demonstrate a reason why different viruses impact asthmatic exacerbation at varying extents. Poly I:C challenge resulted in a dose dependent detection of MCP-1 in HASMC suggesting that stimulation with TLR3 agonists can provoke HASMC to produce monocyte chemotatic agents. Secondly, Poly I:C challenge in three HASMC lines demonstrated, similarly to OSM stimulation, that although these cells respond with the same qualitative trend they do so with varying degrees of sensitivity **[Figure 4B]**. Levels of MCP-4, eotaxin-1, eotaxin-3, TARC, IL-8 and IP-10 protein concentrations were also significantly increased in all three cell lines tested **[Figures 8-14]**. These findings implicate TLR3 challenged HASMC in eosinophilia, neutrophilia., lymphocyte recruitment, activation of mast cells and basophils, Th2 mediated inflammation and airway hyperreactivity [96-98]. mRNA expression for some of the aforementioned cytokines and chemokines were also modulated by Poly I:C at 6 hours of stimulation, however variation in qualitative responses were noted **[Figure 17]**. IP-10 mRNA was up-regulated by Poly I:C in both cell lines implying that TLR3 agonists can induce Th1 responses. Interestingly, IP-10 levels have been found to be increased in asthmatics and can be secreted by HASMC in response to interferons and LPS. Furthermore, its receptor (CXCR3) is expressed on mast cells and eosinophils [105]. These findings suggest that IP-10 secretion by HASMC in response to TLR3 agonists could have potential roles in mast cell and eosinophil recruitment/activation in asthma [105]. Contrary to consistent IP-10 upregulation between cell lines, a variation in eotaxin-1, eotaxin-3, MCP-1 and IL-6 up-regulation was detected in all HASMC lines further suggesting the quantitative

variation between HASMC lines even though a similar qualitative trend was observed. These results could be explained by variation in TLR receptor expression, which will be discussed below.

In regards to LPS challenge, HASMC supernatants also contained dose dependent increases in IL-6 and MCP-1 concentrations **[Figure 5]**. The variation in detectable IL-6 between HASMC lines was greater than the variation seen with TLR3 challenge which may suggest that HASMC have a wider breadth of responses to TLR4 agonists. Interestingly, the HASMC lines with the greatest TLR3 induced IL-6 detection were not the same cell lines that generated supernatants with the greatest IL-6 in response to TLR4 challenge. This suggests that different HASMC lines respond to TLR3 and TLR4 stimulation uniquely. In addition to MCP-1 and IL-6 protein detection, all three cell lines showed significant increases in eotaxin-3 and IP-10 levels in supernatants **[Figures 11 and 14]**. Therefore, similar to TLR3 stimulation, TLR4 challenge may also induce IP-10 induction of mast cell recruitment *in vivo*. However, contrary to TLR3 challenge, eotaxin-3 production was increased in all cell lines (this was not seen with Poly I:C challenge) suggesting that eosinophil and Th2 phenotype mediators are secreted more uniformly between patient HASMC lines in response to LPS. IL-8, MCP-4, eotaxin-1 and TARC were also detected in supernatants in response to LPS, however the qualitative levels were dissimilar between cell lines **[Figures 8-14]**. Taken together, this data further demonstrates the variation of responses in cell lines also observed with both OSM and Poly I:C stimulations. Examining mRNA alterations in response to LPS demonstrated

that the aforementioned cytokine and chemokine mRNA levels were not altered at 6 hours of stimulation **[Figure 17]**. This may indicate that 6 hours is not be the optimal time point to examine mRNA modulation in response to LPS in HASMC, thus examining other time points is be merited in the future. Since protein levels were detected in HASMC supernatants, it is possible that LPS challenge of HASMC lines induces the production of mediators for eosinophila, mast cell and basophil stimulation, and the Th2 phenotype often seen in asthma.

Lastly, it can be noted that the levels of cytokines and chemokines produced in response to LPS were a great deal lower in magnitude than those produced in response to Poly I:C. Similarly to TLR3 agonist challenge, the variation in responses to LPS and Poly I:C could be supported by a variation in TLR expression, which will be discussed later. Furthermore, these two TLRs signal differently since TLR3 is mostly found in endosomes while TLR4 is located on the plasma membrane and requires the formation of complexes with co-factors including, CD14, LPS binding protein and MD2, before initiating downstream signaling [66]. Therefore, the location of the TLRs and the availability of the required co-factor for signaling complexes could also be a reason for the variation of responses to TLR agonist stimulations observed. Based on the above mentioned findings, this data demonstrates that HASMC challenged with TLR3 and TLR4-ligands can produce a variety of pro-inflammatory proteins. In addition, TLR3-ligand challenges induce a greater production of these cytokines and chemokines (with the exception of HASMC-J). This suggests that TLR3 stimulation in most HASMC tested

results in a more robust innate immune response that TLR4 stimulation. This observation supports the suggestion that TLR3 challenge can induce a greater response by HASMC secondary to viral infection in comparison to bacterial infection. These responses could therefore be associated with the predominance of asthma exacerbations associated with viral infections as opposed to bacterial [12].

4.3. Cytokine and Chemokine Production in Response to TLR-ligands and OSM

[Tables 1-3] in the appendix summarizes the regulation of the aforementioned cytokine and chemokine protein and mRNA levels present upon OSM, LPS and Poly I:C stimulations in HASMC. However, examining the alterations that occur when HASMC challenged with TLR agonists are co-stimulated with OSM would aid in claifying the pro-inflammatory role of OSM in HASMC.

To help address this issue, co-stimulations with TLR agonists and OSM were performed in HASMC culture systems *in vitro*. Co-stimulations with OSM and Poly I:C augmented IL-6 detection in all three cell line supernatants in comparison to Poly I:C alone **[Figure 6]**. Although Poly I:C stimulation alone resulted in a variation of IL-6 detection in each cell line, the addition of 0.5ng/ml OSM increased the IL-6 in supernatants to approximately 6000pg/ml in each cell line, which may indicate maximal responses were obtained in the culture system. The addition of OSM in HASMC-J and HASMC-M lines resulted in a synergistic production of IL-6 between Poly I:C and OSM combination. However, an additive response was seen in HASMC-F. Co-stimulation with OSM and LPS in all three cell line supernatants augmented IL-6 detection in comparison

to LPS alone **[Figure 7]**, as seen with Poly I:C. Interestingly, unlike TLR3 challenge, even with the addition of OSM there was still variation in the IL-6 concentrations in the supernatants from the three cell lines. This data suggests that there is greater disparity in LPS stimulation than Poly I:C stimulation in HASMC. However, all three cell lines demonstrate synergistic responses with LPS and OSM co-stimulation. Taken together, these findings indicate that OSM can augment pro-inflammatory responses measured by IL-6 secretion in HASMC challenged with TLR agonists.

To further study the potential role of OSM in viral infections in the lung (specifically the smooth muscle), the levels of an array of cytokines and chemokines were analyzed using HASMC supernatants co-stimulated with OSM and Poly I:C **[Figures 8-14]**. As mentioned previously, Poly I:C dose dependent challenges resulted in a consistent qualitative trend between cell lines where there were increases in detection of MCP-1, MCP-4, IL-6, eotaxin-1, eotaxin-3, TARC, IL-8 and IP-10. In addition to IL-6, the presence of 0.5ng/ml of OSM synergistically amplified MCP-1 and MCP-4 levels in the supernatants (with the exception of HASMCJ). Greater variation between cell lines was identified in co-stimulations, however for the most part a similar qualitative trend was observed. Cytokine and chemokine protein alterations demonstrated a consistent trend of an augmentation of concentrations when OSM was present with the exception of IL-8 which was significantly decreased with the addition of OSM in two cell lines. Previous findings by Richards et al demonstrated that upon stimulation with OSM, IL-1 induced IL-8 was also decreased in lung fibroblasts [110]. Thus, OSM differently affects

IL-8 expression which may be due to altered signaling mechanisms (STATs) or per harps OSM can modulate mRNA stability [110]. Eotaxin-1 and eotaxin-3 alterations were varied between cell lines where in HASMC-J a decrease was observed yet an increase in one of the other cell lines. These findings suggest that HASMC patient lines may respond differently in regards to eotaxin production. TARC and IP-10 levels were only augmented in one cell line implying that similar to eotaxin, variations in responses are produced with co-stimulation. mRNA expression for MCP-1, eotaxin-1, eotaxin-3 and IP-10 were significantly augmented at 6 hours in the presence of OSM in comparison to Poly I:C alone in HASMC-M **[Figure 17]**. However this trend was not observed with IL-6 mRNA expression. Discrepancies between protein and mRNA data could be due to only analyzing 6 hour mRNA expression. Other time points may demonstrate similar trends seen with the protein levels in the supernatants. Lastly, variation in mRNA expression between cell lines further elucidates the variation in sensitivity or response rate in HASMC patient lines. Details of the regulation of proteins and mRNA by Poly I:C and OSM co-stimulation is available in **[Table 3]** in the appendix**.** Taken together these findings indicate that HASMC challenged with TLR3 agonists in the presence of OSM respond with (for the most part) greater levels of cytokines and chemokines involved in the pathogenesis of asthma. Lastly, some of these responses, specifically IL-6, MCP-1 and MCP-4 suggest that OSM and Poly I:C can synergistically induce HASMC responses.

Equivalent experiments were carried out with HASMC lines co-stimulated with OSM and LPS challenge. These were useful in determining if upon bacterial stimulus HASMC respond in the same fashion as in TLR3 stimulation **[Figures 8-14]**. A summary of regulation of protein and mRNA alterations by OSM and LPS in HASMC is available in **[Table 4] in** the appendix**.** As mentioned previously, dose dependent challenges of TLR4 agonist, LPS, resulted in an increased detection of MCP-1, IL-6, eotaxin-3 and IP-10. Similarly to Poly I:C, the addition of 0.5ng/ml OSM resulted in synergistic augmentations of MCP-1 and IL-6 detection in all three cell lines tested. However contrary to Poly I:C , MCP-4, eotaxin-1, eotaxin-3 and TARC consistently demonstrated no changes with co-stimulation in comparison to TLR4-ligand alone. This data suggests that HASMC can elicit a wider range of immune mediator responses upon challenge with Poly I:C in comparison to LPS. Lastly, variation in cell line responses was noted with IL-8 and IP-10 supporting the interpretation that HASMC respond with varying degrees of sensitivity. Similar to TLR3 co-stimulation, mRNA expression for various cytokines and chemokines was also examined. As seen with LPS stimulation alone, 6 hour co-stimulation with OSM did not alter mRNA expression of any of the proteins examined **[Figure 17]**. Consistent with previous suggestions, 6 hours may not be the optimal time point to examine mRNA expression derived from LPS challenged HASMC. As mentioned previously, LPS stimulation in neutrophils results in the production of OSM [51]. Therefore, it could be postulated that during an asthmatic exacerbation due to bacterial stimuli, neutrophils that have already been recruited to the smooth muscle could produce greater amounts of OSM, augmenting the innate immune response in these cells.

It is therefore possible to suggest that the OSM load would continue to increase, creating a positive feedback mechanism where the inflammatory response would become continuously more severe. However, an important question to explore is whether the infection comes before the OSM or whether the OSM before the infection. Based on findings discussed in this thesis as well as with previously published data, it could be proposed that the smooth muscle of asthmatics is recurrently infiltrated with immune cells that do not produce OSM in a dormant (not challenged) state. However upon stimulation with a bacterial, or potentially viral pathogens, the OSM expression is elevated followed by the cycle of augmentation of inflammatory mediators resulting in increased severity of asthmatic symptoms.

The production of type I IFNs by HASMC has yet to be fully determined, however, stimulation of bronchial SMCs with Chlamydia pneumoniae has been shown to produce low levels of IFN-β, signifying its relevance in bacterial respiratory infection [59]. Determining whether type I IFNs can be produced by HASMC in this model was examined by stimulating HASMC with OSM, LPS or Poly I:C as well as with the co-stimulations and investigating for production of IFN-α and IFN-β. ELISAs performed on the supernatants demonstrated that HASMC do not produce detectable IFN-β or IFN-α by ELISA in this model at the level of sensitivity of detection **[Figure 15]**. As mentioned previously, IP-10 can be released by cells in response to IFNs and LPS. However, it could be postulated that in this system HASMC IP-10 production is predominately induced by TLR agonists, since Poly I:C and LPS induced increases of mRNA and

protein levels of IP-10 yet no IFN was detected. IFN bioactivity assays could be completed in the future to confirm or increase the level of sensitivity for IFNs. The apparent lack of type I IFN responses by HASMC is interesting in that TLR agonists induce IFN in other cell types, such as fibroblasts [111]. This may indicate that HASMC do not participate in innate IFN responses typical of other cells.

Although variations can be observed between HASMC lines in response to TLR ligand and OSM stimulation, as well as co-stimulations, these findings still suggest that both TLRs and OSM could potentiate HASMC contribution to asthma and asthma exacerbations by fueling the production of mediators for various cytokines and chemokines. The above findings identify that IL-6, MCP-1, MCP-4, eotaxin (1/3), and IP-10 can be secreted by HASMC upon certain stimuli implicating smooth muscle products in contributing to infiltration of inflammatory cells, such as monocytes, dendritic cells, mast cells, basophils, Th2 cells, and eosinophils.

4.4. HASMC responses to PBMC conditioned media in the presence of OSM antibodies

Various pro-inflammatory cytokines can regulate HASMC *in vitro*. Determining the relative contribution of OSM among a milieu of cytokines may help identify the significance of OSM in such a milieu. **[Figure 16 A]** demonstrates that PBMC challenged with LPS can elicit an extensive pro-inflammatory response confirmed by the detection of IL-6, OSM, IFN-γ, IL-10, IL-12p70, IL-13, IL-2, IL-5, IL-4, IL-8, TNF-α and IL-1β in conditioned medium (data not shown). When assessing IL-6 as a readout for

responses to PBMC conditioned medium by HASMC, upon the addition of an antibody to the OSMRB (1.3ng/ml), IL-6 concentrations were significantly lowered in comparison to media control as well as a mouse IgG control antibody **[Figure 16 B]**. Thus inhibition of the OSMRβ on HASMC decreased responses to an inflammatory cytokine milieu. On the other hand, neutralization to the OSM ligand did not significantly reduce the IL-6 levels when assessed similarly. An explanation for this could be that the antibody to the OSM ligand is not effective. The antibody (R&D systems) was derived against E.coli human OSM, and may not neutralize mammalian expressed OSM efficiently. Since the antibody to the OSMRβ significantly decreased IL-6 detection in HASMC supernatants it can be proposed that in this system that OSM signaling occurs largely through the OSMR complex which is consistent with the lack of LIF modulation of HASMC responses **[Figure 3].** Using an antibody for the LIFR followed by IL-6 detection in supernatants would be helpful to further confirm that in the absence of OSM (due to receptor inhibition), IL-6 levels in HASMC supernatants is significantly decreased. These findings suggest that inhibiting the specific OSMR complex can modify HASMC responses.

4.5. Modulation of ECM Modulators in Response to TLR-ligands and OSM

In addition to their physical properties, ASMCs have been implicated in a variety of inflammatory and immunomodulatory responses. ASM has been shown to secrete various cytokines, chemokines, cell adhesion molecules , and ECM proteins, all of which may contribute to the pathogenesis of asthma [112]. ECM remodeling is a key characteristic of asthma and progresses over time due to a variety of factors such as

deposition of components of the ECM (collagens and fibronection), migration and proliferation of smooth muscle, growth factors, MMPs and TIMPS [2]. The regulation of these factors by OSM and TLR-ligands may contribute to the smooth muscle cell phenotype in asthma.

[Figure 18] demonstrated that upon OSM stimulation, a dose dependent induction of VEGF can be detected in HASMC supernatants, as shown previously by Faffe et al [19]. mRNA expression of VEGF was also significantly elevated in two cell lines by 6 hours of OSM stimulation **[Figure 19].** These findings implicate HASMC and OSM in ECM remodeling likely produced by the angiogenesis factor, VEGF, as it has been shown to be up-regulated in asthmatic airways [19]. HASMC stimulated with Poly I:C or LPS slightly inhibited VEGF levels detected in supernatants. Interestingly, as the levels of Poly I:C increased in the OSM co-stimulation, VEGF levels were decreased. These findings, as well as the decrease in VEGF with Poly I:C alone could suggest that Poly I:C actually inhibits VEGF production by HASMC. This trend was not seen with the co-stimulation with LPS and OSM. Ma and colleagues, were also able to identify that in mice, Poly I:C stimulation inhibited VEGF induced tissue responses, such as angiogenesis, and Th2 inflammation [106] . However this effect was TLR3 independent and instead occurred via the RLG-like helicase (RLH) pathway [106]. Therefore, it could be proposed that in the system used for this thesis, the inhibition of VEGF by Poly I:C could be occurring through a non-TLR dependent pathway such as RLH, Ras, MAPK or IFN pathways or via VEGF receptor regulation by Poly I:C [106]. Investigation of these

pathways and receptors would be useful in pin-pointing the effect of Poly I:C on VEGF inhibition.

Fritz et al, have published that intratracheal administration of OSM using adenoviral constructs in mice increases parenchymal collagen and smooth muscle actin accumulation [27]. Furthermore, unpublished data produced in the laboratory has suggested that OSM can decrease mRNA levels of elastin in total lung RNA preparations. **[Figure 19]** illustrates that the mRNA expression for elastin was decreased by OSM, in HASMC in culture, however collagen and smooth muscle actin was increased after stimulation with OSM for 6 and 24 hours. However, these findings were only present in one cell lines. To confirm this observation additional patient cell lines would need to be examined. If confirmed, this would indicate an OSM induced alteration in the type of ECM produced by HASMC, with less elastin and more collagen in the matrix.

VEGF, collagen 1A1, smooth muscle actin and elastin mRNA expression was also determined for HASMC stimulated with TLR3 and TLR4 agonists in the absence or presence of OSM for 6 hours. The rationalization for analyzing collagen 1A1 instead of other collagens was due to previous findings that showed that ASMCs from asthmatics can produce more collagen type I and fibronectin than those of non asthmatics [31]. **[Figure 20]** demonstrates that stimulation with Poly I:C and LPS alone did not modulate mRNA expression for these factors in more than one cell line. Similarly to OSM stimulation, the role of TLR agonists on collagen, elastin, VEGF and smooth muscle actin mRNA requires additional patient cell line analysis to be better clarified. OSM and

TLR-ligand co-stimulations elicited similar results where variation in patient line responses was noted. Overall, these findings do not definitively implicate OSM or TLR agonists in the alteration of any of these ECM modulators in HASMC with the exception of VEGF.

MMPs and TIMPs are essential in the balance between ECM deposition and degradation, and therefore are associated with ECM remodeling [33]. HASMC stimulated with OSM, Poly I:C and LPS did not alter the detection of MMPs (MMP-1, MMP-2, MMP-3, MMP-10) in supernatants **[Figure 21].** Furthermore, the addition of OSM to the TLR3 and TLR4 agonists did not modulate MMP detection in comparison to the ligands alone. These findings therefore suggest that HASMC stimulated with OSM or TLR agonists tested, do not particiapte in alterations of ECM catabolic enzymes, however analysis of TIMP protein levels would be interesting to determine if HASMC contribute to ECM remodeling through TIMP expression.

4.6. Receptor Modulation in Response to TLR-ligands and OSM

Cytokines such as TNF-α, IL-1β and IFN-γ, have the potential to up-regulate the expression of TLRs in ASMCs , resulting in increased concentrations of cytokines, chemokines and growth factors released from these cells [20]. In addition, results from this thesis have implicated OSM in the augmentation of responses by TLR3 and TLR4 agonists. Taken together these findings propose a rationale for determining if OSM can modulate TLR/OSMRB expression in HASMC. HASMC lines stimulated with OSM for 6 and 24 hours demonstrated that TLR3, CD14 and OSMRB expression can be up-

regulated secondary to OSM stimulation **[Figure 22]**. However this trend was only seen in two out of the three cell lines tested. TLR4 expression was also increased in HASMC-J and HASMC-M **[Figure 23]**, yet OSM induced TLR4 mRNA expression appeared to decrease in HASMC-A. The variance in TLR expression in response to OSM between cell lines could provide a reason for differences in cytokine/chemokine secretion in response to TLR agonists. Of interesting note, TLR3 expression was for the most part greater than TLR4 expression (also shown previously by Sukkar et al [69]) providing potential justification for higher pro-inflammatory protein levels detected in HASMC supernatants stimulated with Poly I:C in comparison to LPS. The up-regulation of TLR3 or TLR4 could therefore be proposed as a mechanism of action in regards to increased cytokine and chemokine production with TLR agonists and OSM co-stimulation.

Based on the findings mentioned above regarding CD14 up-regulation in response to OSM, preliminary experiments were conducted to determine if soluble CD14 could be detected in HASMC supernatants stimulated with OSM. Overall, the observation that soluble CD14 was not detected, nor was exogenous soluble CD14 able to potentiate LPS responses (data not shown) indicated that the CD14 required for TLR4 signaling (specifically LPS) is most likely membrane bound as opposed to the soluble form in HASMC. Flow cytometry was utilized in order to compare protein levels to mRNA expression levels of TLRs and the co-receptor CD14. Flow cytometry for TLR4 could not be performed due to lack of effective antibody. Human TLR4 antibody was found to not be specific for TLR4 when examining protein levels with a flow cytometer. Therefore,

mRNA expression of TLR4 could not be correlated with cell surface protein levels. Collectively, results by FACS showed that CD14 and TLR3 expression is increased upon OSM stimulation in certain HASMC lines **[Figures 24-26]**. This could indicate a potential mechanism of action for higher cytokine/chemokine responses upon co-stimulation. Furthermore, the variation between cell lines may explain the trend that patient HASMC lines respond *in vitro* to OSM and TLR agonists with varying degrees of sensitivity and response rate. In addition, **[Figure 27]** demonstrates that CD14 expression in HASMC is heterogeneous and that OSM up-regulates the percentage of CD14+ cells by 6 hours of stimulation. As mentioned earlier, ASMCs have both structural and immunological roles in asthma [16]. CD14 heterogeneity could imply that only subpopulations in the smooth muscle population can respond to TLR4 agonists. Furthermore, this data could indicate that the cells present in the CD14+ population are more prone to TLR up-regulation or that other HASMC could be recruited into this subpopulation upon OSM stimulation. In regards to asthma exacerbations in response to bacterial infections, one could propose that production of OSM by infiltrating neutrophils and macrophages could up-regulate CD14 expression on HASMC and therefore form more TLR4 signaling complexes. This could result in greater TLR4 functionality in HASMC and thus potentiate enhanced immune responses such as the production of chemotactic agents implicated in asthma pathogenesis.

TLR and OSMRβ mRNA expression was also examined in HASMC stimulated with Poly I:C and LPS in the absence or presence of OSM **[Figure 23]**. As shown

previously by Sukkar et al [69], Poly I:C induces up-regulation of TLR3 (as seen in HASMC-M and HASMC-J). However, the addition of OSM did not augment the expression even though OSM alone induced TLR3 increases **[Figure 22]**. It is possible that TLR3 expression was at maximum capacity in this system and that co-stimulation cannot further induce this expression. Poly I:C also increased mRNA expression of CD14 (two cell lines) and TLR4 (one cell line). Interestingly, in both cell lines tested CD14 expression was augmented by co-stimulation with Poly I:C and OSM. These findings could be further assessed by stimulating HASMC with Poly I:C and OSM followed by LPS challenge to determine if an even greater IL-6 response can be induced by these cells. In regards to LPS challenged HASMC, TLR3, CD14 and TLR4 mRNA expression was not modulated. However, LPS and OSM co-stimulation significantly enhanced CD14 expression in HASMC. These findings, similar to Poly I:C and OSM co-stimulation, provide a potential mechanism of action for OSM augmented cytokine and chemokine production in response to TLR agonist and OSM co-stimulations. OSMRB expression was not altered by Poly I:C or LPS in this model, suggesting that synergy was not due to TLR agonist upregulation of the OSMR complex.

Since it has been observed that OSM can up-regulate TLR3, TLR4 and CD14 expression in HASMC lines, determining if OSM priming to maximize receptor expression can elicit the same response as co-stimulation became a question of interest in this project. **[Figure 28]** illustrates that Poly I:C and LPS challenged cells that received the 6 hour OSM prime showed a significant increase in IL-6 levels in supernatants,

implying an enhanced response. However, Poly I:C and LPS co-stimulated cells that did not receive the OSM prime showed the same levels of IL-6 in supernatants. Overall, these findings indicate that in this model, OSM priming for 6 hours was sufficient to induce an augmented IL-6 response by TLR agonists. In regards to asthma, these findings implicate that the OSM load present in smooth muscle upon release by immune cells such as neutrophils, does not need to be present for a prolonged amount of time to elicit an enhanced response.

4.7. Signaling Pathways Induced by TLR-ligands and OSM

The JAK-STAT pathway has been identified in the expression of VEGF and eotaxin in HASMC in response to OSM [19, 50]. In addition, the gp130 family of cytokines (including OSM) has been shown to signal through the activation of JAK 1, JAK2 and TYK2 and by recruiting STAT3 [38, 43, 49]. In the present studies, HASMC stimulated with OSM resulted in phosphorylation of STAT3, STAT5 and STAT6 **[Figure 29]**. TLR agonists alone did not activate STAT3, STAT5 or STAT6. Overall, these findings implicate STAT3, STAT5 and STAT6 in the production of various cytokines and chemokines in HASMC in response to OSM combinations with TLR agonists, but not TLR agonists alone. Of interesting note, analysis of STAT5 activation in the presence of TLR –ligands and OSM resulted in a greater signal than OSM alone, suggesting that STAT5 phosphorylation may be synergistically induced by the co-stimulations, although this would need to be confirmed with quantitative western blots. If confirmed, this could potentially implicate STAT5 in the synergistic response observed for the cytokines and

chemokines summarized in **[Tables 2 and 3]** upon TLR-ligands and OSM co-stimulation. Future experiments to examine STAT5 requirement in these responses could include siRNA approaches. Activation of the MyD88 pathway or NFκB pathway (discussed below) by TLR-ligands could induce the production of various cytokines and chemokines. At the same time, OSM binding and signaling via the JAK-STAT pathway could enhance the production of these cytokines and chemokines. Examining the role of the MAPK pathway, specifically ERK and JNK, in this system would be helpful to further identify the signaling cascades occurring with OSM and TLR-ligand co-stimulations.

In cells that can produce cytokines and chemokines through NFκB signaling, a variety of stimuli result in the activation of NFκB through the phosphorylation of p65 and the degradation of IκB-α, allowing the activated NFκB to then translocate to the nucleus and induce gene expression [113, 114] . ASMCs have been correlated with the NFκB signaling cascade in regards to the production of IL-8, TGF-β, ICAM-1 and VCAM [16]. In this project, HASMC stimulated with OSM and TLR agonists were examined by Western blotting to identify NFκB activation through p65 phosphorylation and IκB-α degradation. The phosphorylation of the NFκB subunit p65 has been used to determine NFκB activation, as shown by Aul et al, where LPS induced inflammation in smokers lungs was associated with phosphorylation of p65, suggesting NFκB activation [115]. In the present study, p65 was phosphorylated at both 20 minutes and 2 hours in HASMC stimulated with OSM, Poly I:C and LPS, with the exception of the control and OSM

stimulated cells at 2 hours. However the lack of signal with the control and OSM at 2 hours was only seen in one cell line, identifying the need for further cell line examination to determine NFκB regulation via p65 phosphorylation in HASMC. Currently, p65 activation in all stimulations suggests that NFκB is activated in HASMC, even with no challenge shown by the control signal. On the other hand, the NFκB inhibitor, IκB-α, was degraded in the cell lysates stimulated with OSM and TLR-ligand co-stimulations at 2 hours but not at 20 minutes or in the other challenges. These findings suggest that NFκB activation through IκB-α degradation occurs only in the co-stimulation. This indicates a potential signaling mechanism that could result in the increased cytokine and chemokine proteins detection seen in HASMC supernatants after co-stimulation. A possible explanation for this degradation could be the amount of IκB-α mRNA present in the co-stimulated cells, to identify if the co-stimulation results in an alteration of the NFκB expression RT-PCR at 2 hours could be used. If this finding demonstrate that the IκB expression is equivalent to that in the other stimulations it could be suggested that the degradation of IκB could be induced by the co-stimulations. If this is confirmed a potential mechanism of this could be due to the increased expression of TLRs in cells stimulated with OSM which would allow greater TLR downstream signaling and thus NFκB activation.

-CHAPTER 5-

CONCLUSION

ASM has been shown to affect not only the airway caliber and structural integrity of the lung, but can also contribute to the severity of asthma through its ability to participate in airway inflammation. In addition to immunomodulatory and bronchial hyperresponsive roles, ASM can also induce ECM remodeling in the airways through ASM migration, proliferation and production of ECM modulators. Viral and bacterial infections have been shown to be associated with asthma exacerbations which result in increased AHR and therefore severity of symptoms. Furthermore, asthma exacerbations can accelerate the ECM remodeling seen in asthmatic airways. Previous findings have implicated the gp130 cytokine OSM in the production of VEGF and pro-inflammatory mediators by HASMC. Using an *in vitro* model of asthma exacerbations, this thesis explored the functions of OSM in TLR agonist challenged HASMC *in vitro*.

Since it has been previously shown that OSM is expressed by airway macrophages and neutrophils as well as detected in the sputum of asthmatics, it is plausible that the cellular source of OSM in asthma could be the influx of inflammatory cells that are recruited to the airways upon an asthmatic exacerbation. These cells could potentially migrate to smooth muscle where they secrete OSM in response to a variety of

stimuli, one of which is LPS, and potentially augment the production of pro-inflammatory mediators produced by HASMC. Experiments involving HASMC stimulated with OSM in culture were able to demonstrate that OSM can induce dose dependent increases in IL-6, MCP-1, eotaxin-1, and VEGF levels in supernatants. The initiation of the production of these mediators was found to be mostly associated with the OSMR complex since upon inhibition of the OSMRβ with an antibody; IL-6 responses were significantly decreased in supernatants of HASMC challenged with PBMC CM. Furthermore, challenges with TLR3 agonist, Poly I:C, mimicking effects of viral infections could induce the secretion of IL-6, MCP-1, eotaxin-1, eotaxin-3, TARC, IL-8 and IP-10 from these cells. LPS stimulation, a TLR4 bacterial agonist, could elicit similar responses, however with much lower responses for MCP-1, IL-6, eotaxin-3 and IP-10 secretion. Interestingly, co-stimulation with OSM and both ligands resulted in synergistic increases in MCP-1 and IL-6 detection in supernatants, while MCP-4 was also synergistically increased in response to Poly I:C co-stimulation. These findings, as well as additive responses observed with other chemokines upon co-stimulation, implicate OSM and TLR-ligands in a variety of immunomodulatory responses often seen in the pathogenesis of asthma. Analysis of signaling cascades suggest that these responses could be occurring through the activation of STAT3, STAT5 and STAT6. In order to better understand the signaling occurring in this system, investigation of the MAPK pathway as well as the use of quantitative western blots would be useful.

In regards to ECM remodeling in this system, OSM induced VEGF production at both the protein and mRNA levels. However collagen, smooth muscle actin and elastin responses were less clear. Interestingly, VEGF levels were inhibited by TLR ligands, specifically Poly I:C. It could be proposed that this occurs through a TLR3 independent signaling pathway or the regulation of the VEGF receptor. Other ECM modulators such as MMPs were found to not be altered by OSM or the TLR-ligands tested suggesting that they are not involved in the catabolic initiation of ECM degradation. In order to further identify the role of OSM and TLR-ligands on HASMC ECM remodeling, the expression of TIMPs could be examined to determine modulation of ECM deposition.

Of interesting note, HASMC lines responded to these stimulations with the same qualitative trend however at varying degrees of sensitivity, evident by cytokine and chemokine concentrations as well as mRNA expression. Examination of TLR expression in HASMC, at both the mRNA and protein levels, identified that variation in TLR expression was noted between cell lines. This findings could explain why some HASMC lines respond to Poly I:C or LPS with a greater production of cytokines and chemokines. Furthermore, mRNA expression of TLR3 was found to be greater in these cells in comparison to TLR4 expression, with the exception of one cell line. This data are consistent with the findings that Poly I:C challenge resulted in higher levels of cytokines and chemokines in supernatants, which could be explained by TLR3 expression levels. Interestingly, the cell line that produced the highest levels of IL-6 in response to LPS had

the greatest TLR4 mRNA expression, further demonstrating that HASMC line variation in response could be associated with TLR expression.

Upon OSM stimulation, TLR3, TLR4 and CD14 expression was found to be up-regulated; suggesting that OSM co-stimulation with TLR-ligands could result in greater cytokine and chemokine secretion by HASMC in response to increased receptor availability and function. It is well known that LPS requires a TLR4 complex (consisting of CD14, LPS binding protein and MD2) to bind and cause downstream signaling. CD14 expression in HASMC was found to be heterogeneous and co-stimulations with OSM and TLR-ligands significantly increased CD14 mRNA expression. These findings suggest that by increasing the availability of CD14, OSM can induce greater TLR4 functionality and therefore higher levels of cytokine and chemokines responses. Furthermore, it is possible that only a certain population of HASMC can respond to LPS, of which this population is increased with OSM stimulation. Lastly, OSM priming of HASMC demonstrated that these cells do not require consistent OSM fueling to initiate these co-stimulation responses.

Based on the findings mentioned in this thesis, it could be hypothesized that during an asthmatic exacerbation due to pathogens, neutrophils and other immune cells that have been recruited to the smooth muscle could contribute to increased levels of OSM, augmenting the innate immune response in these cells. It is therefore possible that the OSM load would continue to increase since the enhancement of chemotactic agents would result in further cellular infiltration and thus OSM availability. This could create a

positive feedback mechanism where the inflammatory response would continuously progress. It could be proposed that the smooth muscle and airways of asthmatics is constantly infiltrated with immune cells that do not produce OSM in a dormant not challenged state. However upon stimulation with a bacterial or viral, pathogen the OSM production is set in motion followed by the cycle of augmentation of immune mediators resulting in increased severity of asthmatic symptoms. Through the activation and signaling cascades of the JAK-STAT, specifically STAT3, STAT5, STAT6, and NFκB pathways, HASMC could participate in the initiation of an asthmatic allergic response through the recruitment of eosinophils, neutrophils, monocytes, lymphocytes, basophils and mast cells in the airways. In addition, mast cells activation and generation of histamine could be influenced by HASMC secretion of allergic mediators and OSM and TLR-ligand induction of responses by HASMC could be implicated in airway hyperreactivity and the development of a Th2 phenotype often seen in asthma. Lastly, enhanced NFκB activation with OSM and TLR-ligand co-stimulations, as seen with IκB-α degradation could increase the inflammatory responses mentioned above.

Overall, these findings implicate OSM in the augmentation of responses to TLR-ligands in HASMCs. A list of concluding remarks regarding experiments performed in this thesis can be found in **[Table 5].** Pin-pointing the implications of OSM on HASMC in an *in vitro* model of asthma exacerbations could aid in potential therapeutic targets for asthma and asthma exacerbations. Furthermore, OSM has also been implicated in allergic rhinitis and COPD suggesting its role in a variety of airway inflammatory diseases.

-CHAPTER 6-

APPENDIX

TABLE 1.

Human airway smooth muscle cell patient characteristics

Cell Line/ Date of Isolation	Age	Diagnosis	Medication	Smoker (Y/N)	FEV1/ L FEV1/VC %
HASMC-M March 11/2010	80	Mild COPD	-	-	2.42 61%
HASMC-J June 24/2010	75	COPD Adenocarcinoma	Eltroxin, Micardis, Hydroclorothiazide, vitalux, ventolin, advair, spiriva, vitamin D, calcium, glucosamine	YES	1.31 51%
HASMC-F February 23/2010	69	Adenocarcinoma with BAC component	Atenolol, losec, vitalux, clonazepam, lorazepam, calcium, vitamins	NO	1.9 75%
HASMC-A April 5/2005	82	Stroke Mild COPD Emphysema Squamous cell carcinoma	Apro-Warfin, ASA, digoxin, Lipitor, effexor, metoprol, furosemide, glucosamine	YES	- -

TABLE 2.

Cytokine and Chemokine production in HASMC in response to OSM

	HASMC-A		HASMC-J		HASMC-M	
	Increase/Decrease	Conc./Time	Increase/Decrease	Conc./Time	Increase/Decrease	Conc./Time
MCP-1						
Protein	N/A	N/A	Increased	0.5ng/ml	Increased	0.5ng/ml
mRNA	Increased	6 Hour	Increased	6 Hour	No Change	N/A
MCP-4						
Protein	N/A	N/A	No Change	N/A	No Change	N/A
mRNA	N/A	N/A	N/A	N/A	No Change	N/A
IL-6						
Protein	N/A	N/A	Increased	2ng/ml	Increased	2ng/ml
mRNA	Increased	6 Hour	Increased	6 Hour	N/A	N/A
Eotaxin-1						
Protein	N/A	N/A	No Change	N/A	Increased	0.5ng/ml
mRNA	Increased	6 Hour	Increased	6 Hour	No Change	N/A
Eotaxin-3						
Protein	N/A	N/A	No Change	N/A	No Change	N/A
mRNA	Increased	24 Hour	Increased	24 Hour	No Change	N/A
TARC						
Protein	N/A	N/A	No Change	N/A	Increased	0.5ng/ml
mRNA	N/A	N/A	N/A	N/A	N/A	N/A
IL-8						
Protein	N/A	N/A	No Change	N/A	No Change	N/A
mRNA	N/A	N/A	N/A	N/A	N/A	N/A
IP-10						
Protein	N/A	N/A	No Change	N/A	Increased	2ng/ml
mRNA	Increased	6 Hour	Increased	6 Hour	No Change	N/A

N/A= Data not available

TABLE 3.

Cytokine and Chemokine production in HASMC in response to Poly I:C and OSM

	HASMC-A		HASMC-J		HASMC-M	
	Increase/Decrease	Additive/ Synergistic	Increase/Decrease	Additive/ Synergistic	Increase/Decrease	Additive/ Synergistic
MCP-1						
Protein- Poly I:C	Increased	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	Increased by OSM	Synergistic	Increased by OSM	Additive	Increased by OSM	Synergistic
mRNA- Poly I:C	N/A	N/A	No Change	N/A	Increased	N/A
mRNA- Poly I:C + OSM	N/A	N/A	No Change by OSM	N/A	Increased by OSM	Synergistic
MCP-4						
Protein- Poly I:C	Increased	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	Increased by OSM	Synergistic	No Change by OSM	N/A	Increased by OSM	Synergistic
mRNA- Poly I:C	N/A	N/A	N/A	N/A	N/A	N/A
mRNA- Poly I:C + OSM	N/A	N/A	N/A	N/A	N/A	N/A
IL-6						
Protein- Poly I:C	N/A	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	N/A	N/A	Increased by OSM	Synergistic	Increased by OSM	Synergistic
mRNA- Poly I:C	N/A	N/A	No Change	N/A	Increased	N/A
mRNA- Poly I:C + OSM	N/A	N/A	No Change by OSM	N/A	No Change by OSM	N/A
Eotaxin-1						
Protein- Poly I:C	Increased	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	Increased by OSM	Synergistic	Decreased by OSM	N/A	No Change by OSM	N/A
mRNA- Poly I:C	N/A	N/A	Increased	N/A	No Change	N/A
mRNA- Poly I:C + OSM	N/A	N/A	No Change by OSM	N/A	Increased by OSM	Additive
Eotaxin-3						
Protein- Poly I:C	N/A	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	N/A	N/A	Decreased by OSM	N/A	Increased by OSM	Additive
mRNA- Poly I:C	N/A	N/A	Increased	N/A	No Change	N/A
mRNA- Poly I:C + OSM	N/A	N/A	No Change by OSM	N/A	Increased by OSM	Synergistic
TARC						
Protein- Poly I:C	Increased	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	Increased by OSM	Synergistic	No Change by OSM	N/A	No Change by OSM	N/A
mRNA- Poly I:C	N/A	N/A	N/A	N/A	N/A	N/A
mRNA- Poly I:C + OSM	N/A	N/A	N/A	N/A	N/A	N/A

IL-8						
Protein- Poly I:C	Increased	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	Decreased by OSM	N/A	Decreased by OSM	N/A	Increased by OSM	Synergistic
mRNA- Poly I:C	N/A	N/A	N/A	N/A	N/A	N/A
mRNA- Poly I:C + OSM	N/A	N/A	N/A	N/A	N/A	N/A
IP-10						
Protein- Poly I:C	Increased	N/A	Increased	N/A	Increased	N/A
Protein- Poly I:C + OSM	No Change by OSM	N/A	No Change by OSM	N/A	Increased by OSM	Additive
mRNA- Poly I:C	N/A	N/A	Increased	N/A	Increased	N/A
mRNA- Poly I:C + OSM	N/A	N/A	No Change by OSM	N/A	Increased by OSM	Synergistic

N/A= Data not available

TABLE 4.

Cytokine and Chemokine production in HASMC in response to LPS and OSM

	HASMC-A		HASMC-J		HASMC-M	
	Increase/Decrease	Additive/ Synergistic	Increase/Decrease	Additive/ Synergistic	Increase/Decrease	Additive/ Synergistic
MCP-1						
Protein- LPS	Increased	N/A	Increased	N/A	Increased	N/A
Protein- LPS + OSM	Increased by OSM	Synergistic	Increased by OSM	Synergistic	Increased by OSM	Additive
mRNA- LPS	N/A	N/A	No Change	N/A	No Change	N/A
mRNA- LPS + OSM	N/A	N/A	No Change by OSM	N/A	No Change by OSM	N/A
MCP-4						
Protein- LPS	No Change	N/A	Increased	N/A	No Change	N/A
Protein- LPS + OSM	No Change by OSM	N/A	No Change by OSM	N/A	No Change by OSM	N/A
mRNA- LPS	N/A	N/A	N/A	N/A	N/A	N/A
mRNA- LPS + OSM	N/A	N/A	N/A	N/A	N/A	N/A
IL-6						
Protein- LPS	N/A	N/A	Increased	N/A	Increased	N/A
Protein- LPS + OSM	N/A	N/A	Increased by OSM	Synergistic	Increased by OSM	Synergistic
mRNA- LPS	N/A	N/A	No Change	N/A	No Change	N/A
mRNA- LPS + OSM	N/A	N/A	No Change by OSM	N/A	No Change by OSM	N/A
Eotaxin-1						
Protein- LPS	No Change	N/A	Increased	N/A	No Change	N/A
Protein- LPS + OSM	No Change by OSM	N/A	No Change by OSM	N/A	No Change by OSM	N/A
mRNA- LPS	N/A	N/A	No Change	N/A	No Change	N/A
mRNA- LPS + OSM	N/A	N/A	No Change by OSM	N/A	Increased by OSM	Synergistic
Eotaxin-3						
Protein- LPS	N/A	N/A	Increased	N/A	Increased	N/A
Protein- LPS + OSM	N/A	N/A	No Change by OSM	N/A	No Change by OSM	N/A
mRNA- LPS	N/A	N/A	No Change	N/A	No Change	N/A
mRNA- LPS + OSM	N/A	N/A	No Change by OSM	N/A	Increased by OSM	Synergistic
TARC						
Protein- LPS	No Change	N/A	Increased	N/A	No Change	N/A
Protein- LPS + OSM	No Change by OSM	N/A	No Change by OSM	N/A	No Change by OSM	N/A
mRNA- LPS	N/A	N/A	N/A	N/A	N/A	N/A
mRNA- LPS + OSM	N/A	N/A	N/A	N/A	N/A	N/A

IL-8						
Protein-LPS	Increased	N/A	Increased	N/A	No Change	N/A
Protein-LPS + OSM	Decreased by OSM	N/A	No Change by OSM	N/A	No Change by OSM	N/A
mRNA-LPS	N/A	N/A	N/A	N/A	N/A	N/A
mRNA-LPS + OSM	N/A	N/A	N/A	N/A	N/A	N/A
IP-10						
Protein-LPS	Increased	N/A	Increased	N/A	Increased	N/A
Protein-LPS + OSM	Increased by OSM	Additive	No Change by OSM	N/A	No Change by OSM	N/A
mRNA-LPS	N/A	N/A	No Change	N/A	No Change	N/A
mRNA-LPS + OSM	N/A	N/A	No Change by OSM	N/A	No Change by OSM	N/A

N/A= Data not available

TABLE 5.

-LIST OF CONCLUSIONS-

1. OSM stimulation, in comparison to IL-11, IL-31, and LIF, resulted in a greater IL-6 response in supernatants when cells were co-stimulated with OSM and TLR-ligands. . Priming HASMC with OSM for 6 hours was sufficient to induce an augmented IL-6 response by TLR-ligands and OSM.
2. HASMC lines stimulated with increasing concentrations of OSM resulted in a dose dependent increase in IL-6, MCP-1, VEGF, eotaxin-1, TARC and IP-10 levels in supernatants. IL-6, MCP-1, eotaxin-1, eotaxin-3, IP-10 and VEGF mRNA levels were also elevated at 6 h.
3. HASMC lines challenged with Poly I:C resulted in increased levels of IL-6, MCP-1, MCP-4, eotaxin-1, eotaxin-3, TARC, IL-8 and IP-10 in supernatants. mRNA expression at 6 hours demonstrated increases in IP-10, eotaxin-1, eotaxin-3, MCP-1 and IL-6 levels.
4. HASMC lines challenged with LPS resulted in increased levels of IL-6, MCP-1, eotaxin-3 and IP-10 in supernatants. mRNA expression at 6 hours did not reveal any alterations in these cytokines and chemokines suggesting it was not the optimal time point.
5. In response to OSM, Poly I:C and LPS, HASMC lines responded with a similar quantitative trend however with varying degrees of sensitivity. Greater variations in responses were seen with HASMC challenged with LPS in comparison to Poly

I:C. This is supported by the TLR mRNA expression observed, where HASMC express greater TLR3 than TLR4 expression, with the exception of HASMC-J which has higher TLR4 expression and LPS induced responses.

6. Co-stimulations with OSM and Poly I:C augmented IL-6, MCP-1, and MCP-4 levels in supernatants in comparison to Poly I:C alone. Some of these responses were synergistic while others were additive. mRNA expression for MCP-1,eotaxin-1, eotaxin-3, and IP-10 were significantly up-regulated with co-stimulation.
7. Co-stimulation with OSM and LPS augmented IL-6 and MCP-1 levels in supernatants in comparison to LPS alone. Some of these responses were synergistic while others were additive. mRNA expression was not analyzed at an optimal time point since no alterations were noted.
8. IFN-α and IFN-β could not be detected in HASMC supernatants in response to OSM or TLR-ligands.
9. HASMC challenged with PBMC conditioned media elicited an IL-6 response which could be significantly decreased with the addition of an antibody to the OSMR. This findings as well as the lack of LIF modulation on HASMC suggests that OSM signals through the OSMR in these experiments.
10. Modulation of collagen 1A1, smooth muscle actin, elastin and MMPs by OSM or TLR-ligands was not seen in HASMC lines.

11. OSM stimulation increased TLR3, TLR4, CD14 and OSMRβ mRNA expression. TLR3 and CD14 cell surface protein expression detected by flow cytometry was also elevated in response to OSM.

12. HASMC use membrane bound CD14 for LPS signaling and CD14 expression is a heterogenous subpopulation of HASMC. In response to OSM, this subpopulation could be increased and CD14 mRNA expression was augmented with OSM and TLR-ligand co-stimulations. Cell lines with greater CD14 expressing cells responded with higher responses to LPS stimulation.

13. HASMC stimulated with OSM activated STAT3, STAT5 and STAT6 through phosphorylation. NFκB activation could be seen with TLR-ligand and OSM co-stimulations through IκB-α degradation.

-CHAPTER 7-

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