ONCOSTATIN M REGULATION OF HASMC AND ITS EXPRESSION BY PBMC

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ABSTRACT

Asthma is a complex respiratory condition that has markedly increased in prevalence over the past 5 decades. It is classified as a chronic inflammatory disease of the airways due to overzealous immunological pathways resulting in increased immune cell infiltration, changes in the extracellular matrix/airway structural integrity and airway hyper-responsiveness. Commonly, persons with asthma experience exacerbations associated with bacterial and/or viral infections that significantly increase the morbidity and rate of hospitalization of individuals with this condition. The mechanisms that cause these exacerbations are not yet clear. It is postulated here that Oncostatin M (OSM) is released by macrophage cells in response to viral/bacterial products and contributes to the pathology in exacerbations through stimulating human airway smooth muscle cell (HASMC) pro-inflammatory responses, modulation of extracellular matrix (ECM) protein expression and HASMC proliferation.

The findings of this thesis showed that OSM indeed could stimulate HASMC production of various cytokines including: IL-6, monocyte chemotactic protein (MCP)-1, MCP-3, Eotaxin-1, Eotaxin-3, IL-8 and vascular endothelial growth factor (VEGF). Furthermore, OSM could act in a synergistic manner with IL-4, IL-13 or IL-17A in the selective expression of: IL-6, MCP-1, Eotaxin-1, Eotaxin-3, IL-8 and VEGF by cultured HASMC. Elevation in MCP-1 and IL-6 expression was sensitive to p38 and STAT inhibition indicating the engagement of these signaling pathways. OSM did not consistently modulate changes in ECM remodeling proteins or proliferation of these cells *in vitro*.

The findings also demonstrate that toll-like receptor (TLR)-4 and TLR-7/8 ligands could markedly elevate OSM steady state mRNA and protein expression in cultured peripheral blood mononuclear cells (PBMC) that were differentiated towards macrophages with M-CSF but not cells differentiated in the absence of M-CSF.

Collectively, these *in vitro* results suggest a mechanism for viral/bacterial-induced asthma exacerbations that involves elevated OSM production by infiltrating monocytes in response to these pathogens and subsequent augmentation of HASMC pro-inflammatory responses. These include synergistic expression of select chemotactic factors that would recruit inflammatory cells and contribute to acute asthma exacerbations.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AHR	Airway Hyper responsiveness
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
ASMC	Airway Smooth Muscle Cells
BALF	Bronchoalveolar Lavage Fluid
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complementary DNA
CM	Conditioned Media
Col1A1	Collagen 1A1
cRPMI	Complete RPMI
CT	Threshold cycle
DC	Dendritic Cell
DNA	Deoxyribonucleaic Acid
dsRNA	Double stranded RNA
ECM	Extracellular Matrix
ELISA	Enzyme-Link Immunosorbent Assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
G-CSF	Granulocyte- Colony stimulating Factor
GM-CSF	Granulocyte Macrophage- Colony stimulating Factor
Gp130	Glycoprotein 130
Gp130R	Gp130 Receptor
HAoSMC	Human Aortic Smooth Muscle Cells
HASMC	Human Airway Smooth Muscle Cells
HDM	House dust mite
hiNOS	Human induced Nitric Oxide Synthase
HRV	Human Rhinovirus
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
IL-17RA	IL-17 Receptor A
IL-17RC	IL-17 Receptor C
IL-6ST	IL-6 Signal Transducer
IP-10	Interferon gamma-induced protein 10
IR	Infrared
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,

	alpha
JAK-STAT	Janus Kinase- Signal Transducers and Activators of Transcription
JNK	c-Jun N-terminal kinase
LIF	Leukemia Inhibitory Factor
LIFR	LIF Receptor
LPS	Lipopolysaccharide
M-CSF	Macrophage -Colony Stimulating Factor
mAB	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MAPKK	MAPK Kinase
MAPKKKK	MAPKK Kinase
MCP	Monocyte chemotactic Factor
mLST8	MTOR associated protein, LST8 homolog
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
mTORC	Mammalian Target of Rapamycin complex
MyD88	Myeloid differentiation primary response gene (88)
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NKT	Natural Killer T cells
NLS	Nuclear localization Signal
NO	Nitric Oxide
OSM	Oncostatin M
OSMRβ	Oncostatin M Receptor beta
OVA	Ovalbumin
Pam2CSK4	Palmitic acid (2)-Cys-Ser-Lys-Lys-Lys-Lys
PAMP	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PDAR	Pre-developed Assay reagents
PHA	Phytohaemagglutinin
PI3K	Phosphatidylinosinsitol-3 Kinase
PIKK	phosphatidylinosinsitol kinase related kinase
PMSF	phenylmethylsulfonyl fluoride
PPAR	Peroxisome Proliferator-Activated Receptor
qRT-PCR	Quantitative Reverse Transcription- Polymerase Chain Reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RHD	rel Homology Domain
RIPA	Radioimmunopreciptation Assay

RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
SCF	Stem Cell Factor
SD/SEM	Standard Deviation/ Standard Error of the Mean
SDS-PAGE	Sodium dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SH2	Src-Homology 2
shc	SH2- and collagen-homology-domain-containing protein
SHP2	SH2-domain-containing tyrosine phosphatase
SmGM	Smooth Muscle Growth Medium
SOS	Son of Sevenless
ssRNA	Single stranded RNA
STAT	Signal Transducer and Activators of Transcription
TARC	Thymus and activation regulated chemokine
TBS	Tris Buffered Saline
TBS-T	TBS plus 0.015% Tween-20
Th	T-helper cell
TIMP1	Tissue inhibitors of metalloproteinase
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factors
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	Thymic stromal lymphopoietin
TYK	Tyrosine Kinase
UNG	Uracil-N-glycosylase
VEGF	Vascular Endothelial Growth Factor

-CHAPTER 1-

INTRODUCTION

1.1 Asthma

Asthma is a chronic lung inflammatory condition that affects approximately 10 percent of the population. It is also one of the most common chronic conditions worldwide, particularly in industrialized nations, and is heterogeneous in nature. (1-6). The exact causes and mechanisms contributing to this condition differs substantially between its subtypes (atopic, non-atopic, eosinophilic, non-eosinophilic) and the severity observed between individuals(1–3). The fundamental feature of asthma, in all forms, is airway hyper responsiveness (AHR) due to responses to environmental or endogenous stimuli (1, 3–5). Responses generated by the recognition of these stimuli result in narrowing of the airways due to airway smooth muscle hyper-contraction, airway edema, cell infiltration and overproduction of mucous by goblet cells (6-8). As a result, patients with asthma experience wheezing, dyspnea, tightness in the chest, coughing and overall airway dysfunction(1–3, 6, 7). In severe asthma, increases in extracellular matrix around the airways further compromise lung function(8, 9).

In atopic asthma, allergic responses are a result of airway hyper responsiveness to antigens of which the individual has become sensitized to such as ragweed, cat dander or house dust mite (HDM)(4, 10–12). Damage to airway epithelium by microbes and environmental irritants likely cause the initial signals for the recruitment and maturation of antigen presenting cells such as dendritic cells (DC) and macrophages in antigen

sensitization(4, 13, 14). Various other cell types have been shown to be involved in asthma pathogenesis including: B cells, T_H2, T_H17 and NKT cells (2, 5, 15, 16). T_H2 polarized responses have been shown to be key in both atopic and non-atopic asthma. T_H2 pro-inflammatory cytokines: IL-4, IL-13 and IL-5 induce isotype switching in B cells to produce IgE, eosinophilic infiltration, mobilization of mast cells and increased mucus secretion (5, 6, 12, 15, 17). Mast cells produce a variety of lipid mediators and cytokines that may interact with structural cells such as smooth muscle or epithelial cells involved in hyperactivity(18, 19). Alveolar macrophages have been shown to induce mast cell release of histamine (20), a mediator involved in allergic responses. Studies have also shown some neutrophilic infiltration in severe asthmatics from increased T_H17-mediated responses. These include the production of IL-17A and IL-22, which have been implicated in other chronic inflammatory conditions due to their regulation of CXC chemokines(2, 8, 21–26). It is apparent that the cytokine profiles in asthma are very complex and requires further investigation because of the level of elevated cytokines observed in asthma.

1.2 Asthma Exacerbations/Severe Asthma

A common complication in patients with asthma, in addition to other chronic lung inflammatory conditions, is exacerbations due to bacterial or viral infections of the respiratory tract. Acute asthma exacerbations result in increased AHR severity and exaggeration of other asthma sequelae. Hence, acute asthma exacerbation is of significant clinical interest since it is the major factor resulting in increased morbidity,

hospitalizations and mortality in patients with asthma (27–30). Severe asthmatic patients have chronic severe symptoms that are associated with airway remodeling, altered airflow obstruction and potential increased cellular infiltrations(2). Individuals with severe asthma have increased risks of severe exacerbations, adverse reactions to treatment and chronic morbidity in patients with steroid-resistance(31). Therefore understanding the mechanisms that induce and exacerbate atopic and non-atopic asthmatic disease is of significant importance to patients and the health care system(30).

1.2.1 Viral Infection-induced Asthma exacerbations

Viruses and allergens appear to interact in the induction of asthma but also in the exacerbations of established asthma in a complex manner. Viral respiratory infections are the most frequent causes of exacerbations in both children (up to 80%) and adults (50%) (30, 32, 33). Human rhinovirus (HRV)(28), influenza, respiratory syncytial virus (RSV), coronavirus and metapneumovirus are the most common viral-induced causes of asthma exacerbations(30, 31, 34). Rhinovirus, a positive sense RNA virus(29), appears to be the most commonly implicated, representing about 67% of all virus infection-induced asthma exacerbations(29, 35). It has been suggested that these viruses enhance symptoms by increasing the sensitivity of the airways, increasing airway infiltration by both neutrophils and eosinophils(22, 27, 28), and by inducing increased type III interferon (IFN-λ)(37). However, the mechanisms of these particular viruses in asthma exacerbation are not fully clear.

1.2.2 Bacterial Pulmonary Infections in Asthma Exacerbations

Some bacterial infections can also induce asthma exacerbations, although apparently to a lesser degree than viral infections. Three main bacteria have been implicated in these cases: *Chlamydia pneumoniae*, *Mycoplasma pneumonia*(27, 29, 38) and *Haemophilus influenzae*(31, 39). *Chlamydia pneumoniae* is a gram-negative obligate intracellular bacterium that infects the lungs and is a major cause of pneumonia. *Mycoplasma pneumonia* is the smallest free-living organism and lacks a cell wall(27). Both these bacteria have also been detected in cases with acute bronchitis and exacerbations of asthma (27, 29). *Haemophilus influenzae,* a gram negative bacteria, has been isolated from the airways of asthmatic patients with increased neutrophilia (31, 39). Infections with *Haemophilus influenzae* appear to drive an IL-17-mediated response resulting in the increased number of neutrophils as described in an *in vivo* study using OVA-sensitized mice (39). Patients with neutrophilic forms of asthma also tend to have greater severity in their symptoms and steroid-resistance(31). However, the mechanisms in which bacterial infections induce alterations in asthma are also not fully clear.

1.3 Human Airway smooth muscle cells (HASMC)

The pulmonary airways consist of bands of submucosal smooth muscle that wraps around the circumference of the airway lumen. Airway bronchoconstriction in asthmatics is abnormally exaggerated due to AHR resulting in excessive airway narrowing. It is clear that airway smooth muscle contribute to the pathophysiological responses of asthma due to its role in contractile dysfunction, however, airway smooth muscle cells are also

involved in airway remodeling including smooth muscle cell hyperplasia and hypertrophy. HASMC also exhibit immune-modulatory capabilities by playing a role in regulating inflammation through the secretion of numerous cytokines and chemokines(40). For instance, *in vivo* studies in mice showed that eotaxin, an eosinophil chemotactic protein that can be derived from airway smooth muscle cells, was elevated both in protein and mRNA in asthmatic airways when compared to controls(41, 42). This chemokine contributes significantly in asthmatic inflammation by facilitating eosinophil activation and accumulation. In addition, RANTES, a CC chemokine with chemotactic activity for eosinophils, memory T cells and monocytes is also secreted by HASMC. This release of RANTES is observed in response to TNF-α, IL-1α and platelet activating factor stimulation in cultured HASMC(43) .These cells can also produce IL-8 (a neutrophil chemokine), TARC (a T cell chemokine) and stem cell factor (SCF), which attract and regulates mature mast cells (18, 44). HASMC exposure to IgE immune complexes induces IL-5 secretion in cultured HASMCs, which promotes the survival of eosinophils(40, 45). Therefore, it is evident that HASMC can play a significant role in the pathophysiology of asthma and will be the main focus throughout this thesis.

1.4 Immune cells and Immune regulation in Asthma

Asthma and allergic responses are the result of a dysregulated immune system in the respiratory mucosa (15). As mentioned previously, there appears to be many roles of select cells of the immune system which include innate cells such as dendritic cells, eosinophils, neutrophils and macrophages; and cells of the adaptive immune system: T

cells and B cells(4, 13, 14). An essential feature of asthma or allergic airway inflammation is the airways becoming sensitized to an inhaled stimulus such as an allergen(4, 14, 15). Disruption or damage of the epithelial layer likely facilitates the sensitization process by causing the release of many immune modulating factors, one of which is thymic stromal lymphopoietin (TSLP) from the epithelium(4, 14, 46, 47). The release of these factors results in the activation/maturation of dendritic cells (DC) and macrophages(46) that are situated below the airway epithelium leading to the internalization of the stimuli or allergen(14, 15) and eventually antigen presentation to cells of the adaptive immune system: T cells and B cells(14, 15). At the same time, cells of the innate immune system are also recruited through various chemotactic factors, released by resident macrophages or dendritic cells, to the site of damage or inflammation. Below, is a summary of the roles of select immune cells and their recruitment to the lungs as these are relevant aspects to this thesis: monocytes/macrophages, T lymphocytes and granulocytes.

1.4.1 Monocytes/Macrophages

Macrophages arise from monocytes, which circulate the blood and egress through the endothelial layer of blood vessels eventually differentiating into tissue macrophages or dendritic cells (48–50). Human monocytes are divided into subsets based on their expression of two cell surface markers, CD14 (co-receptor involved in the recognition of LPS through TLR4) and CD16 (FcγRIII) as reviewed in ref. (48, 50). $CD14^{++}$ $CD16^{-}$ subsets are referred to as classical or inflammatory monocytes which comprise approximately 95% of the monocytes found in human peripheral blood(48, 50). This

subset also expresses high levels of CCR2, a chemokine receptor for MCP-1/CCL2, MCP-3/CCL7 and MCP-5/CCL12 (48, 50, 51) which are major monocyte chemo-attractant proteins(52) The other subsets are collectively referred to as $CD16^{+}$ monocytes, which include $CD14^{+}$ $CD16^{++}$ (known as non-classical monocytes) that express higher levels of MHC class II molecules and resemble mature tissue macrophages and $CD14^{++}$ $CD16^{+}$ (referred to as intermediate monocytes) (48, 50). Although there are differing subsets, these blood monocytes all have the capacity to differentiate into macrophages and express CD115, Macrophage-Colony Stimulating Factor Receptor (M-CSFR)(48, 50). As stated previously, MCP-1/CCL2, MCP-3/CCL7 and MCP-5/CCL12 are common chemotactic factors that recruit monocytes from the bone marrow(48, 50, 51). In addition to these chemokines, there are several chemokines involved in the recruitment and trafficking of monocytes as reviewed in Shi & Pamer (2011), for instance: Fractalkine/CX_3CL1 is a chemokine that effectively recruits both monocytes and T cell through CX_3CR1 chemokine receptor; Macrophage inflammatory protein (MIP)-1α/CCL3 and RANTES/CCL5 recruit leukocytes including monocytes to inflamed tissues through their interaction with CCR1 and CCR5(50).

Macrophages are an important cell type of the innate immune system as they have critical roles in inflammation and host defense(48, 49). Interestingly, the lungs of asthmatic patients have been shown to have an increased numbers of macrophages(53) and may play a role in the chronic inflammation due to their increased infiltration into the airway mucosa observed in patients with steroid-resistance (4). Pulmonary macrophages can be derived from blood monocytes that have been recruited to the lungs or through a

self-renewing population of macrophages resident in the lungs(31). These macrophages found within the lungs of asthmatics have been shown to be involved in the modulation of airway smooth muscle contraction through inducing histamine release from basophils and mast cells(20, 31), impairment of functional β-adrenergic responses through the release of reactive oxygen species such as nitric oxide(NO)(46) , which has been demonstrated to be elevated as measured in the exhaled breaths of asthmatics(12) and lastly generation of thromboxane A2 resulting in increased cholinergic neurotransmission(31). In addition to their affects on airway smooth muscle contraction, there is evidence that demonstrates that alveolar macrophages have been found to be activated in asthmatic inflammation upon allergen challenge and increased secretion of cytokines such as Tumor necrosis factor- alpha (TNFα) and IL-6 by these cells(54). Macrophages from atopic asthmatics have also been shown to be able to stimulate $CD4^+$ T cells from the peripheral blood to secrete Th2 cytokines (55) which is further discussed below.

1.4.2 T Lymphocytes

T lymphocytes are one of two cells of the adaptive immune system of lymphoid lineage(56). T cells serve as crucial cells within the immune system specifically through three main effector functions: killing/cytotoxicity, activation and regulation (56). Not surprisingly, T cells are also highly involved in asthma and allergic airway inflammation through their secretion of various immune-modulating proteins(14, 16). Like monocytes, various chemokines are involved in the recruitment of naïve and effector T cells, some of which also recruit monocytes, such as: MCP-1/CCL2, CCL1, RANTES/CCL5, TARC/CCL17 and lastly CCL22. Many of these chemokines can be secreted from both

stromal cells such as epithelial cells and immune cells such as macrophages or T cells themselves during an allergic response or inflammation(57). T cells can be grouped as $CD4^+$ or $CD8^+$. Most $CD4^+$ T cells are referred to as T helper (T_H) cells, which have the potential to differentiate into several phenotypes: T_H1, T_H2, T_H9, T_H17, T_H22, T_H3/Tr1 and lastly T_{reg}(5, 16, 56). T_H1, T_H2 and T_H17 phenotypes are described further.

T_H1 are often involved in bacterial/viral infections. These cells types arise when APC such as a dendritic cells secrete increased levels of IL-12 and IFNγ in response to the infection, causing the activation of T-bet, the transcription factor that induces T_H1 phenotypes and eventual cytokine release which include: IFNγ and TNFα(56).

T_H2 are activated in response to helminths infections, however, it has been well established that these cells are central in allergy(15, 56, 58–60). The development of a T_H2 phenotype is favored when naïve T cells recognize IL-4 inducing activation of GATA3, a transcription factor that highly induces T_H2 responses, and STAT6 through IL-4 signaling(56, 60). These cells release copious amounts of IL-4, IL-5 and IL-13, cytokines often referred to as pro-allergic cytokines due to their prominent roles in allergy and have been shown to be highly elevated in atopic asthmatics(7, 58–62). These cytokines will be further discussed below and serve as a major aspect of this thesis.

T_H17 cells have been implicated in many inflammatory conditions but mostly in auto-immunity(63, 64). Like the phenotypes described above, differentiation of naïve T cells into the T_H17 phenotype required the recognition of select cytokines: IL-6 and TGF-β in the absence of IL-4 and IL-12. This results in the activation of STAT3, which is required for this differentiation(65) and increased expression of the transcription factor:

RORγT (56, 66). RORγT drives the expression and release of T_H17-type cytokines including but not limited to: IL-17A, IL-17F and IL-6 (64, 67, 68). Several studies have demonstrated an increase role IL-17-type cytokines in asthma(22, 23, 25, 26). Due to the increased evidence of T_H17- type cytokines in asthma, IL-17A, the most characterized T-$_H17$- cytokine, plays an essential part of this thesis and will be further discussed below.

1.4.3 Granulocytes

Granulocytes or polymorphonuclear cells consist of cells that have been shown to play important roles in asthma and allergic disease. These cells include: eosinophils and neutrophils. Eosinophils have been shown to be elevated in numbers during asthma exacerbations(43, 69). The recruitment of eosinophils from the bone marrow to the lungs and airway lumen occurs in response to eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26(42, 70, 71). Eosinophils can also be recruited through RANTES/CCL5, CCL3 and MCP-3/CCL7 through CCR3 chemokine receptors highly expressed on the surface of these cells(69). In addition, IL-5, a T_H2 cytokine that is elevated in asthma, is highly involved in the recruitment, development and activation of eosinophils. Once recruited to the lungs, these cell types have been involved in secretion of various cytokines (IL-4, IL-5 and IL-13), chemokines (CCL11 and CCL22), matrix metalloproteinases (MMP) and leukotrienes (72, 73).

Although eosinophils play an essential role in asthma, up to 50% of asthmatic have non-eosinophilic inflammation, of which, 20% have severe neutrophilic inflammation(39, 74). Neutrophils play essential roles within the immune system as they are the first cells recruited to sites of infection or inflammation(56, 74). In asthma,

neutrophil numbers have been shown to be elevated in sputum of patients with severe asthma, especially in those with low eosinophils as opposed to individuals with mild/moderate asthma that show levels comparable to healthy controls(74). Neutrophil trafficking is mediated by pro-inflammatory mediators, primarily IL-8/CXCL8, TNFα and other leukocyte chemokines mentioned previously(26, 39, 74). IL-8 levels have also been shown to be elevated in the sputum of severe asthmatics (26, 75–77). Similar to eosinophils, once neutrophils are recruited to the lungs, these cells are involved in further cytokine and chemokine secretion including: IL-8, IL-6, IL-1β and TNFα. Neutrophils also secrete leukotrienes, reactive oxygen species and various proteases that include MMP-9, collagenase and elatases that alter the extracellular matrix(74).

The cells mentioned above all secrete various cytokines and chemokines that have been shown play essential roles in asthma pathology and are reviewed in the subsequent sections.

1.5 Pro-inflammatory cytokines

As summarized above, macrophages, T cells, eosinophils and neutrophils all secrete cytokines that are involved in asthma (5, 31, 54, 78). Cytokines are small molecular weight proteins that are part of a group of immune modulatory molecules (56). Many cytokines can be described as either pro-inflammatory or anti-inflammatory or combination of both (16, 50, 56). Release of pro-inflammatory cytokines will promote inflammation through facilitating processes such as cellular infiltration and edema, one such example is TNFα (56). Anti-inflammatory cytokines are those that regulate or

diminish the process of inflammation and are often involved in wound healing, for instance IL-10 (5, 16, 56). Below is a summary of various cytokines, of which, the majority are pro-inflammatory, that are involved in asthma and atopic airway disease.

1.5.1 Gp130 cytokines and OSM

The gp130 family of cytokines (also referred to as the IL-6 family) are grouped together based on their shared similarities in structure and functional activity(79–81). Members include: IL-6, Leukemia inhibitory factor (LIF), ciliary neurotropic factor (CNTF), Oncostatin M (OSM), interleukin-11, cardiotrophin-1 (CT-1)(79–82) and IL-31(83). With the exception of IL-31, gp130 cytokines bind to cell membrane receptor complexes that require the gp130 signal transducing subunit (79, 82). The various receptor complexes also require receptor chains specific for each of the different ligands(82, 84–87). For instance, functional receptors for IL-6 and IL-11 include a homodimer of two gp130 molecules in addition to IL-6Rα and IL-11Rα, respectively(82, 84–87), whereas OSM and LIF require heterodimers of OSMRβ/gp130 and LIFR/gp130 (respectively) to signal within the cell(79–82, 85–87). These cytokines activate genes involved in cell differentiation, survival, apoptosis, proliferation, acute phase response, inflammation (both pro- and anti-inflammatory) and hematopoiesis. They often share biological activity due to the use of the common gp130 subunit (81, 82). Signal transduction involves the activation of Janus kinases (JAK), tyrosine kinases that lead to the activation of the transcription factor STAT (signal transducers and activators of transcription). These cytokines also signal through MAPK (mitogen-activated protein kinase) pathways (79, 81, 82). LIF and OSM, in human systems, are the most similar in

structure, function and genetic sequence. Likely due to their structural similarities, OSM (human homolog) is able to signal through both the LIFR complex (LIFR and gp130) and OSMR (OSMR and gp130)(82, 85).

The gp130 family of cytokines may be implicated in asthma due to their roles in inflammation and inducing cell proliferation and maturation as reviewed in ref.(86). Human airway smooth muscle cells (HASMC), an important cell type in the responses in asthma, have been examined for responses to some of the gp130 cytokines including LIF, IL-6 and IL-11 in addition to Oncostatin M (40, 42).

Oncostatin M

Oncostatin M (OSM) is a multifunctional cytokine that is part of the gp130 cytokine family and has been the subject of various reviews(85–90). These have indicated that: the human homologue is a glycoprotein of 28 kDa secreted by activated T cells(91, 92), monocytes(93), dendritic cells(94), macrophages(95–97), mast cells(98) and neutrophils (99, 100); OSM activates intracellular signaling pathways involving gp130 and both LIFR and OSMR which can activate the JAK-STAT, MAPK and PI3'K pathways (87); OSM can exhibit very diverse biological activities on a wide variety of cell types including modulation of growth, inflammatory responses, tissue remodeling(101, 102), liver development, and hematopoiesis(88, 103–105). OSM activity in inflammation is evident, however, its precise roles in asthma are not clear(88, 103).

There are many cytokines that have been found to be elevated in asthmatics(9, 26, 43, 54, 77, 86, 106). OSM expression at the protein and mRNA levels, was significantly

elevated in the sputum of asthmatic patients and appeared to correlate with severity of asthma (9). The major cell types involved in OSM production (macrophages, neutrophils and T cells (14, 39, 54)) are also elevated in patients with asthma(107–109). OSM promotes proliferation of both smooth muscle cells and fibroblast *in vitro*, which may contribute to asthma pathology(42, 110). In addition to contributing to tissue remodeling, OSM is also involved in the production of various chemokines and cytokines(9, 41, 103, 111–115). For instance, OSM has been shown to synergize with IL-4/IL-13 in the production of eotaxin-1 from smooth muscle cells(41, 113). This chemokine attracts and promotes eosinophil infiltration(41). OSM causes the release of tissue remodeling proteins such as TIMP-1 that also may contribute to asthma pathogenesis(41, 88, 101, 112, 113). Whether OSM functions in asthma exacerbations or its mechanisms of action in airway inflammation is not yet clear.

1.5.2 T_H2 Cytokines

It has been well-established that T_H2 type cytokines such as IL-4, IL-5 and IL-13 are elevated in tissues of asthmatics(7). These cytokines are produced by innate-like lymphoid cells and eosinophils, in addition to T_H2 cells, as summarized previously (14, 60). Dysregulation of T_H2-driven responses leading to inflammation is observed in both atopic and non-atopic asthma (3, 7,55, 56). These T_H2 polarized responses induce the major pathophysiologic features of asthma, including bronchoconstriction, airway hyper-responsiveness (AHR), and airway inflammation. IL-4, IL-5 and IL-13 induce pulmonary allergic responses involving the development, migration, and activation of inflammatory cells, such as eosinophils and mast cells. These cytokines also promote production of

allergen-specific antibodies (IgE), goblet-cell hyperplasia, and an increase in vascular permeability. This cocktail of cytokines produced induce isotype switching by B cells to produce IgE, eosinophil infiltration, the mobilization of mast cells and increased mucus secretion by goblet cells (14, 58, 59).

Interleukin-4 and IL-13 induce cell signaling through their interactions with specific cell surface receptors. Both IL-4 and IL-13 signal through the IL-4Rα and IL-13Rα1 chains(116–118), however IL-4 is also able to signal through as second complex consisting of IL-4Rα and the common γ (γc) chains(119). Binding of IL-4 or IL-13 to their specific IL-4R complexes leads to the activation of the JAK-STAT (STAT-6) pathway, ERK (MAPK) and PI3'K-Akt pathways(120). Specific details of cell signaling are described further below.

1.5.3 T_H17 cytokines

T_H17 cells and some innate-like cells secrete T_H17 pro-inflammatory cytokines, IL-17A, IL-17F and IL-22. These cytokines are also elevated in asthma and other chronic inflammatory diseases (8, 64, 121). Specifically, IL-17A has been shown to induce lung inflammation by stimulating cells of the innate immune system and neutrophilic airway inflammation in mice (8, 22). Both IL-17A and IL-22 regulate CXC chemokines and the production of granulocyte colony-stimulating factor (G-CSF), which stimulates the bone marrow in the production of granulocytes (8, 21). Studies have also shown an increase in IL-17A protein levels in the serum and sputum of asthmatics(23, 77). IL-17A interacts with a receptor complex of IL17RA/IL17RC subunits which are generally expressed on a wide variety of cell types(68, 122) which include lung epithelial cells (21) and lung

fibroblasts (22) inducing the production of pro-inflammatory cytokines, chemokines and matrix metalloproteinase. The result is increased recruitment of neutrophils and macrophages, leading to tissue inflammation(17, 20, 21, 60). Interestingly, the levels in which IL-17A and IL-17F are detected in asthmatics may be an indicator of the severity of the condition (23, 124). It is evident that there is a complex cytokine profile observed in asthmatics and much more work is required to completely understand the roles of these cytokines and how these cytokines interact in asthma pathology.

1.6 Toll-like Receptors

Cytokine secretion by many cells of the immune system, particularly those of the innate system, requires the recognition of pathogens through innate pattern recognition receptors(125). Toll-like receptors (TLR) are one group of pattern recognition receptors that are crucial in mounting innate immune responses(125). TLRs are a large family of receptors with approximately 11 members (as reviewed in ref. (125, 126)). These receptors have differing roles and functions in innate immune recognition and recognize broad, highly conserved microbial products referred to as pathogen associated molecular patterns (PAMPS). Upon recognition, signal transduction occurs to activate many genes involved in immune responses. As indicated in the review(125), TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell membranes of cells. TLR2 is a major receptor to recognize gram-positive bacteria by recognition of their cell membrane components including peptidoglycan and lipoteichoic acid (127). TLR2 also recognizes lipoprotein from viral particles and parasites. TLR5 recognizes bacterial flagella and TLR6

recognizes diacyl lipoproteins (127, 128). TLR4 is crucial for recognition of gram-negative bacteria through recognition of LPS (125, 129, 130). There are four TLRs that are located intracellularly and can recognize nucleic acids including TLR3 that recognizes double stranded RNA, TLR7 and TLR8 which recognize single stranded RNA (ssRNA) particularly guanosine and uridine-rich ssRNA (128, 131). TLR9 responds to bacterial DNA in the form of unmethylated CpG dinucleotides (128, 131).

In the airways, many TLRs are expressed on a variety of cell types including: epithelial cells, smooth muscle cells, macrophages, mast cells, eosinophils and dendritic cells(125, 126). Specific TLRs may be involved in the pathogenesis of asthma. For instance, TLR3 ligands along with TSLP may promote increased T_H17 responses(132). In addition, stimulation of cultured HASMC with ligand for TLR2, TLR3 and TLR4 results in increased production of IL-8, IL-6 eotaxin and ICAM-1(133) and increased IFN-alpha-induced cytokines(127). Thus, recognition by TLR may be an important component in the pathogenesis of asthma and asthma exacerbations.

1.7 Cell Signaling Pathways

There are many cellular signaling pathways that may be involved in asthma and asthma exacerbations. Determination of the mechanisms and cross-talk between these pathways will be helpful in the understanding and treatment of acute asthma exacerbations. Below are summarized the major signaling pathways for cytokines that are the focus of this thesis, although there are many other important mediators of cell activity in asthma.

1.7.1 JAK/STAT

The Janus Kinase (JAK) family is a group of evolutionarily conserved set of tyrosine kinase proteins (as reviewed in ref. (84, 134, 135)). There are four JAK proteins in mammalian systems: JAK1, JAK2, JAK3 and TYK2. In cytokine signaling, JAKs bind directly to the intracellular domains of a cytokine receptor signaling chains and catalyze ligand-induced auto-phosphorylation and phosphorylation on the tyrosine residues of cytokine receptor. This phosphorylation of the intracellular domain of the receptor creates a docking site for the STAT (Signal Transducing and Activators of Transcription) proteins. There are seven STAT proteins found in mammals (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6), as reviewed in (134, 136, 137). STAT proteins contain an N-terminal oligomerization domain, a DNA binding domain, an SH2 domain (Src -Homolgy 2), a conserved tyrosine residue and a C–terminus transactivation domain. Some of the STAT proteins contain a conserved serine residue in which its phosphorylation is required for maximal activation. Docking to the phosphorylated intracellular portion of the receptor is through the SH2 domain. Phosphorylation of the STAT protein occurs by the JAK kinases, leading to the dissociation of the STAT proteins from the receptor and allows homo- or heterodimerization and nuclear translocation. Once the STAT proteins translocate to the nucleus, they act as transcription factors(134, 135, 137).

Several cytokines signal through the JAK-STAT pathway. Interferon type cytokines (Type I and Type II interferon), which include IFNα/β and IFN-gamma, signal through this pathway. Other cytokines, which are collectively, referred to as type 1

cytokines including the classical immunological cytokines: IL-2, Il-7, IL-9, IL-13 and TSLP also utilize JAK-STAT in target cells but may differ in the extent of specific STAT proteins activated (STAT1-STAT6). In addition, hematopoietic cytokines such as IL-3, IL-5, GM-CSF and stem cell factor (SCF) (134, 135, 137). The gp130 family of cytokines activates the JAK-STAT pathway with significant activation of STAT3(81, 82, 85, 90). OSM can also activate STAT 5 and STAT1 (79, 81, 103). OSM activation of these STAT proteins leads to the production of VEGF and eotaxin in HASMC (112, 115, 138). STAT2 and STAT6 activation occurs typically through type I interferon and IL-4 and IL-13, respectively. In addition, IL-4 treatment of cultured HASMC release eotaxin in a STAT-6 dependent manner(41, 113). Although many features of asthma can be attributed to the JAK-STAT signaling cascade, there has been limited evidence of specific cell signaling pathways involved in asthma exacerbations.

1.7.2 MAPK

The mitogen-activated protein kinase (MAPK) signaling pathways are central in key cellular processes and are also evolutionarily conserved (as reviewed in ref. (139, 140)). The MAPK pathway consists of three kinase/phosphatase cycles that consists of MAPK (ERK1/2, JNK1/2, P38), MAPK kinase (MEK1/2, MKK 3/4/6/7) and MAPK kinase kinase (Raf-1, MEKK1-4). Most activators of the MAPK pathway begin signaling through activating the receptors that assemble into a complex and activate a MAPKKK usually through GTPase. This is then followed by subsequent phosphorylations leading to the phosphorylation and activation of a MAPK that translocates to the nucleus where it regulates gene transcription(140).

Dimerization of the gp130 family cytokine receptors activates JAK-STAT and MAPK pathways. Gp130 cytokines activate ERK1/2, a MAPK responsible for maintaining cell survival, JNK and p38(79, 80, 85, 115, 139). LIFR-gp130 receptor complex activation of MAPKs is initiated by SHP2 (SH2-domain-containing tyrosine phosphatase) binding to tyrosine residues on both gp130 and LIFR subunits. The OSMR-gp130 complex induces activation of Ras-Raf-ERK1/2 through Shc (SH2- and collagen-homology-domain-containing protein) binding to a tyrosine residue on the cytoplasmic portion of OSMR subunit. ERK activation is induced upon recognition of OSM, LIF and growth factors in HASMC in the expression of IL-8, RANTES, eotaxin and VEGF(41, 112, 115, 141). MAPK p38 activation is seen in LPS induced TNF secretion in macrophages(142) and in the regulation of TLR-4 induced pro-asthmatic changes in airway smooth muscle cells (130). LPS also induced IL-6 production in cultured HASMC when stimulated with IL-1β and TNF-α through activation of p38 and ERK1/2 MAPKs(130). Examinations of the MAPKs pathways in the recognition of other TLR ligands and cytokine synergy may assist in understand mechanisms of asthma exacerbations.

1.7.3 PI3'K/mTOR/AKT and Rapamycin

mTOR (mammalian target of Rapamycin) is a serine/threonine protein kinase that belongs to the phosphatidylinosinsitol kinase related kinase (PIKK) family (as reviewed in refs 73,74). mTOR protein is part of the PI3'K/ AKT/ mTOR signaling pathway in which PI3'K phosphorylates and activates AKT which then can activate mTOR. However, mTOR can be activated by autophosphorylation and can subsequently

phosphorylate AKT. AKT regulates the cell cycle by phosphorylating and inactivating regulators of the cell cycle. mTOR is also activated by MAPK proteins and cellular stimuli such as growth factors, stress signals, nutrients, energy and amino acid abundance(143, 144). mTOR exists in two distinct complexes: mTORC1 and mTORC2. mTORC1 (mTOR complex 1), consisting of mTOR, raptor and mLST8, regulates protein translation, cell growth, intracellular transport, and metabolism(143, 144). mTORC1 is sensitive to rapamycin, a macrolide antifungal bacterial product that inhibits mTOR activity(145). Rapamycin is also used as an immunosuppressant due to its ability to inhibit IL-2 responses in B and T cell activation(146). Rapamycin also prevents IL-12-induced IFNγ production in peripheral blood T cells due to inhibited mTOR phosphorylation of STAT3 on serine 727, a site that requires phosphorylation for maximal activation.(147). In the same manner, mTOR also regulates the activation of STAT1- dependent hiNOS (human inducible Nitric oxide synthase) in response to LPS in lung epithelial cell lines. Inhibition of mTOR activity resulted in inhibition of STAT 1 phosphorylation at serine 727(136). mTOR may also be activated in response to IL-4 since rapamycin inhibits IL-4 induced dendritic cell maturation(148). Therefore, examination of mTOR pathways may provide key mechanisms in asthma exacerbations and smooth muscle hyperplasia observed in some asthmatics.

1.8 Thesis Objectives, Rationale and Hypotheses

Goal: ***The overall aim of this thesis was to examine the potential roles of OSM in bacterial/viral-induced acute asthma exacerbations.***

1.8.1 Rational and hypothesis-Objective 1

Previous publications have characterized OSM's capacity to act in a synergistic manner with various cytokines (41, 112, 113, 138). It has been shown that OSM synergizes with IL-4/IL-13 and IL-1β in the production of eotaxin-1(41) and VEGF(138), respectively, by human airway smooth muscle cells. Furthermore, previous work in the laboratory has demonstrated that OSM is capable of enhancing MCP-1 and IL-6 production by HASMC stimulated with TLR3 and TLR4-ligands, suggesting that OSM can also modulate cytokine and chemokine production in response to TLR agonists (149). Recent work has also demonstrated some synergy between OSM and TLR-3 agonist, poly (I:C), in the production of MCP-1, eotaxin-1, eotaxin-3, IP-10, IL-6 and MCP-4 in HASMC, in addition, OSM in combination TLR-4 agonist, LPS, in the production of MCP-1 and IL-6(149).

Using cultured HASMC, this thesis examined HASMC expression of various pro-inflammatory mediators in response to OSM and cytokines elevated in asthma: IL-4, IL-13 and IL-17A. The synergistic responses observed in cultured HASMC may be the result of multiple mechanisms. One of which may be due increased cell signaling leading to increased activation of transcription factors, which leads to increased expression of these cytokines/chemokines. It is proposed that the cell signaling networks are to be examined as a potential mechanism that contributes to the OSM-induced synergistic responses by HASMC. Alternatively, the observed responses by HASMC may be a result of increased expression of related receptors. Work completely previously in the laboratory has demonstrated that that OSM and IL-4/IL13 synergy in the production of eotaxin-1 in

fibroblasts is associated with the marked elevation of IL-4Rα and IL-13Rα1 stimulated by OSM(113). Similar findings were found in HASMC(41) in which OSM induced increased production of IL-4Rα. Previous work in the laboratory has also shown an up-regulation of TLR3 in response to OSM and poly (I:C) in HASMC(149). Therefore, examining IL-4Rα and IL-17RA, other receptors involved in response to OSM with IL-4, IL-13 or IL-17A is another potential mechanism in which OSM-synergistic responses by HASMC is observed.

Patients with severe asthma appeared to have substantial thickening of the airway sub-epithelium and airway smooth muscle. There appears to be structural changes in asthma. Smooth muscle cell hyperplasia may be an important response to viral infections or to the increased cytokine production in the environment(40). This objective serves to examine the possibility of increased proliferation of airway smooth muscle cells in response to OSM that may manifest in the form of hyperplasia, which may be a critical response in the physiology of asthma exacerbations.

Hypothesis:

It is more specifically hypothesized in this thesis objective that OSM synergizes with select cytokines involved in asthma (IL-4, IL-13, IL-17A) in cultured HASMC in the expression of IL-6 and MCP-1 protein and mRNA. OSM synergy with these cytokines could lead to elevated levels of other inflammatory cytokines or chemotactic factors. It is also postulated that the synergistic responses in HASMC is associated with increased STAT3 activation and receptor subunit up regulation. In addition, it is hypothesized that

OSM modulates HASMC proliferation and elevates expression of various extracellular matrix proteins.

To test these hypotheses, HASMC cultures were stimulated *in vitro* with recombinant proteins (OSM, IL-4, IL-13, IL-17A) at designated concentrations. HASMC responses were evaluated through detection of protein levels in culture supernatants quantified using enzyme-link immunosorbant assay (ELISA) and mRNA analysis using qRT-PCR (TaqMan). HASMC cell signaling were evaluated using total cell lysates and immunoblots to semi-quantify levels of phosphorylated proteins. To examine alterations in HASMC proliferation, MTT proliferation assays were used *in vitro*. Lastly, changes in extracellular matrix remodeling proteins were examined at the mRNA level for changes relative to un-stimulated cells using qRT-PCR (TaqMan)

1.8.2 Rational and Hypothesis- Objective 2

OSM is a cytokine that can be produced by macrophages, neutrophils, dendritic cells and T cells(94, 98, 99, 150). In asthma, it is hypothesized that macrophages are a major source of OSM leading to pathology. Other reports have demonstrated increase cytokine production in macrophages upon TLR activation. For instance, stimulations of monocytes/macrophages with LPS (TLR4 ligand) induces increased expression of TNFα and hiNOS (31, 136). In addition, previous work completed in the laboratory demonstrated that peripheral blood mononuclear cells from human subjects could be cultured and stimulated with LPS (TLR4 ligand). Stimulation of these peripheral mononuclear cells led to increased production of OSM and IL-6.

Hypothesis:

It is hypothesized that monocyte derived macrophages will markedly increase OSM expression upon stimulation with TLR ligands. It is postulated that this increase in OSM expression may be a driving factor in the pathology in asthma exacerbations.

To test this hypothesis, human peripheral blood mononuclear cells from healthy donors were cultured, differentiated towards macrophages and stimulated with various TLR agonist and stimuli. OSM protein levels were assayed and total RNA were examined for modulation of OSM mRNA steady state levels.

-CHAPTER 2-

MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 HASMC Isolation and culture

Human airway smooth muscle cells (HASMC) were isolated from large airways obtained from patients undergoing thoracic surgery at St Joseph's Healthcare Hamilton after obtaining signed consent and with the approval of the hospital Research Ethics Board. Smooth muscle cells were isolated from disease-free areas of the large airways and expanded in RPMI supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, 0.1% Fungizone (purchased from Life Technologies) and 1% L-Glutamine and incubated at 37°C and 5% CO_2. Experiments were performed with cells between passages 4-8.

2.1.2 Peripheral Blood Mononuclear Cell Isolation and culture

Peripheral blood mononuclear cells were obtained from healthy donors with signed consent with the approval of the local Research Ethic Board. Each donor was anonymously coded for all experiments to conceal identity of the individual. Total volume of approximately 50 ml per donor was collected into six 8.5 ml blood collection tubes (BD Vacutainer ACD solution A, cat# 364606) purchased from BD Biosciences (Franklin Lakes, NJ). Blood samples were spun down using Beckman Allegra Centrifuge set to 1500 rpm at room temperature (25°C) for 5 minutes. Plasma layer (top yellow

layer) was removed and store for later use. Remainder of blood samples were poured into 50 ml falcon tubes and diluted (1:1) with PBS containing 1% FBS and gently mixed. Diluted blood samples (15- 30 mL) were poured into Leucosep tubes (cat# 227290P VWR, Mississauga, ON) above porous membrane containing 15 mL Ficoll-Paque (Lymphoprep, cat# 7851, Stem Cell, Vancouver BC) and spun in centrifuge at 2150 rpm (no brake) for 10 minutes at room temperature. Buffy coat layer (thin white layer) containing PBMCs were transferred to new 50 ml falcon tube and diluted layer with PBS, gently mixed and centrifuged for 10 minutes at1500 rpm at room temperature. Supernatants were removed and cell pellet re-suspended in 10-15 mL PBS.

Cell numbers were determined using a hemacytometer and equal volume of trypan blue. Total PBMC cells were seeded onto tissue culture plates and/or positively selected for $CD14^+$ cells using CD14 positive selection magnetic sorting kit (EasySep Human $CD14^+$ Kit, cat # 18058, Stem Cell, Vancouver BC) used as per manufacturer's protocols and incubated for 3 or 7 days in the presence or absence of 10 ng/ml human recombinant M-CSF (cat# 216-MC-005) purchased from R&D systems Inc. (Minneapolis, MN) in conditions of 37°C and 5% CO_2.

2.1.3 Human Aortic Smooth Muscle Cell (HAoSMC) Culture

Human Aortic smooth muscle cells (HAoSMC) were purchased from Lonza Group Ltd (Basel, Switzerland). Cells were cultured in Smooth muscle growth medium (SmGM) purchased from Lonza Group Ltd (Cat# CC-3181) supplemented with 5% FBS and SmGM bulletkit (cat# CC-4149) containing 0.1% insulin, 0.5% hFGF (human Fibroblast Growth Factor), 0.1% gentamycin and 0.1% hEGF (human Epidermal Growth

Factor). Cells were passaged up 8 passages according to manufacturer's protocols and incubated at 37°C and 5% CO_2.

2.1.4 Cytokine stimulation

HASMC were lifted off 75cm^2 or 150 cm^2 upon confluence of 80-90% using 0.5% Trypsin-EDTA diluted 10X with sterile 1X PBS. Cells were plated at cell density of 10,000- 15,000 cells per well in 96-well tissue culture plate and incubated overnight. Cells were washed with 1X phosphate-buffered saline (PBS) and incubated for 3 hours with fresh RPMI containing 2% FBS. Cells were then stimulated with the indicated cytokines in 2% FBS containing media, and 18-24 hour supernatant were later decanted and stored for future analysis by ELISA. Recombinant human cytokines (OSM, LIF, IL-31, IL-11, IL-6, IL-17A, IL-4, and IL-13) were purchased from R&D systems and prepared as directed.

2.1.4 TLR-Ligand Stimulation

Human PBMC plated at 150,000 cell/well in 96-well plate were cultured as described previously and washed twice in warm PBS and stimulated with either 20 ng/ml LPS, 2 ug/ml CpG or Class C CpG, 2 ug/ml Poly (I:C), 5 ng/ml Pam2CSK4, 2uM prostaglandin E_2, 2 ug/ml CL075 or 10 ug/ml PHA or left unstimulated (control) for 24 or 48 hours. Cell supernatants were spun down at 1500 rpm to remove cellular debris and stored at -20°C for future use. Unmethylated CpG (a TLR-3 ligand) was donated by the Ashkar Lab and synthesized at MOBIX (McMaster University). Long polymer Poly (I: C) was purchased from General Electric (GE). Prostaglandin E_2 (cat# 538905) was purchased from EMD Millipore (Darmstadt, Germany). LPS (TLR-4 ligand), PHA and

short polymer Poly (I:C) (cat# 0913-10MG) were purchased from Sigma-Aldrich (St. Louis, MO). CL075 (a TLR 7/8 agonist) (cat# tlrl-c75), Pam2CSK4 (a TLR 2 agonist) (cat# tlrl-pm2s) and CpG ODN Class C CpG oligonucleotide (a TLR 3 ligand) (cat# tlrl-2395) were purchased from Invivogen (San Diego, CA).

2.1.5 Pharmacological Inhibition

The STAT-3 inhibitor, Stattic, was purchased from Abcam Biochemical (Toronto, Canada) and used at the indicated concentrations applied for 30 minutes before stimulation with cytokines. The p38 inhibitor (SB 203580), ERK inhibitor (PD 98059) and AKT inhibitor (Akt X) were purchased from Calbiochem (San Diego, CA). Cells were pre-treated for 1 hour with 10 uM SB 203580 or 5uM AKT X inhibitor and 2 hours with 25 uM PD 98059 or a vehicle control (DMSO) prior to cytokine stimulation.

2.1.6 Proliferation Assay

HASMC cultures were plated in 96 well plates at density of 15,000 cell/cm^2. Cells were either serum derived for 3 hours using 2% FBS containing RPMI and subsequently stimulated with 5 ng/ml IL-4, IL-13 or IL-17A (or left unstimulated) in the presence or absence of 1 ng/ml OSM or 5 ng/ml OSM. Cells were incubated for 48 hours (2 Days) or 7 days. MTT assays using tetrazolium dye (20 uL per well) were added to each well. Cells were incubated for 3 hours at 37°C and 5% CO_2 in the dark. Cells were spun down at 1200 rpm for 5 minutes to remove cell supernatants. Tissue culture grade DMSO was added (100uL) to each well to solubilize formazan crystals. Optical density of each sample was determined using SpectroMax at 450nm. Graphs illustrate mean optical density of each sample ± SEM of 4 replicates.

2.2 Protein Analysis

2.2.1 Enzyme-Link Immunosorbent Assays (ELISA)

The level of cytokine proteins in the cell culture supernatants were quantified by ELISA using Duoset antibody pairs for human IL-6 (cat# DY206), TNFα (cat# DY210) and OSM (cat# DY295) purchased from R&D Systems Inc. and used as per manufacturer's protocols. MCP-1/CCL2 levels were assessed using human MCP-1/CCL2 ELISA Max Deluxe kit (cat# 438805) purchased from BioLegend (San Diego, CA). Limit of detection of each ELISA was 12 pg/ml or less.

2.3 Cell Signaling Analysis

2.3.1 Cell Lysates

HASMC were plated at a cell density of 90,000 cells per well in 6-well tissue culture plates (Falcon) and incubated overnight. Growth medium was removed and cells were washed with PBS, and incubated for 3 hours in media containing 2% FBS. Cells were then stimulated in fresh 2% media with media containing indicated cytokines for 20 minutes or indicated time. Cells were washed twice in ice cold PBS and then lysed using 200 µL (per well) of ice cold Radioimmunopreciptitation assay (RIPA) buffer composed of 1% Igepal CA-360 (Sigma-Aldrich), 0.5% Sodium deoxycholate (Sigma-Aldrich), 0.1% Sodium dodecyl sulfate (SDS) in PBS plus added inhibitors: 1mM Sodium Orthovanadate, 0.1 mg/mL Phenylmethylsulfonylflouride (PMSF), 5 µg/mL Aprotinin, and 1 mM dithiothreitol (DTT) and incubated for 2 minutes on ice. Each well containing cell lysates was scraped using cell scrapers and transferred to collection tubes where cell

lysates incubated for 1 hour on ice. Cells were then sheared by passing the cells through a 21-gauge needle 20 times. The sheared lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C and transferred (supernatants only) to new tubes to be stored at -80°C for future use.

Protein concentrations of total cell lysates were determined using Bradford Assay. Total cell lysates were prepared as a 1:32 or 1:64 dilution (diluted in distilled water) and compare to bovine serum albumin (BSA) standard (0-14ug BSA). Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, Cat# 500-0006) was added to each sample to make a 20% final concentration. The absorbance of each sample was determined using SpectroMax spectrometer at 595nm.

2.3.2 Immunoblots

After determining relative protein concentrations of each cell lysate sample, 15 μg of total protein was prepared in sample containing distilled water and 5X reducing buffer (10% SDS, 25% 1M Tris (pH 6.8), 0.001% bromophenol blue, 5% β-mercaptoethanol, 50% glycerol and ddH_2O) and boiled at 90°C for 5 minutes. Denatured cell lysate were loaded onto 8% SDS-PAGE along side pre-stained PageRuler protein ladder (Thermo Scientific, Waltham, MA, Cat# 26616) and ran at constant voltage of 120 V for 60 minutes to separate proteins on SDS-PAGE. Separated proteins were transferred to nitrocellulose membrane (Pall Corp. Port Washington, NY) by electrical "wet" transferring method at 400 mA (constant ampere) for 65 minutes.

Blots (nitrocellulose membranes containing transferred proteins) were blocked for 1 hour at room temperature using Licor odyssey blocking buffer (Mandal, Guelph, Canada) and were then probed for the indicated phosphorylated or non-phosphorylated proteins as indicated in figures, at 4°C overnight. Primary antibodies specific for p-Y-STAT-1, STAT 1, p-Y-STAT3 STAT3, p-Y STAT5, STAT5, p-Y-STAT6, p-T/Y-p38, p38, p-T/Y-JNK, p-S-Akt, P-T-Akt and Akt were purchased from Cell Signaling Technology (New England Biolabs Ltd., Canada). Primary antibodies specific for Actin and STAT6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies were diluted 1:1000 in odyssey buffer and were detected using Licor anti-Rabbit or anti-Goat IRDye infrared secondary antibodies at 1:5000 dilution (Mandal, Guelph Canada) and imaged using Licor odyssey infrared scanner (Mandal).

2.3.3 Densitometry Analysis

ImageJ software (National Institutes of Health) was used to analyze band intensity through densitometry. Immunoblot images were imported into the software and each band was selected and compared to the indicated loading control band in the corresponding lane. Band intensity was calculated by software and expressed as fold-change relative to control or unstimulated samples (Lane 1).

2.4 RNA Analysis

2.4.1 RNA Isolation

Once cells reached 70-80% confluence in 75cm^2 cell culture flasks, HASMC were serum deprived for 3 hours (removed 10% FBS RPMI medium and replaced with fresh

2% FBS RPMI medium) followed by stimulation with 2ng/ml OSM and/or 5ng/ml of IL-4, IL-13 or IL-17A for 6 or 18 hours. For experiments examining the affects of STAT-3 inhibition, 30-minute pre-incubation of 2.5 uM of Stattic was used in combination with or without the listed cytokines and incubated for 6 or 24 hours. Cytokine concentrations were diluted in 2% FBS RPMI medium and 10 ml was added to each designated flask. After the specified time point has elapsed, supernatants were removed from cells and RNA was isolated using Ambion PureLink RNA mini kit (Life Technologies, Burlington, ON-Cat. # 12183018A) using manufacturer's instructions.

For PBMC RNA, total PBMC was isolated as described previously and plated in 100 mm^2 dishes at 5 $x10^6$ cells per dish in cRPMI containing 10 ng/ml recombinant M-CSF and cultured for 7 days. The M-CSF containing RPMI was removed and cells were treated with designated concentrations of stimuli (refer to TLR-ligand stimulations) in fresh cRPMI (without M-CSF) for 24 hours or left untreated (Control). Total RNA was prepared as per manufacturer's protocols using PureLink RNA Mini kit (Life Technologies, Burlington, Canada).

2.4.2 Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Genomic DNA was removed from all RNA samples prepared using Ambion DNA-free kit (Life Technologies, Burlington, ON- Cat. #AM1906) as directed by manufacturer's protocol. RNA concentrations and contamination levels were determined using NanoVue spectrophotometer (General Electric, GE). Reverse transcription of RNA was performed using Invitrogen Superscript II Reverse Transcriptase kit (Life

Technologies, Burlington, Cat# 18064014) and was carried out as outlined in manufacturer's protocols. The resulting cDNA was diluted to 2 ng/ml and stored at -20°C for future use.

The diluted cDNA (2ng/ml) was used and prepared to be analyzed by quantitative real-time PCR (qRT-PCR TaqMan). Samples were prepared in duplicates or triplicates in 96-well PCR plate (FroggaBio, North York, ON, Cat# 3426-00) with Taqman Universal Master Mix containing uracil-N-glycosylase (UNG) manufactured by Applied Biosystems (Foster City, CA, Cat#4440038) and predetermined assay reagents (PDAR) purchased from Life Technologies (Burlington, ON) for MCP-1/CCL-2, IL-6, eotaxin-1, eotaxin-3, IL-8, VEGFa (Vascular Endothelial Growth Factor), IL-17RA, IL-17RC, IL-4 receptor alpha (IL-4Rα), gp130/IL-6ST, OSM receptor β (OSMRβ), OSM, MMP-1, MMP-3, MMP-9, TIMP-1 and collagen 1A1 (Col1A1). PDAR for β-Actin (ACTB) manufactured by Applied Biosystems (Foster City, CA, Cat#4333762F) was used as the endogenous control gene. Diluted cDNA, PDAR for indicated genes, Taqman Universal master mix and nuclease free water were added to PCR plate in proportions directed by manufacturer's protocols. Plate was placed, read and analyzed in ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) using a ΔΔCT studies which was operated by Sequence Detector version 2.1 software. The resulting data was corrected to β-Actin threshold cycle value (CT value) and expressed at fold change relative to control (unstimulated) with error bars representing Standard Error of the Mean (SEM) between replicates unless otherwise stated.

2.4.3 Nanostring

HASMC RNA for 6 hours (n=3) and 18 hours (n=2) stimulations were isolated as described previously using PureLink RNA mini kit (Life Technologies). RNA quality was assessed using Agilent Bio-Analyzer (Agilent Technologies). RNA gene expression was assessed using Nanostring conducted by Christine King (Farncombe Metagenomics Facility). Gene expression was graphed as average nanostring counts normalized to β-Actin (endogenous control). Human nanostring probes were designed by Kjetil Ask (MIRC, McMaster University) using reference sequences for MCP-1/CCL2, MCP-3/CCL7, IL-6, VEGFa, Eotaxin-1/CCL11, TSLP, OSM, STAT1, STAT3, STAT4, STAT6, TIMP1, MMP1, MMP3 and MMP9.

2.5 Statistical Analysis

GraphPad Prism version 5.0 for Macs (GraphPad Software, San Diego, CA) was used for graphing and for statistical analyses. Figures represent mean values ± SEM. The student's two-tailed *T*-test was used for the indicated experiments. One- or Two-way analysis of variance (ANOVA) was used to assess statistical differences between means. When one-way ANOVA was used, Tukey post-test was used, unless otherwise stated, to compare means from multiple groups. When Two-way ANOVA was used, Bonferroni post-hoc was used. Difference between means were considered statistically significant at (Confidence Interval) CI of $P<0.05$.

-CHAPTER 3-

RESULTS-PART 1

AUTHOR'S PREFACE TO CHAPTER 3

Many of the results illustrated and described within this chapter have been put into a manuscript and submitted to Respiratory Research on June 18th 2014. The author of this thesis is also first author of the submitted manuscript. The following figures have been incorporated into the submitted manuscript or it's supplementary: **Figure 1-7, 9, 11,13,15,17, 20, and 22.**

Select figures and data illustrating HAoSMC responses have also been submitted into a published paper: **Schnittker, D., Kwofie, K., Ashkar, A., Trigatti, B., and Richards, C. D. (2013) Oncostatin M and TLR-4 ligand synergize to induce MCP-1, IL-6, and VEGF in human aortic adventitial fibroblasts and smooth muscle cells.** ***Mediators of Inflammm,:*****317503** Please be advised that the figure illustrated in this thesis is a different figure than the figures illustrated in the published paper and that the author of this thesis is also second author of the published paper cited above due to her work with HAoSMC and HAAF responses to OSM.

-CHAPTER 3-

RESULTS-PART 1

Previous work done in the laboratory has shown OSM synergy with IL-4, IL-13 and IL-17 in the production of IL-6 by cultured HASMC (C. Richards, personal communication). The production of MCP-1 by HASMC was next examined, since MCP-1 along with other chemokines have been shown to be significantly elevated in the bronchoalveolar lavage fluid of allergic asthmatics when compared to normal individuals (51). In addition, several studies have illustrated the potential significant roles that MCP-1 may play in lung inflammation, cell infiltrations and airway hyper-responsiveness (reviewed in ref. 83). For these reasons, examination of MCP-1 production along with other chemokines by HASMC will be an integral part of examining the responses induced by OSM synergy.

3.1 HASMC responses to OSM and select cytokines

3.1.1 OSM synergizes with IL-4, IL-13 and IL-17 in the production of MCP-1 by Human Airway Smooth Muscle cells (HASMC)

The cell line HASMC 8N2 (refer to **Table 1**) was cultured and assessed for cytokines responses. In **Figure 1** is evidence that the production of MCP-1 by HASMC 8N2 was stimulated by OSM in a synergistic manner. Treatments of IL-4, IL-13 and IL-17A alone induced MCP-1 levels in HASMC supernatants to approximately 300 pg/ml. However, in the presence of OSM, the levels of MCP-1 by these cells significantly increased to concentrations between 1500- 2500 pg/ml. Treatments of HASMC by OSM

alone induced approximately 600 pg/ml; whereas the treatment of IL-4, IL-13 and IL-17A in combination with OSM induced MCP-1 levels substantially greater than the sum of each cytokine alone, which was defined as synergistic. Statistical analysis illustrated that the MCP-1 levels associated with combinations of OSM with IL-4/IL-13/IL-17A stimulations to be significant when compared to levels by either cytokines alone.

3.1.2 OSM synergizes with IL-17A in production of VEGF and IL-8

Figure 2 shows results assessing supernatants of another HASMC cell line (HASMC 8N1) where significant levels of VEGF (**Fig 2A)** and IL-8 (**Fig.2B)** were detected in cultures treated with IL-17A in combination with 1 ng/ml OSM. This was more pronounced when the cells were treated with 5 ng/ml of OSM. Unlike what was observed with MCP-1 levels, OSM did not synergize with IL-4 or IL-13 in the production and release of VEGF and IL-8 indicating selective responses by HASMC to OSM/IL-17A combination.

3.1.3 OSM and IL-17A act synergistically in a dose-dependent manner

Next, HASMC responses were examined when increasing concentrations of recombinant IL-17A and OSM were used. The levels of detected MCP-1 (**Fig 3A**) and IL-6 (**Fig 3B**) significantly increased with increasing concentrations of both IL-17A and OSM stimulation. The detected MCP-1 and IL-6 levels were greatest when cultures were stimulated with 10 ng/ml of both IL-17A and OSM. In addition, levels of MCP-1 (**Fig 3A**) and IL-6 (**Fig 3B**) were significantly greater in cultures stimulated with IL-17A and OSM combinations than either cytokine alone. Based on this data, which has also been confirmed in two other HASMC cell lines (data not shown), the synergy that was

observed between IL-17A and OSM occurred in a dose-dependent manner. Previous work in the laboratory also demonstrated that synergy between OSM and IL-4/IL-13 occurred also in a dose-dependent manner (submitted manuscript, Kwofie *et al.* 2014)

3.1.4 OSM synergizes with IL-4, IL-13 and IL-17A in the expression of selected chemokines/cytokines at the mRNA level

In **Figure 4-5**, HASMC 8N2 were stimulated with 5 ng/ml IL-4, IL-13 and IL-17A with or without 2 ng/ml OSM and incubated for 6 hours (**Fig. 4)** or 18 hours **(Fig. 5).** Here, the results illustrate that OSM interacts with IL-4, IL-13 and IL-17A synergistically in the expression of MCP-1, at both 6 hours (**Fig 4A)** and 18 hours (**Fig. 5A)**. Stimulations of the cytokines alone resulted in relatively lower induction of MCP-1 mRNA levels: OSM alone induced an approximately 9-fold increase in MCP-1 mRNA steady state levels when compared to the unstimulated control; IL-4 stimulation induced 5-fold increase while IL-13 treatment resulted in no fold change relative to the unstimulated control; IL-17A stimulation induced slightly higher MCP-1 mRNA levels than baseline or untreated control at approximately 2-fold induction. When the combinations of OSM/IL-4 and OSM/IL-13 stimulations were used, a 20-25-fold induction was observed, which was greater than the induction caused by either cytokine alone. OSM/IL-17A stimulations resulted in an approximately 50-fold induction of MCP-1 mRNA steady state levels relative to the unstimulated control, this was also a substantial elevation when compared to the levels induced by either cytokine alone (**Fig4A)**. When cells were stimulated for 18 hours, similar patterns were observed

although with greater levels observed (**Fig. 5A).** OSM stimulation alone resulted in a 33-fold induction of MCP-1; an approximate 6-fold and 8-fold induction were observed with IL-13 and IL-4 stimulations alone (respectively); and a 3.5-fold induction of MCP-1 mRNA was observed when cultures were stimulated with IL-17A. Stimulations of HASMC cultures with the combinations of OSM with IL-4, IL-13 or IL-17A resulted 110-140 –fold increase of MCP-1 mRNA levels relative to the unstimulated control, with OSM/IL-17A stimulation inducing the greatest fold change between the three combinations. These results are consistent with protein level trends demonstrated in **figure 1**, which illustrated synergistic stimulation of MCP-1 by OSM and either IL-4, IL-13 or IL-17A.

IL-6 mRNA steady state levels also demonstrated synergistic elevation at 6 hours (**Fig 4B)** and 18 hours **(Fig 5B).** OSM and IL-4 treatments alone stimulated an approximately 5-fold induction meanwhile IL-13 and IL-17A stimulations resulted in about 12-fold and 8-fold induction, respectively, in IL-6 mRNA levels. Stimulation of HASMC cultures with OSM/IL-4 combination however, resulted in a 36-fold induction; OSM/IL-13 stimulation resulted in 20-fold induction, which was slightly higher than the sum of either cytokine alone; and a 133-fold increase in IL-6 mRNA levels were observed when cultures were stimulated with the OSM/IL-17A combination (**Fig 4B)**. Stimulation for 18 hours illustrated pronounced elevation in IL-6 mRNA levels than what was observed at 6 hours (**Fig. 5B**). OSM and IL-13 stimulations alone resulted in 12-13 fold induction of IL-6 mRNA; IL-4 treatment alone resulted in a 30-fold induction; and IL-17A alone stimulated a 4-fold induction in IL-6 mRNA which was lower than what was

observed at 6 hours (**Fig. 4B & 5B).** Stimulation with the OSM/IL-4 combination resulted in an approximate 150-fold increase in IL-6 steady state mRNA; OSM/IL-13 stimulation induced 220-fold increase; and OSM/IL-17A stimulated an induction of approximately 1280-folds relative to the unstimulated control.

When IL-8 steady state mRNA levels were examined, only select stimulations induced elevated IL-8 expression. At 6 hours stimulation, OSM, IL-4 and IL-13 treatments alone resulted in minimal IL-8 induction. When cultures were stimulated with OSM in combination with either IL-4 or IL-13, IL-8 expression was observed to be below baseline, similar to what was observed with OSM treatment alone. In contrast, IL-17A stimulated HASMC cultures resulted in a 15-fold induction of IL-8 mRNA levels. When stimulated with the OSM/IL-17A combination, a 17-fold induction was observed (**Fig. 4C)**. At 18 hours (**Fig. 5C)**, IL-8 mRNA levels were lower in magnitude than what was observed at 6 hours stimulations, however, a similar pattern were observed. OSM, IL-4 and IL-13 treatments alone resulted in baseline IL-8 expression. The same was observed with OSM/IL-4 and OSM/IL-13 combinations. IL-17A treatments alone resulted in a 2-fold induction in IL-8 mRNA steady state levels (**Fig 5C)** which was lower than the 15-fold induction observed at 6 hours (**Fig 4C).** IL-17A in combination with OSM augmented IL-8 mRNA levels in a synergistic manner (9-fold induction compared to baseline). Therefore, in IL-8 expression, OSM synergized with IL-17A and did not do so with IL-4 or IL-13 at 18 hours.

When VEGF expression was examined, 6-hour stimulations did not show synergistic responses by the cultured HASMC (**Fig 4D**). OSM stimulation elevated

VEGF expression 3.5-fold meanwhile, IL-4 IL-13 and IL-17A treatments alone resulted in baseline expression. The combinations of OSM/IL-4 and OSM/IL-17A stimulations stimulated a 3-3.5-fold induction in VEGF expression, the same as OSM alone while OSM/IL-13 treatments resulted in 1.5-fold induction in VEGF steady state mRNA, which was lower than OSM treatment alone (**Fig 4D**). At 18-hours stimulations (**Fig 5D**), OSM treatment alone resulted in a 5-fold induction in VEGF mRNA while IL-4 and IL-17A treatments alone resulted in baseline expression and a 2-fold induction by IL-13 treatment was observed. When HASMC cultures were treated with OSM/IL-4 and OSM/IL-13 combinations, approximately 6-fold inductions in VEGF steady state mRNA were observed which were levels that were the sum of either cytokine alone. On the other hand, OSM/IL-17A treated cell cultures showed a 14-fold induction in VEGF expression, which was greater than the sum of both OSM and IL-17A alone. Therefore, VEGF expression was observed to be synergistic at 18 hours stimulation with OSM/IL-17A combination but not at 6-hours stimulation nor with the other combinations used.

The mRNA levels of Eotaxin-1 (CCL11) were noticeably augmented with OSM and either IL-4 or IL-13 when in combination at both 6 hours (**Fig. 4E)** and 18 hours **(Fig. 5E)**. At 6 hours, OSM alone induced a 35-fold induction in eotaxin-1/CCL11 mRNA meanwhile; IL-4 and IL-13 stimulated a 475-fold and a 140-fold induction in eotaxin expression, respectively. However, the OSM/IL-4 combination induced an approximately 1800-fold elevation in eotaxin-1 and 1100-fold induction was observed by OSM/IL-13 stimulated cell cultures. This elevation was clearly synergistic in nature. In contrast, IL-17A stimulated only 5-fold induction in eotaxin-1, which was substantially

lower than the levels observed with the other treatments but IL-17A in combination with OSM did induce a 90-fold increase in eotaxin-1 (**Fig. 4E)**. These results indicated that OSM synergized with IL-4 and IL-13 in addition to IL-17A, although to a substantially lesser degree (much lower than IL-4 alone), in the expression of eotaxin-1 at 6 hours. At 18 hours (**Fig 5E**), similar results were observed as the 6-hour stimulations. OSM elevated eotaxin-1 mRNA levels 85-fold whereas IL-4 and IL-13 stimulations resulted in 525-fold and 230-fold induction (respectively) in eotaxin-1 expression. In contrast to what was observed at 6 hours, IL-17A in combination with OSM did not result in synergistic expression of eotaxin-1 in marked contrast to IL-4 and IL-13 however, IL-17A alone induced a 5-fold increase in eotaxin-1 mRNA steady state level (**Fig 5E)**, consistent with what was observed at 6 hours stimulation (**Fig 4E**).

When eotaxin-3 (CCL26) mRNA was examined, similar trends were observed as eotaxin-1 where eotaxin-3 was markedly induced by the T_H2 cytokines (IL-4 and IL-13) but in contrast, neither OSM nor IL-17A induced eotaxin-3 expression (**Fig 4F & 5F)**. IL-4 and IL-13 stimulations alone induced a 50-fold and 55-fold elevation, respectively, in eotaxin-3 expression relative to the unstimulated control at 6 hours (**Fig 4F)**. At 18 hours stimulation, IL-4 and IL-13 treatments induced further expression of eotaxin-3; 180-fold elevation was observed with IL-4 treatment while 70-fold induction was observed for IL-13 alone. Although IL-13 induced marked expression of eotaxin-3 mRNA, there was no observed synergistic expression when IL-13 was combined with OSM in either time point (**Fig 4F & 5F)**. In fact, levels of eotaxin-3 were reduced to 30-folds induction at 6-hours when HASMC cultures were stimulated with the OSM/IL-13 combination (**Fig 4F).** In

marked contrast, OSM/IL-4 stimulations resulted in synergistic elevation in eotaxin-3 expression at both time points; approximately 70-fold induction at 6 hours (**Fig 4F)** and 600-fold induction at 18 hours (**Fig 5F**). Stimulations with OSM in combination with IL-17A did not induce any expression of eotaxin-3 at either time points tested (**Fig 4F & 5F).** Thus, OSM did selectively synergize with IL-4 in the expression of eotaxin-3 but not with IL-13, nor IL-17A.

In **Figures 6-7**, a second cell line, HASMC 8N1, was examined for mRNA expression at 6 hours (**Fig. 6)** or 18 hours **(Fig. 7)** in response to OSM, IL-4, IL-13 and/or IL-17A**.** Similar trends were observed in this cell as HASMC 8N2 described above (**Fig 4 & 5**). Here, the results illustrate that OSM acts with IL-4, IL-13 and IL-17A synergistically in the expression of MCP-1 (**Fig 6A & Fig 7A**). OSM alone induced 10-fold induction of MCP-1 mRNA while IL-4, IL-13 and IL-17A treatments alone induced a 5-fold, a 4-fold and a 2-fold elevation in MCP-1 mRNA, respectively. When HASMC cultures were stimulated with OSM in combination with IL-4, IL-13 or IL-17A, MCP-1 steady state mRNA levels were noticeable elevated; OSM/IL-4 and OSM/IL-13 stimulations induced an approximately 40-fold increase in MCP-1 mRNA; OSM/IL-17A stimulated a 24-fold elevation in MCP-1 mRNA relative to the unstimulated control at 6 hours (**Fig 6A**). At 18 hours, synergistic expression of MCP-1 mRNA was only observed with OSM/IL-4 and OSM/IL-17A combinations (19-fold and 20-fold induction, respectively), whereas OSM, IL-4 and IL-17A stimulations alone induced 10-fold, 6-fold and 2-fold elevation in MCP-1 mRNA, respectively. The stimulation of OSM/IL-13 did not induce synergistic expression at this time point (**Fig 7A**). When examining IL-6

steady state mRNA levels, synergistic expression was also observed at both 6 hours (**Fig 6B**) and 18 hours (**Fig 7B**). At 6 hours, OSM stimulation alone induced 3-fold elevation in IL-6 mRNA while IL-4, IL-13 and IL-17A stimulations alone induced a 7- fold, 4-fold and a 1.5-fold increase in IL-6 steady state mRNA, respectively. When HASMC cultures were stimulated with OSM in combination with IL-4, IL-13 or IL-17A, there was an observed elevation of 23-fold, 25-fold and 12-fold (respectively) (**Fig 6B**). At 18 hours, similar results were observed (**Fig 7B**). However, at this time point, OSM/IL-17A combination stimulated a 30-fold induction in IL-6 mRNA level which was greater than the levels observed at 6-hours but both clearly synergistic elevation (**Fig 6B & 7B**).

When IL-8 mRNA levels were examined at 6 hours, there was no synergistic expression induced by the indicated treatments. In fact, OSM at 6 hours in this cell line induced 3-fold induction in IL-8 mRNA while IL-4, IL-13 and IL-17A induced up to 3 – fold induction or no regulation at all (IL-4 stimulation alone). When combinations of OSM with IL-4, IL-13 or IL-17A were used to stimulate the HASMC cultures, up to a 3-fold induction of IL-8 mRNA was observed (**Fig 6C)**. These results differed somewhat was observed in the HASMC 8N2 cell line (**Fig 4C**), however, at18 hours of stimulation (**Fig 7C**), the results were similar between both cell lines. **Figure 7C** shows baseline expression by all treatments with the exception of OSM/IL-17A treatment which induced a 7-fold increase in IL-8 steady state mRNA (**Fig 7C**), therefore illustrated synergistic expression of IL-8 mRNA when OSM is combined with IL-17A but not with either IL-4 or IL-13. These results were also consistent with the protein levels observed in **figure 2B**.

VEGF mRNA levels at 6 hours demonstrated additive expression when HASMC cultures were stimulated with OSM combined with IL-4, IL-13 or IL-17A in this particular cell line (**Fig 6D)**. In contrast, at 18 hours, VEGF steady state mRNA was synergistically elevated when cell cultures were stimulated with OSM/IL-17A combination causing a 10-fold induction in mRNA. OSM and IL-17A treatments alone induced 6.5-fold and baseline induction (respectively) in IL-8 mRNA, thus indicating synergistic expression. Combinations of OSM with either IL-4 or IL-13 did not induce synergistic elevation in VEGF (**Fig 7D**). These results also were consistent with the observations previously described showing synergistic elevation in VEGF protein when HASMC cultures were stimulated with OSM/IL-17A combination (**Fig 2A**).

Eotaxin-1 mRNA levels in HASMC 8N1 demonstrated very similar trends as the previous cell line (HASMC 8N2) at both 6 hours and 18 hours. At 6 hours, OSM alone induced a 20-fold elevation in eotaxin-1 expression whereas IL-4 and IL-13 stimulations alone resulted in a 180-fold and 80-fold induction, respectively, in eotaxin-1 steady state mRNA levels. When OSM was combined with either IL-4 or IL-13, there was marked elevation in eotaxin-1 mRNA levels of 870-fold induction and 600-fold induction, clearly illustrating synergy. IL-17A treatment alone resulted in baseline expression, however when combined with OSM, 34-fold induction of eotaxin-1 mRNA was observed (**Fig 6E**) which was also synergistic. At 18 hours (**Fig 7E)**, similar trends were observed as those at 6 hours however; the magnitude in fold induction was slightly less at 18 hours (**Fig 7E**).

In examining eotaxin-3 expression, synergistic expression was observed when HASMC 8N1 cultures were stimulated with OSM in combination with either IL-4 or IL-

13 at both 6 hours (**Fig 6F**) and 18 hours (**Fig 7F**). At 6 hours, IL-4 and IL-13 stimulations alone induced a 66-fold and a 30-fold increase, respectively, meanwhile OSM/IL-4 and OSM/IL-13 combinations resulted in a 83-fold and a 48-fold elevation (respectively) in eotaxin-3 steady state mRNA levels. Neither OSM or IL-17A nor the combination of the two cytokines induced eotaxin-3 expression (**Fig 6F**). At 18 hours (**Fig 7F**), the same trend was observed although greater fold changes were observed at this time point compared to 6-hour steady state levels. These results are similar to those observed in HASMC 8N2, with the exception of the OSM/IL-13 stimulation, which resulted synergistic expression in the mRNA levels in HASMC 8N1(**Fig 6F& 7F**) but did not in HASMC 8N2 (**Fig 4F& 5F**).

The data presented in **Figure 4 - Figure 7** demonstrate that OSM synergy with IL-4, IL-13 or IL-17A was observed at the mRNA level and was selective in the cytokine/chemokine expression by HASMC. As complementary analysis to qRT-PCR, HASMC responses to OSM alone were examined using nanostring technology at both 6 hours and 18 hours (**Fig 8**) to confirm previous results and examine other responses. Here, MCP-1 (**Fig 8A**), IL-6 (**Fig 8B**), VEGF (**Fig 8C**) and eotaxin-1 (**Fig 8D**) where elevated, as indicated by elevated nanostring counts, by OSM stimulation in multiple HASMC experiments (n=4) at 6 hours and similarly at 18 hours (n=2), which confirmed previous observations in **Figure 4-7**. MCP-3 mRNA levels were also elevated upon OSM stimulations by HASMC at both 6 hours and 18 hours (**Fig 8E**). TSLP steady state mRNA levels had a slight elevation, although not statistically significant, when HASMC cultures were stimulated with OSM (**Fig 8F**). OSM mRNA levels were also examined to

indicate whether recombinant OSM stimulation could induce HASMC expression of OSM mRNA. No OSM expression was observed by the cultured HASMC, as indicated by the nanostring counts of 20 or less (**Fig 8G**).

3.1.5 OSM but not other gp130 cytokines induce marked responses in cultured HASMC

To test if the responses observed were unique to OSM amongst the other gp130 cytokines, HASMC 8N1 cell cultures were treated with OSM, LIF, IL-11, IL-31 and IL-6 to compare OSM to other members of this family. HASMC 8N1 cell signaling responses were tested using standard immunoblot methods after 20 minute-treatments with human recombinant OSM, LIF, IL-11, IL-31 and IL-6 all at 10 ng/ml (relevant molarity also shown- **Figure 9)** as described in material and methods.

Figure 9 shows that OSM activated STAT1, 3, and 5, p38 and JNK (MAPK) in HASMC as assessed by the increased phosphorylated form of these molecules. The other gp130 cytokines did not or did so minimally. Densitometry analysis **(Figure 9B)** illustrated this quantitatively. These observations were observed in multiple HASMC cell lines (data not shown) and was also demonstrated in Human Aortic Smooth Muscle Cells (HAoSMC) (**Figure 10A,** modified from Schnittker, D, Kwofie, K et al (129))**,** albeit these cells activated STAT6 to higher level and JNK to a lesser degree when compared to HASMC based on the detected level of phosphorylation in immunoblots**.** Although HAoSMC responded similarly to HASMC when treated with OSM, no synergistic responses to OSM with IL-4, IL-13 or IL-17 in the levels of IL-6 in the cell supernatants were detected (**Fig 10B)**.

3.1.6 IL-4, IL-13 and IL-17A in combination with OSM activate STATs and MAPK cell signaling pathways in multiple HASMC cell lines

In **Figure 11** three HASMC cell lines (HASMC 8N1, HASMC 8N2 and HASMC 6N) were treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media only) with or without 1 ng/ml OSM and incubated for 20 minutes. HASMC cell signaling was assessed using immunoblots as described previously.

Figure 11A illustrates western blots from one of three cell lines that demonstrate activation of multiple cell signaling pathways where densitometry analysis was completed and averaged to all three **(Fig 11B).** STAT 3 activation was observed as indicated by increased detection of phospho-STAT-3 at tyrosine 705 when HASMC cultured were treated with OSM alone or in combination with IL-4, IL-13 or IL-17A (**Fig. 11A and B)**. Activation of the AKT pathway was also observed through the increased detection of phosphorylated AKT at serine 473 when cells were treated with OSM alone or in combination with the indicated cytokines. Levels of phosphor-p38 (MAPK) appeared to be augmented when cells were stimulated with IL-17A and OSM together when compared to either treatment alone and this was statistically significant in all cell lines based on densitometry analysis.

To determine if OSM may also modulate signaling through regulating the expression of STAT proteins, multiple HASMC experiments were used to assess mRNA expression of four STAT proteins: STAT1, 3, 4 and 6 at 6 hours and 18 hours using nanostring technology (**Figure 12**). Here, OSM stimulation resulted in elevated mRNA levels of STAT 1(**Fig 12A**) and STAT3 (**Fig 12B**) at both 6 hours and 18 hours. STAT 4

and STAT6 resulted in no significant change in the mRNA levels as indicated by the nanostring counts (**Fig 12 C& D**).

3.2 Examination of potential mechanisms of OSM synergy in cultured HASMC

The synergistic responses observed in cultured HASMC could be the result of many possible mechanisms, one of which may be due increased cell signaling leading to increased activation of transcription factors. This would lead to increased expression of relevant and possibly selected cytokines/chemokines. Thus, the cell signaling networks that may contribute to the OSM-induced synergistic responses by HASMC were assessed. Alternatively, the observed responses by HASMC may be a consequence of increased expression of related receptors. Work done previous within the laboratory has demonstrated that the OSM and IL-4/IL13 synergy in the stimulation of eotaxin-1 in fibroblasts was correlated to the marked elevation of IL-4Rα and IL-13Rα1 stimulated by OSM(113). Similar findings were found in HASMC (41) in which OSM induced increased production of IL-4Rα. Previous work in this laboratory has also shown an up-regulation of TLR3 in response to OSM and poly (I:C) in HASMC(149). This examination of receptors involved in response to OSM or IL-4, IL-13 or IL-17A was assessed at the RNA level as an indication of another potential mechanism in which OSM-synergistic responses by HASMC is observed.

3.2.1 OSM synergy with IL-4, IL-13 and Il-17A is likely not through modulation of mTOR activity

Since previous evidence suggested that OSM activates AKT pathway (**Fig 9A & 11A**), OSM synergy with IL-4, IL-13 or IL-17A maybe rapamycin sensitive. Upon examination with two HASMC cell lines and increased concentration of rapamycin (**Fig 13A-C)**, there was no consistent indication that rapamycin, an inhibitor of mTOR, reduced OSM-induced synergistic responses by HASMC.

3.2.2 Determining optimal concentration of Stattic (STAT inhibitor)

To assess the effects of STAT signaling in HASMC cells, the STAT-3 inhibitor, Stattic, was chosen. To determine concentrations of the STAT-3 inhibitor, Stattic, that would not alter cell viability, the agent was applied at increasing doses (0 – 10 uM). The responses by two HASMC cell lines were examined through detection of IL-6 and MCP-1 protein in cell supernatants (**Figure 14). Figure 14A** illustrates decreased levels of MCP-1 and IL-6 by HASMC 8N2 pre-treated (30 minute pre-incubation) with increasing concentrations of Stattic -with or without 1 ng/ml OSM. **Figure 14B** also shows that HASMC 8N1 responded with decreasing production of MCP-1 and IL-6 in response to the increasing concentrations of Stattic. Upon examination of cell viability and reduction in IL-6 and MCP-1 levels, it was determined concentration that of 1.25uM, 2.5 uM and 5.0 uM were acceptable concentrations (minimal viability change) to use for further studies using HASMC cell lines.

Figure 15 indicates the observation that Stattic abrogated OSM-induced STAT1, STAT3 and STAT5 activation at the concentrations used. OSM-induced STAT3

activation appeared less sensitive to the Stattic application than that of STAT1 and STAT5 activation. However, 5 uM Stattic treatment resulted in a complete reduction in the activation of STAT3 by OSM.

In **Figure 16,** STAT3 and STAT1 activation were examined in HASMC 8N1 and HASMC 8N2 (passage 5) to directly compare the responses between each cell line. Similar to what was observed in **Fig 15**, STAT 1 activation was abrogated using the lowest concentration of Stattic (1.25uM) in both cell lines. STAT-3 activation was minimally reduced using 1.25 uM Stattic but at 2.5 uM a substantial reduction was observed in STAT 3 activation.

3.2.3 Responses by cultured HASMC are reduced by treatments of Stattic (STAT inhibitor), SB (p38 selective inhibitor) and PD (ERK MAPK inhibitor)

In **Figure 17**, HASMC 8N1 were cultured in the presence of 1.25- 5 uM Stattic for 30 minutes. Cells were subsequently treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml OSM. Cells were incubated for 18 hours and ell supernatants were examined for levels of MCP-1 and IL-6 using standard ELISA protocols.

At the lowest concentration of Stattic (1.25uM), significant reduction in the levels of IL-6 (**Fig 17A)** by cells treated with IL-17A and OSM in combination was observed. However, there were no observed changes with the other combinations. In the production of MCP-1 **(Fig, 17B),** the synergy between IL-17A and OSM was significantly reduced (more than 50% reduction) only when in combination. This drop in MCP-1 production is

not observed with either cytokine alone. IL-4 and OSM synergy in the production of MCP-1 was also significantly reduced however, the magnitude observed here was clearly less than the reductions observed in cells treated with IL-17A/OSM. This suggests that the synergic responses by HASMC to IL-17A/OSM are substantially more sensitive than the other combinations to STAT inhibition. When cells were exposed to 2.5 uM Stattic (**Fig. 17D**), a further decrease in the induction of MCP-1 in HASMC was observed. OSM/IL-17A combination showed the greatest reduction (by approximately 70%) in MCP-1 production whereas OSM/IL-4 and OSM/IL-13 combinations showed about a 50% reduction in MCP-1 levels. IL-6 production at 2.5uM **(Fig. 17C)** appeared to be significantly reduced by 50-60% only in OSM/IL-17A combinations whereas the other combinations did not have any diminished IL-6 production in the presence of 2.5 uM. It was also observed that this concentration of Stattic resulted in significant reduction in IL-6 levels when cells were treated with IL-17A alone. At 5uM, an even further decrease in IL-6 production was observed **(Fig.17E)** most notably in cells treated with OSM/IL-17A combination. MCP-1 levels **(Fig. 17F)** were substantially reduced regardless of the cytokine treatment.

Figure 18 shows MCP-1 levels in the cell culture supernatants of a second cell line (HASMC 8N2). Marked reductions were observed in the levels of MCP-1 in the supernatants of cells treated with IL-4, IL-13 or IL-17A in combination with OSM if Stattic was present at either 1.25 uM (**Fig 18A**) or 2.5 uM (**Fig 18B**). Consistent with what was observed in **Figure 17,** it appeared that IL-17A/OSM synergistic induction of

MCP-1 was more sensitive to Stattic than the other combinations**.** Cells exposed to 2.5 uM of Stattic **(Fig. 18B)** had supernatant levels of MCP-1 nearly abolished.

Above are described results that indicate that inhibition of STAT3 through the use of Stattic could significantly reduce HASMC responses, specifically synergistic responses to OSM and IL-4, IL-13 or IL-17A in terms of MCP-1 and IL-6 protein detection in cell supernatants. To determine if this was reflected at the mRNA level, RNA from cell cultures after 24 hours were assessed. In **figure 19,** HASMC 8N2 cells were cultured as previously described and treated with 5 ng/ml IL-4, IL-13 or IL-17A with or without 1 ng/ml OSM in the presence or absence of Stattic (2.5 uM) in which cells were pre-incubated for 30 minutes prior to stimulation. Cells were incubated for 24 hours Total RNA isolated after 24 hours was analyzed for MCP-1 (**Fig 19A**) and IL-6 (**Fig 19B**).

Here, stimulation of HASMC 8N2 with the indicated cytokines for 24 hours showed synergistic expression of MCP-1 **(Fig 19A),** although the magnitude was substantially less that the fold induction that was observed previously at both 6 (**Fig 4A**) and 18 hours (**Fig 5A**). In terms of IL-6 expression, mRNA levels were also substantially lower at 24 hours when compared to 6 and 18 hours (**Fig 4B & 5B**) with the exception of OSM/IL-17A-induced IL-6 expression which resulted 200-fold elevation in IL-6 mRNA **(Figure 19B)**. More importantly, the treatment of these cells with 2.5 uM of Stattic tended to reduce OSM/IL-4 and OSM/IL-17A -induced MCP-1 expression, specifically, an approximate 50% reduction **(Figure 19A).** IL-6 expression induced by OSM/IL-17A combination was reduced by approximately 25% when cells were treated with 2.5uM Stattic **(Figure 19B)**. The reduction in MCP-1 expression by 2.5 uM Stattic mirrored

what was observed at 18 hours at the protein level indicating that protein levels after 24 hours may have been further reduced but may need to be examined as well.

To assess modulation through other cell signaling pathways, HASMC responses in the presence or absence of a p38 inhibitor (SB 203580), an ERK inhibitor (PD 98059) and an Akt inhibitor (Akt X) were examined. In **figure 20**, HASMC responses to OSM synergy with IL-4, IL-13 or IL-17A were assessed in the same manner described in **figure 17**. In **figure 20A,** markedly diminished levels of IL-6 were observed in the supernatants of HASMC 8N1 when cells were pre-treated with SB203580. This reduction in IL-6 suggested the possibility that the concentration of inhibitor was excessive. However, when MCP-1 levels were later examined within these supernatants (**Fig 20B)**, results showed that the p38 inhibitor (at this concentration) selectively and significantly reduced OSM/IL-17A synergistic induction of MCP-1 by approximately 30%. Other combinations did not have significant effect in the reduction of MCP-1 associated with the use of the p38 inhibitor. Next assessed was ERK (MAPK) by using PD98059, an ERK inhibitor. Here, **(Fig.20C**) significant reductions in the stimulation of IL-6 were observed when HASMC cultures were exposed to the ERK inhibitor. In the assessment of MCP-1 levels (**Fig 20D)**, OSM/IL-17A synergistic induction of MCP-1 in HASMC 8N1 was reduced by approximately 30% whereas the other combinations had no significant reduction when the ERK inhibitor was applied. Next, Akt cell-signaling pathway was examined by utilizing Akt X, an inhibitor of Akt activation. In the induction of IL-6 (**Fig, 20E)** and MCP-1 **(Fig.20F),** Akt X did not seem to alter the levels of IL-6 in

the cell supernatants however; MCP-1 levels were reduced significantly in all treatments according to statistical analysis.

Based on the substantial reduction in IL-6 levels when SB203580 was applied, levels of IL-6 protein in HASMC 8N1 supernatants were assessed after treatments of three lower concentrations of SB 203580. When the cells were exposed to 1 uM of SB **(Fig. 21A),** significant effects were observed when cells were treated with OSM/IL-4 and OSM/IL-17A combinations resulting in an approximately 50% reduction in OSM/IL-17A-induced IL-6 levels. At the higher concentrations of SB 203580 (**Fig 21B-C)** further reductions in the detection of IL-6 were observed.

3.2.4 OSM synergy was associated with IL-4Rα induction but neither IL-17RA nor IL-17RC were regulated.

To continue examining the mechanisms associated with OSM-induced synergistic responses in HASMC, whether OSM could up-regulate the receptors for IL-4 and IL-17A signaling was examined. Two HASMC cell lines, HASMC 8N1 and HASMC 8N2 were treated for 6 hours and subsequently processed for RNA levels (**Figure 22**). It was observed that OSM induced a 2.5 to 3-fold increase in the expression of IL-4Rα **(Fig. 22E)** which is consistent with previous publications(41, 113) this suggests that OSM synergy with IL-4 and IL-13 was due to receptor up-regulation which primes the cells to IL-4 and/or IL-13(113) therefore leading to increased STAT-6 activation and subsequent synergistic eotaxin-1 expression(41, 42). In contrast neither IL-17RA nor IL-17RC mRNA was regulated in either cell line by OSM stimulation however; there was a 4-fold

increase in IL-17RA mRNA expression in both cell lines when treated with IL-4. Expression of OSMR and gp130 were examined as well. OSM induced a 3-fold increase in gp130 expression and 4-fold increase in OSMR expression while the other cytokines did not alter OSMR nor gp130 expression. Therefore, these results indicated that OSM did not regulate IL-17A receptor chains but did confirm previous publications regarding IL-4Rα.

3.3 HASMC proliferation and changes in extracellular matrix protein expression in response to OSM

Patients with severe asthma show substantial thickening of the airway sub epithelial smooth muscle (151) and thus, the following experiments examined whether OSM (or IL-4/IL-13/IL-17A) altered proliferation of airway smooth muscle cells or extracellular matrix proteins or enzymes *in vitro.*

3.3.1 HASMC proliferation is altered by OSM but not further with OSM in combination with IL-4, IL-13 or IL-17A

In **Figure 23,** HASMC were cultured as previously described. Cells were serum derived, using 2% FBS RPMI for 3 hours and subsequently stimulated with 5 ng/ml IL-4, IL-13 or IL-17A (or left unstimulated) in the presence or absence of 1 ng/ml OSM and 5 ng/ml OSM. Cells were incubated for 48 hours (2 Days) **(Fig.23A)** or 7 days **(Fig 23B)**. MTT assays using tetrazolium dye and cell counts using trypan blue (data not shown) were completed as an indication of HASMC cell numbers and thus proliferation.

Here, it observed that only cells in the presence of OSM had statistically significant increases in formazan measurements (correlating with cell number). There was no observed alteration in cell number due to synergistic effects of OSM in combination with IL-4, IL-13 or IL-17A **(Fig 23)**.

3.3.2 HASMC expression of extracellular matrix remodeling proteins at both 6 hours and 18 hours

To explore whether these cytokines can affect expression of various extracellular proteins that may play a role in severe asthma, HASMC mRNA levels of collagen 1A1 (Col1A1), Tissue Inhibitors of Matrix Metalloproteinases-1 (TIMP-1), Matrix Metalloproteinase-1 (MMP-1), MMP-3 and MMP-9 at both 6 Hours (**Figure 24 and 26)** and 18 hours (**Figure 25 and 27).** HASMC RNA was isolated from HASMC 8N2 (**Fig. 24 and 25**) and HASMC 8N1 (**Fig. 26 and 27)** as previously described. In **figure 24**, it was observed that OSM treatment of HASMC 8N2 caused a slight induction of Collagen 1A1, approximately 1.4 fold induction, whereas IL-4, IL-13 and IL-17A resulted in no change in mRNA levels at 6 hours. TIMP-1 expression in HASMC was elevated (2-fold increase) by OSM while IL-4, IL-13 and IL-17A resulted in baseline expression at this time point. MMP-1 expression by the cells at 6 hours all resulted in baseline expression with the exception of OSM treated cells that had a slight increase in the mRNA level. Cells treated with OSM markedly elevated MMP-3 expression but IL-4, IL-13 and IL-17A induced expression remained close to baseline.

At 18 hours, more distinct trends in the expression of these genes were observed **(Figure 25).** Here, at 18 hours, a clear elevation in the expression of collagen by IL-17A

treated cells (3 fold increase) was observed while IL-4 and IL-13 treatments resulted in baseline expression. OSM treated cells also showed close to baseline expression but slight elevated at about 1.3 fold induction. Similar to that observed at the 6-hour time point, OSM treated HASMC doubled TIMP-1 expression whereas IL-17A induced a 3.5-fold increase in the mRNA steady state levels. IL-13 and IL-4 treated cells showed baseline expression of TIMP-1 mRNA. In MMP expression (MMP-1 and MMP-3), slight induction by OSM (approximate 1.5 fold induction) was observed whereas IL-17A treated HASMC resulted in suppression of MMP-1 and MMP-3 mRNA expression.

In HASMC 8N1 cells (**Figure 26- 27)** some consistent results were observed. At 6 hours, OSM stimulated slight elevations in TIMP-1 **(Fig 26B)** and MMP-3 (**Fig 26D)** steady state mRNA whereas the other stimulations resulted in baseline expression which was similar to HASMC 8N2. However, collagen 1A1(**Fig 26A)** and MMP-1 **(Fig 26C)** mRNA levels appeared to be reduced upon OSM stimulation in this particular cell line. At 18 hours, similar trends to HASMC 8N2 were observed. TIMP-1 (**Fig 27B)** and MMP-3 (**Fig 27D)** mRNA levels were elevated by OSM stimulation (7.5-fold and 4-fold induction, respectively) while the other treatments resulted in baseline steady state mRNA levels (**Fig 27B & 27D)**. Below baseline levels were observed with IL-13 and IL-17A stimulations (**Fig 27A).** A 3-fold induction in MMP-1 mRNA levels were observed with OSM stimulation (**Fig 27C)** which was in marked contrast to 6 hours MMP-1 expression (**Fig 26C).** Treatments of IL-4, IL-13 or IL-17A resulted in 2-fold induction of MMP-1 steady state mRNA (**Fig 27C)**. Between two HASMC cell lines, consistent elevation by OSM stimulation was observed in TIMP-1 expression and MMP-3 expression through

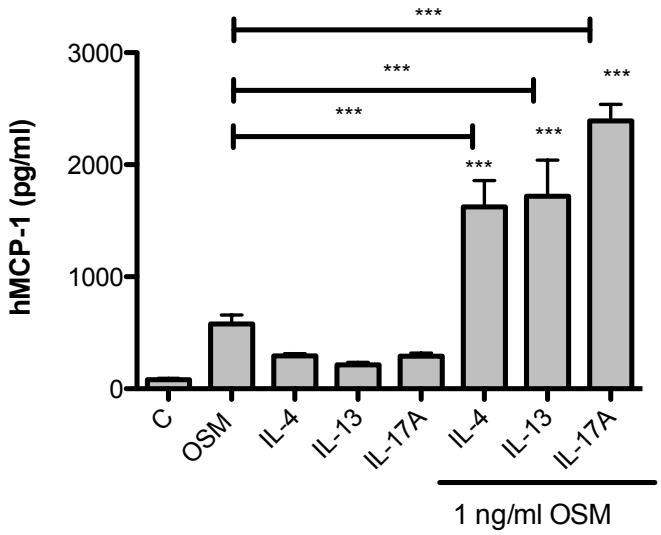
qRT-PCR. When mRNA levels were examined using nanostring technology, TIMP-1 mRNA levels appeared to be elevated by OSM at 6 hours and more pronounced at 18 hours (**Fig 28A)**. MMP-1 and MMP-3 mRNA levels were not modulated by OSM as detected by nanostring counts (**Fig 28B & 28 C**) when averaging responses of three cell lines. There was no expression of MMP-9 by these cells as detected by nanostring (**Fig 28D)** and qRT-PCR (data not shown).

FIGURE 1- OSM acts synergistically with IL-4, IL-13 and IL-17A in the stimulation of MCP-1 in HASMC

HASMC 8N2 were cultured in 96-well plates. Sub-confluent cells were serum-deprived in 2% FBS RPMI for 3 hours. Cells were then treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml OSM. Cells were incubated for 18 hours. Cell supernatants were collected and analyzed for levels of MCP-1. Graph illustrates mean ± SEM and one-Way ANOVA with Tukey post-hoc analysis where ***=P<0.0001 when comparing samples to unstimulated control and when comparing OSM with IL-4/IL-13/IL-17A to OSM alone (above lines).

FIGURE 2- OSM synergy with IL-17A but not IL-4 and IL-13 in the stimulation of IL-8 and VEGF production. HASMC 8N1 were plated in 96-well plates. Sub-confluent cells were serum deprived for 3 hours using 2% FBS RPMI. Cells were then treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml or 5 ng/ml OSM. Cells were incubated for 18 hours. Cell supernatants were collected and analyzed for levels of (A) VEGF and (B) IL-8. Graphs illustrate mean ± SEM and One-Way ANOVA with Tukey post-hoc analysis (**=P<0.001; ***=P<0.0001) when comparing samples to unstimulated control (and when comparing OSM in combination with IL-4/IL-13/IL-17A to OSM alone (above lines).

FIGURE 1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3000
2000
1000
0
***
***
***
***
***
***
C
OSM
IL-4
IL-13
IL-17A
IL-4
IL-13
IL-17A
1 ng/ml OSM

FIGURE 2

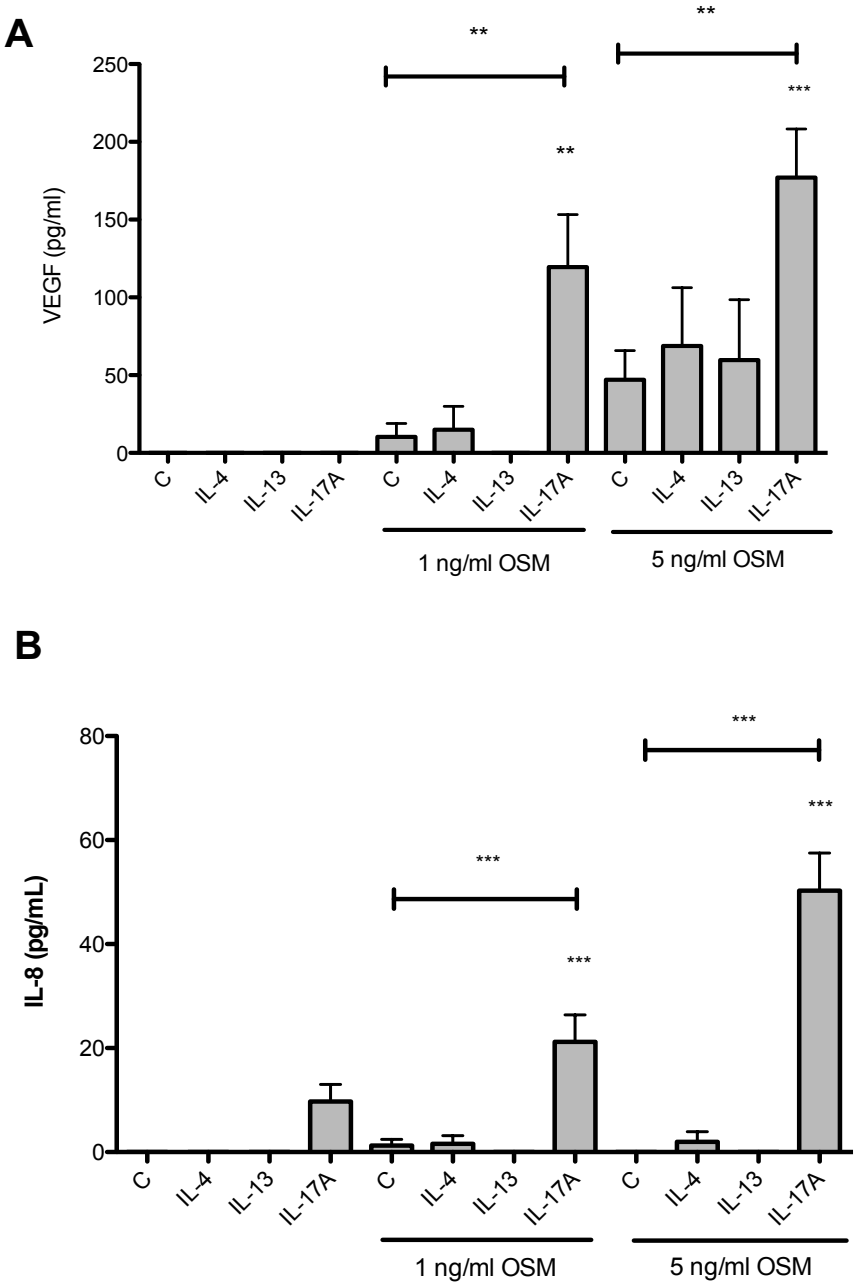
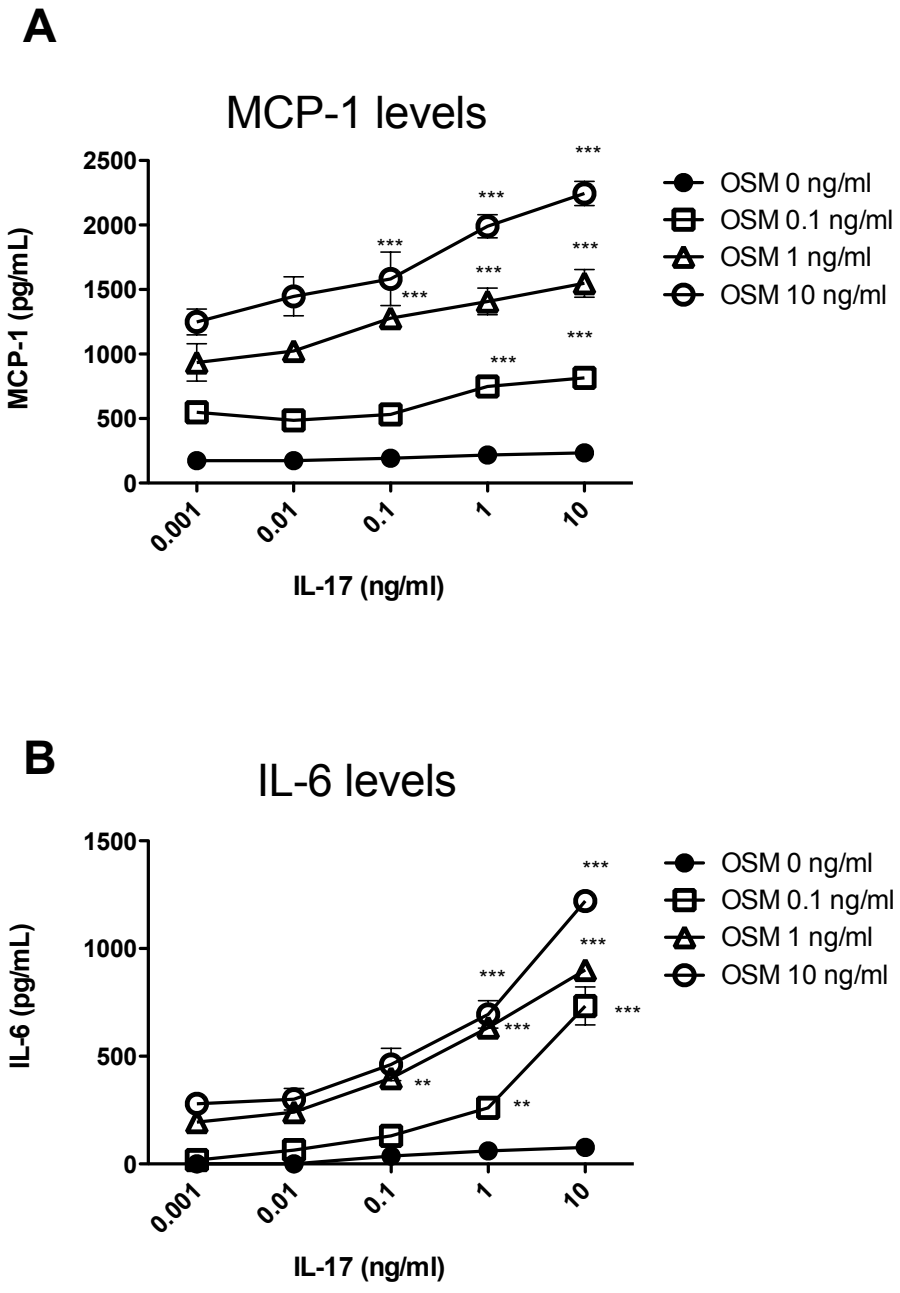

A
VEGF (pg/ml)
250
200
150
100
50
0
**
**
**
***
C
IL-4
IL-13
IL-17A
C
IL-4
IL-13
IL-17A
C
IL-4
IL-13
IL-17A
1 ng/ml OSM
5 ng/ml OSM
B
IL-8 (pg/mL)
80
60
40
20
0
***
***
***
***
C
IL-4
IL-13
IL-17A
C
IL-4
IL-13
IL-17A
C
IL-4
IL-13
IL-17A
1 ng/ml OSM
5 ng/ml OSM

FIGURE 3- OSM synergy with IL-17A in the stimulation of MCP-1 and IL-6 production is in a dose-dependent manner. HASMC 8N1 were plated in 96-well plates. Sub-confluent cells were serum deprived for 3 hours using 2% FBS RPMI. Cells were then treated with increasing concentrations of recombinant human IL-17A (0.000-10 ng/ml) with the indicated concentrations of recombinant human OSM. Cells were incubated for 18 hours. Supernatants from each well were collected and analyzed for (A) MCP-1 and (B) IL-6 levels. The graph shows the mean ±SEM with a two-Way ANOVA with Bonferroni post-hoc analysis (***=P<0.001) when comparing either cytokine (OSM or IL-17A alone)

FIGURE 4- OSM synergy with IL-4, IL-13 and IL-17A in the stimulation of selective cytokines/chemokines by HASMC 8N2 at mRNA level at 6 hours. Cultured HASMC 8N2 treated with 1 ng/mL of recombinant OSM with 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 6 hours. Total RNA was isolated as described in methods. Gene expression were analyzed using qRT-PCR (Taqman) for (A) MCP-1 expression, (B) IL-6 expression, (C) IL-8 expression, (D) VEGF expression, (E) Eotaxin-1 expression and (F) Eotaxin-3 expression shown as mean fold change relative to control using human β-Actin as the endogenous control.

FIGURE 3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MCP-1 levels
MCP-1 (pg/mL)
2500
2000
1500
1000
500
0
0.001
0.01
0.1
1
10
IL-17 (ng/ml)
OSM 0 ng/ml
OSM 0.1 ng/ml
OSM 1 ng/ml
OSM 10 ng/ml
B
IL-6 levels
IL-6 (pg/mL)
1500
1000
500
0
IL-17 (ng/ml)

FIGURE 4

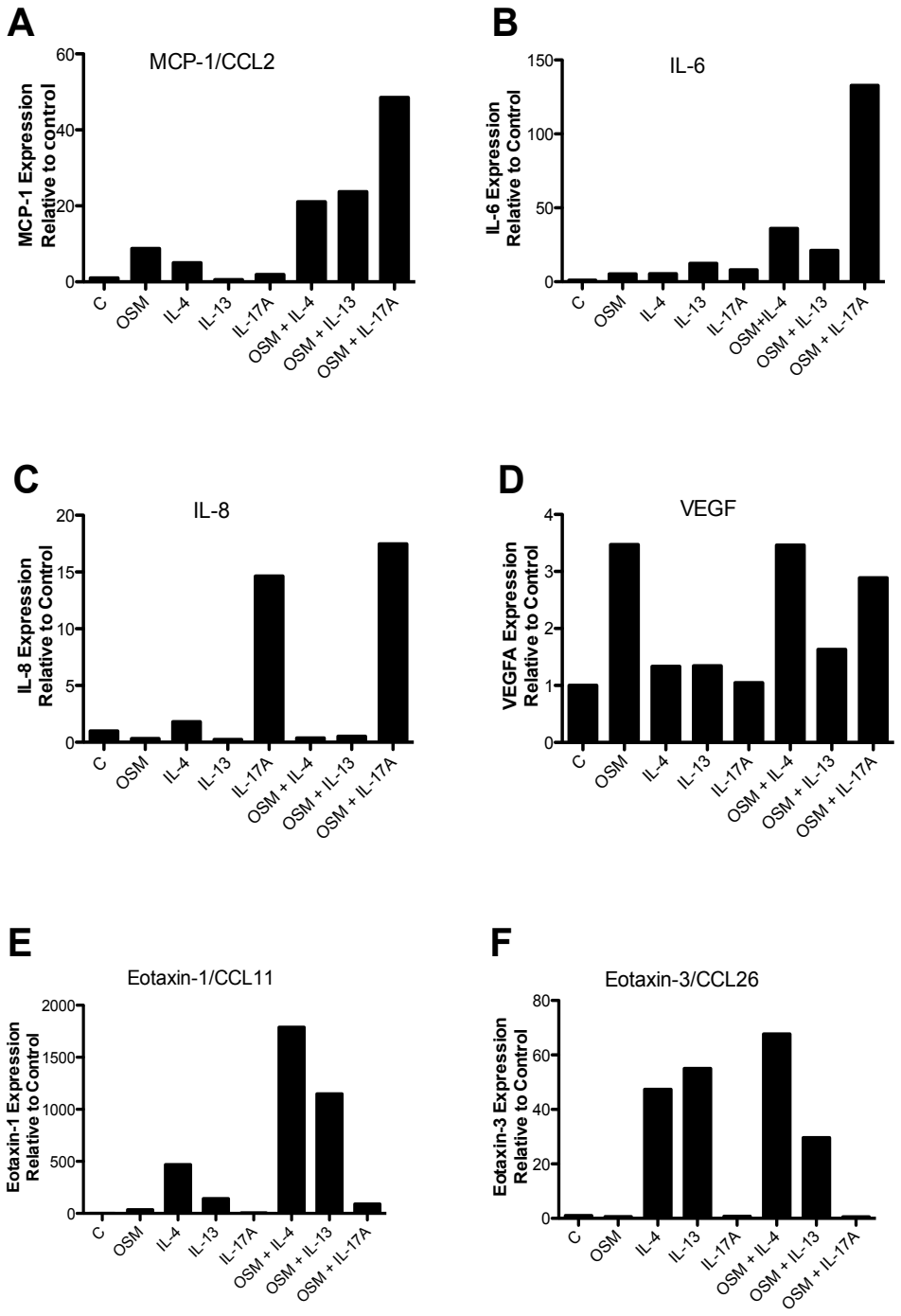

A
MCP-1/CCL2
MCP-1 Expression Relative to control
60
40
20
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
B
IL-6
IL-6 Expression Relative to Control
150
100
50
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM + IL-13
OSM + IL-17A
C
IL-8
IL-8 Expression Relative to Control
20
15
10
5
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
D
VEGF
VEGFA Expression Relative to Control
4
3
2
1
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
E
Eotaxin-1/CCL11
Eotaxin-1 Expression Relative to Control
2000
1500
1000
500
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
F
Eotaxin-3/CCL26
Eotaxin-3 Expression Relative to Control
80
60
40
20
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A

FIGURE 5- OSM synergy with IL-4, IL-13 and IL-17A in the production of selective cytokines/chemokines by HASMC 8N2 at mRNA level at 18 hours. Cultured HASMC 8N2 treated with 1 ng/mL of recombinant OSM with 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 18 hours. Total RNA was isolated as described in methods. Gene expression were analyzed using qRT-PCR (Taqman) for (A) MCP-1 expression, (B) IL-6 expression, (C) IL-8 expression, (D) VEGF expression, (E) Eotaxin-1 expression and (F) Eotaxin-3 expression shown as mean fold change relative to control using human β-Actin as the endogenous control.

FIGURE 6- OSM synergy with IL-4, IL-13 and IL-17A in the production of selective cytokines/chemokines by HASMC 8N1 at mRNA level at 6 hours. Cultured HASMC 8N1 treated with 1 ng/mL of recombinant OSM with 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 6 hours. Total RNA was isolated as described in methods. Gene expression were analyzed using qRT-PCR (Taqman) for (A) MCP-1 expression, (B) IL-6 expression, (C) IL-8 expression, (D) VEGF expression, (E) Eotaxin-1 expression and (F) Eotaxin-3 expression shown as mean fold change relative to control using human β-Actin as the endogenous control.

FIGURE 5

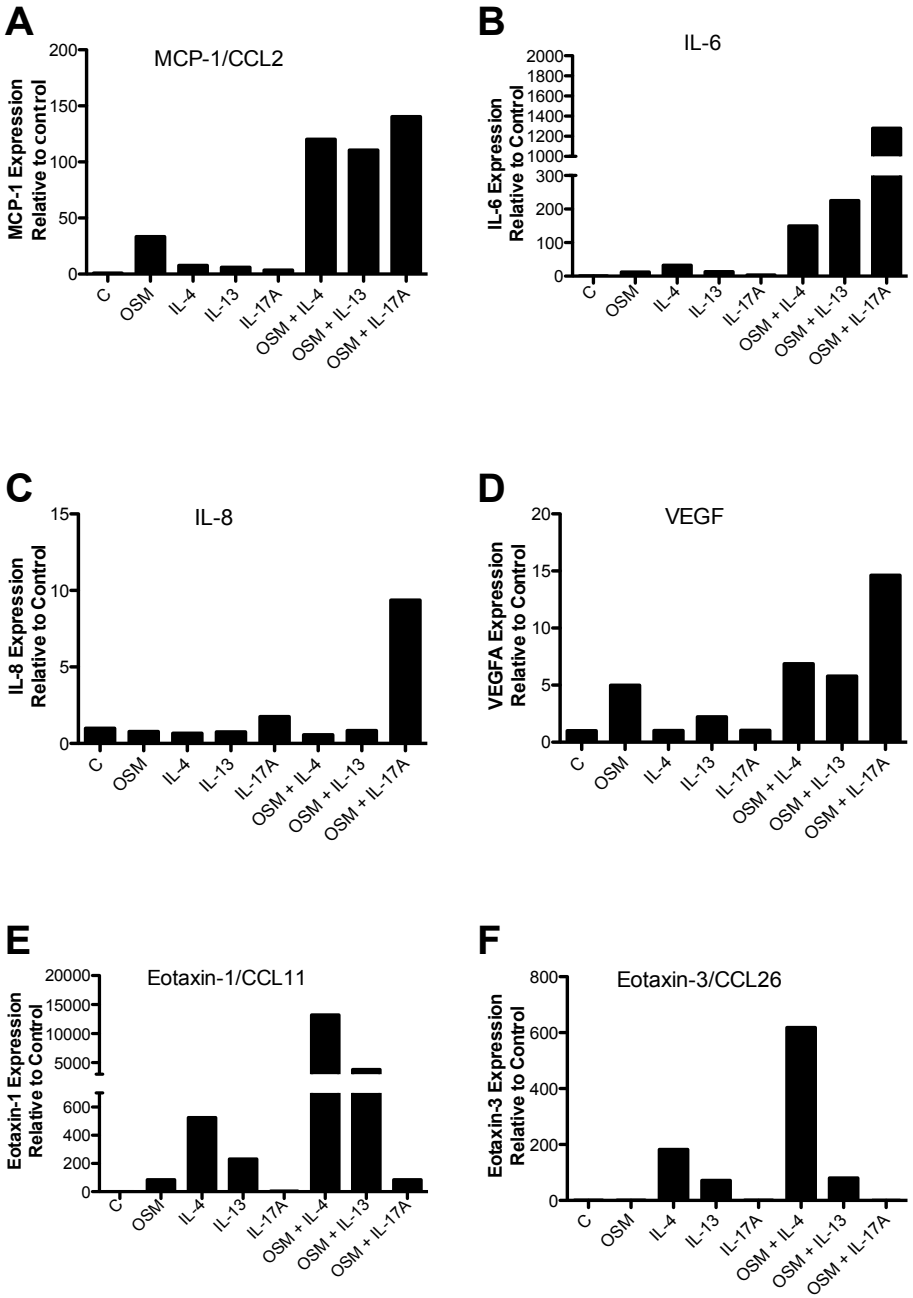

A
MCP-1/CCL2
MCP-1 Expression Relative to control
200
150
100
50
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
B
IL-6
IL-6 Expression Relative to Control
2000
1800
1600
1400
1200
1000
300
200
100
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
C
IL-8
IL-8 Expression Relative to Control
15
10
5
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
D
VEGF
VEGFA Expression Relative to Control
20
15
10
5
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
E
Eotaxin-1/CCL11
Eotaxin-1 Expression Relative to Control
20000
15000
10000
5000
600
400
200
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A
F
Eotaxin-3/CCL26
Eotaxin-3 Expression Relative to Control
800
600
400
200
0
C
OSM
IL-4
IL-13
IL-17A
OSM + IL-4
OSM + IL-13
OSM + IL-17A

FIGURE 6

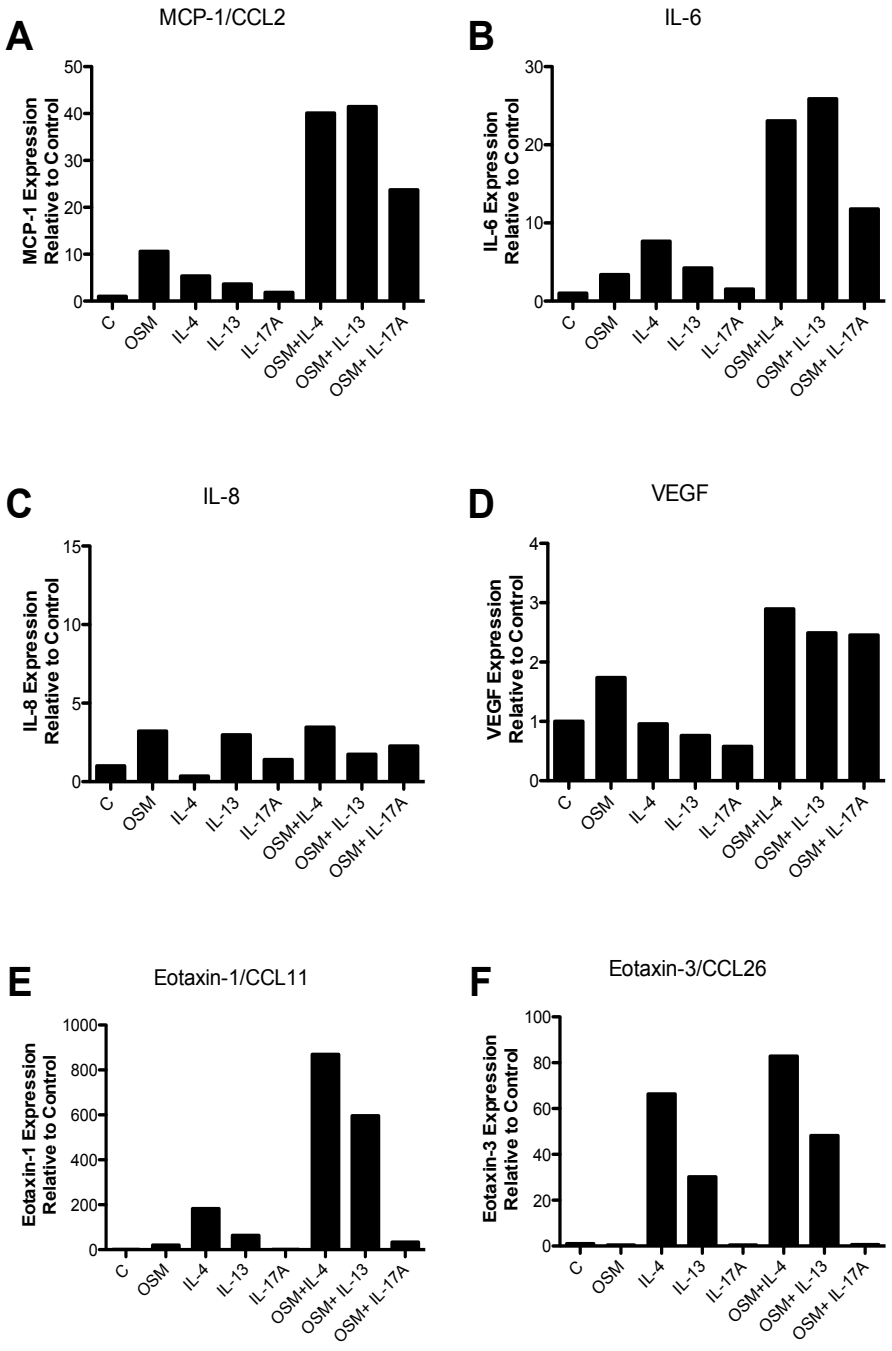

A
MCP-1/CCL2
MCP-1 Expression Relative to Control
50
40
30
20
10
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
B
IL-6
IL-6 Expression Relative to Control
30
20
10
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
C
IL-8
IL-8 Expression Relative to Control
15
10
5
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
D
VEGF
VEGF Expression Relative to Control
4
3
2
1
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
E
Eotaxin-1/CCL11
Eotaxin-1 Expression Relative to Control
1000
800
600
400
200
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
F
Eotaxin-3/CCL26
Eotaxin-3 Expression Relative to Control
100
80
60
40
20
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A

FIGURE 7- OSM synergy with IL-4, IL-13 and IL-17A in the production of selective cytokines/chemokines by HASMC 8N1 at mRNA level at 18 hours. Cultured HASMC 8N1 treated with 1 ng/mL of recombinant OSM with 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 18 hours. Total RNA was isolated as described in methods. Gene expression were analyzed using qRT-PCR (Taqman) for (A) MCP-1 expression, (B) IL-6 expression, (C) IL-8 expression, (D) VEGF expression, (E) Eotaxin-1 expression and (F) Eotaxin-3 expression shown as mean fold change relative to control using human β-Actin as the endogenous control.

FIGURE 8- OSM induces elevation of select chemokine mRNA in multiple HASMC experiments. Multiple HASMC RNA experiments were stimulated with 2 ng/ml OSM or left untreated and mRNA levels were assessed using nanostring for MCP-1 (A), IL-6 (B), VEGFa (C), CCL11 (D), MCP-3 (E), TSLP (F), and OSM (G). n=3 for cells treated for 6 hours; n=2 for cells treated for 18 hours. Graph illustrates average nanostring count ±SEM and statistical analysis (*=$P<0.05$) using an unpaired T test examining 6-hour groups only.

FIGURE7

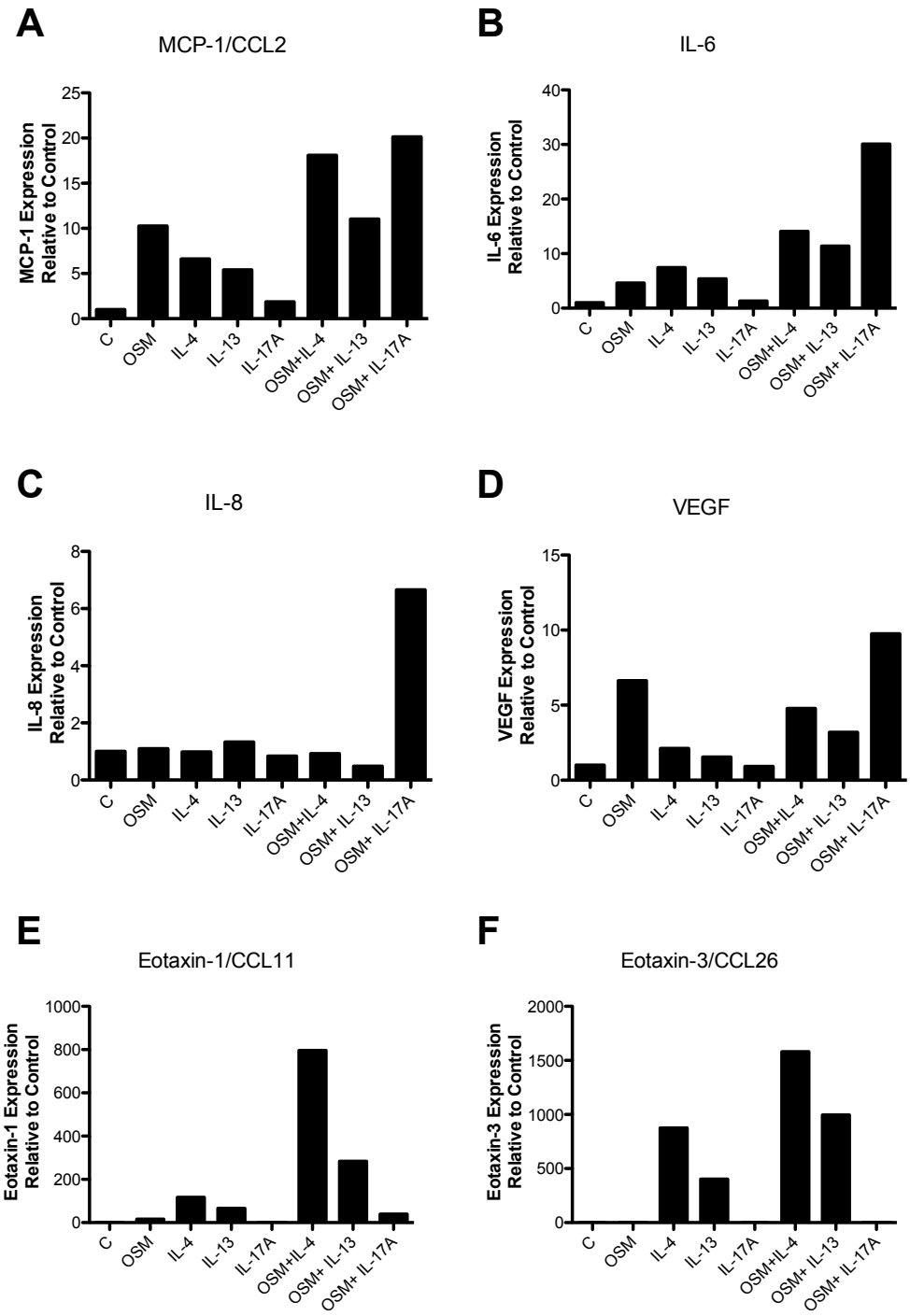

A
MCP-1/CCL2
MCP-1 Expression Relative to Control
25
20
15
10
5
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
B
IL-6
IL-6 Expression Relative to Control
40
30
20
10
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
C
IL-8
IL-8 Expression Relative to Control
8
6
4
2
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
D
VEGF
VEGF Expression Relative to Control
15
10
5
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
E
Eotaxin-1/CCL11
Eotaxin-1 Expression Relative to Control
1000
800
600
400
200
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A
F
Eotaxin-3/CCL26
Eotaxin-3 Expression Relative to Control
2000
1500
1000
500
0
C
OSM
IL-4
IL-13
IL-17A
OSM+IL-4
OSM+ IL-13
OSM+ IL-17A

FIGURE 8

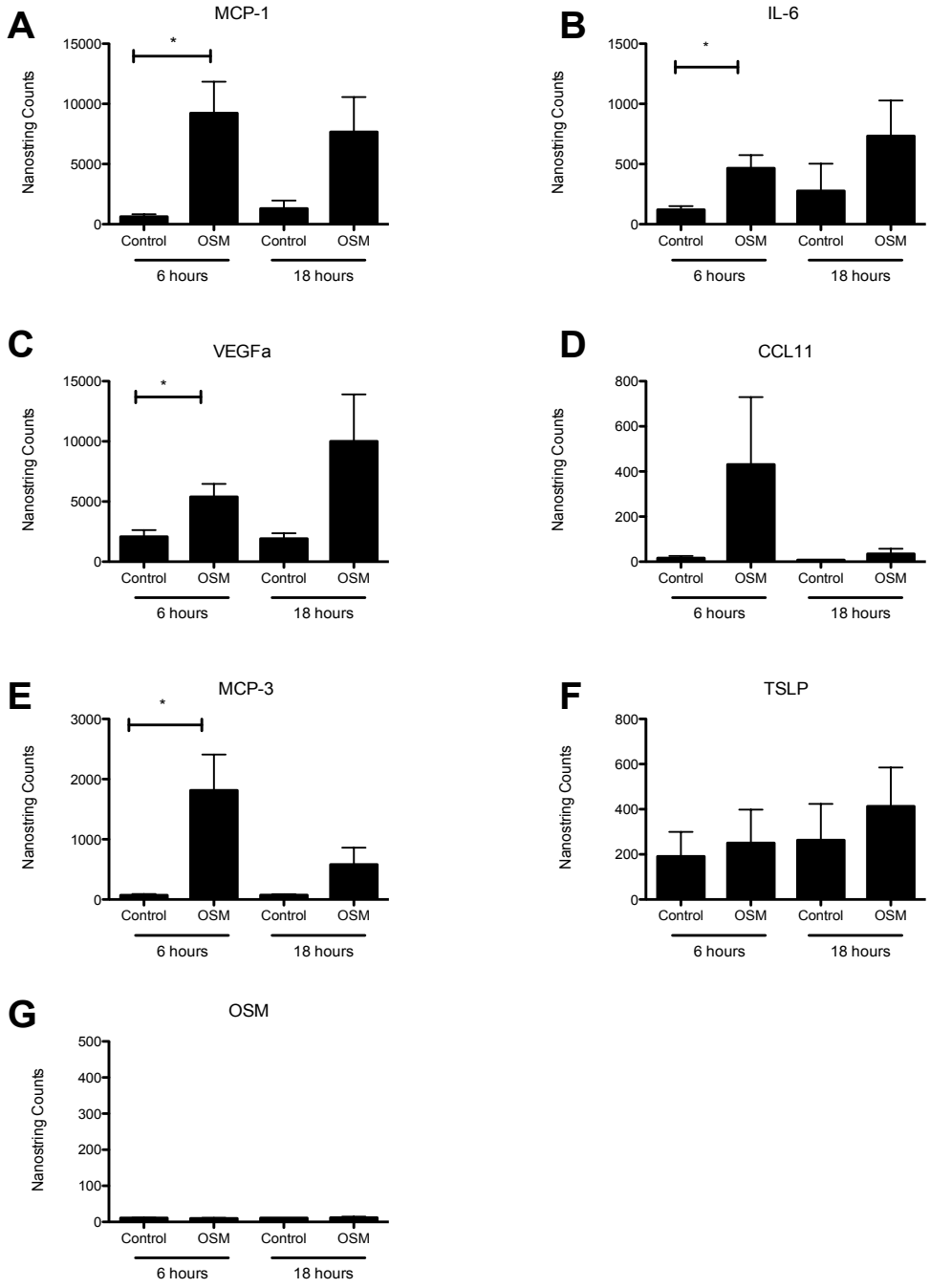
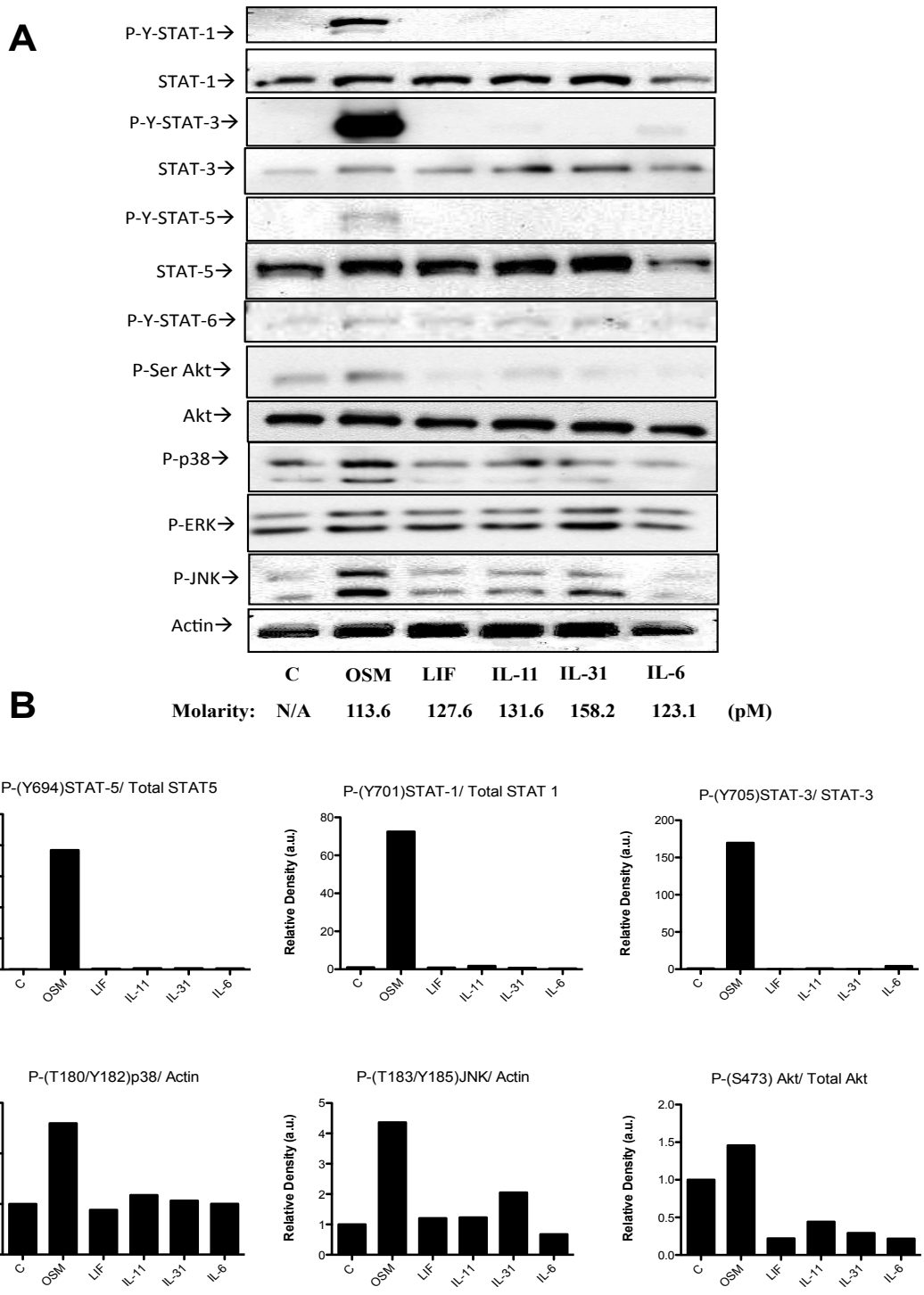

A
MCP-1
Nanostring Counts
15000
10000
5000
0
*
Control
OSM
Control
OSM
6 hours
18 hours
B
IL-6
Nanostring Counts
1500
1000
500
0
*
Control
OSM
Control
OSM
6 hours
18 hours
C
VEGFa
Nanostring Counts
15000
10000
5000
0
*
Control
OSM
Control
OSM
6 hours
18 hours
D
CCL11
Nanostring Counts
800
600
400
200
0
Control
OSM
Control
OSM
6 hours
18 hours
E
MCP-3
Nanostring Counts
3000
2000
1000
0
*
Control
OSM
Control
OSM
6 hours
18 hours
F
TSLP
Nanostring Counts
800
600
400
200
0
Control
OSM
Control
OSM
6 hours
18 hours
G
OSM
Nanostring Counts
500
400
300
200
100
0
Control
OSM
Control
OSM
6 hours
18 hours

FIGURE 9 -OSM, and not other gp130 cytokines, induces marked responses in HASMC. HASMC 8N1 were cultured *in vitro* and treated with 10 ng/ml of recombinant human OSM, LIF, IL-11, IL-31, or IL-6 (or media alone) and incubated for 20 minutes. Total cell lysates were prepared for standard immunoblot methods. (A) Membrane blots were probed for phosphorylated STAT1 (Tyr 701), STAT3 (Tyr 705), STAT5 (Tyr 694), STAT 6 (Tyr 641), JNK and total STAT1, STAT3 and STAT5. Molarity calculated based on predict molecular masses provided by R&D systems (B) Densitometry analysis of P-STAT1, PSTAT-3 and P-STAT-5 using ImageJ.

FIGURE 10- Human Aortic Smooth Muscle Cells (HAoSMC) signaling responses to OSM and select cytokines. HAoSMC were cultured as previously described *in vitro* (A) HAoSMC were treated with 10 ng/ml of OSM, LIF, IL-11, IL-31, or IL-6 (or media alone) and incubated for 20 minutes in 6-well plates. Total cell lysates were prepared for standard immunoblot methods. Membrane blots were probed for phosphorylated STAT1 (Tyr 701), STAT3 (Tyr 705), STAT5 (Tyr 694), STAT 6 (Tyr 641), JNK, ERK, Akt and relevant total proteins. (B) HAoSMC cells were plates in 96-well plates at 10,000 cells/ well. Cells were treated as described previously with 5 ng/ml IL-4, IL-13 or IL-17A and/or 1 ng/ml OSM. Mean levels of IL-6 ± SEM. ***=P<0.0001 One-way ANOVA with Tukey post hoc

FIGURE 9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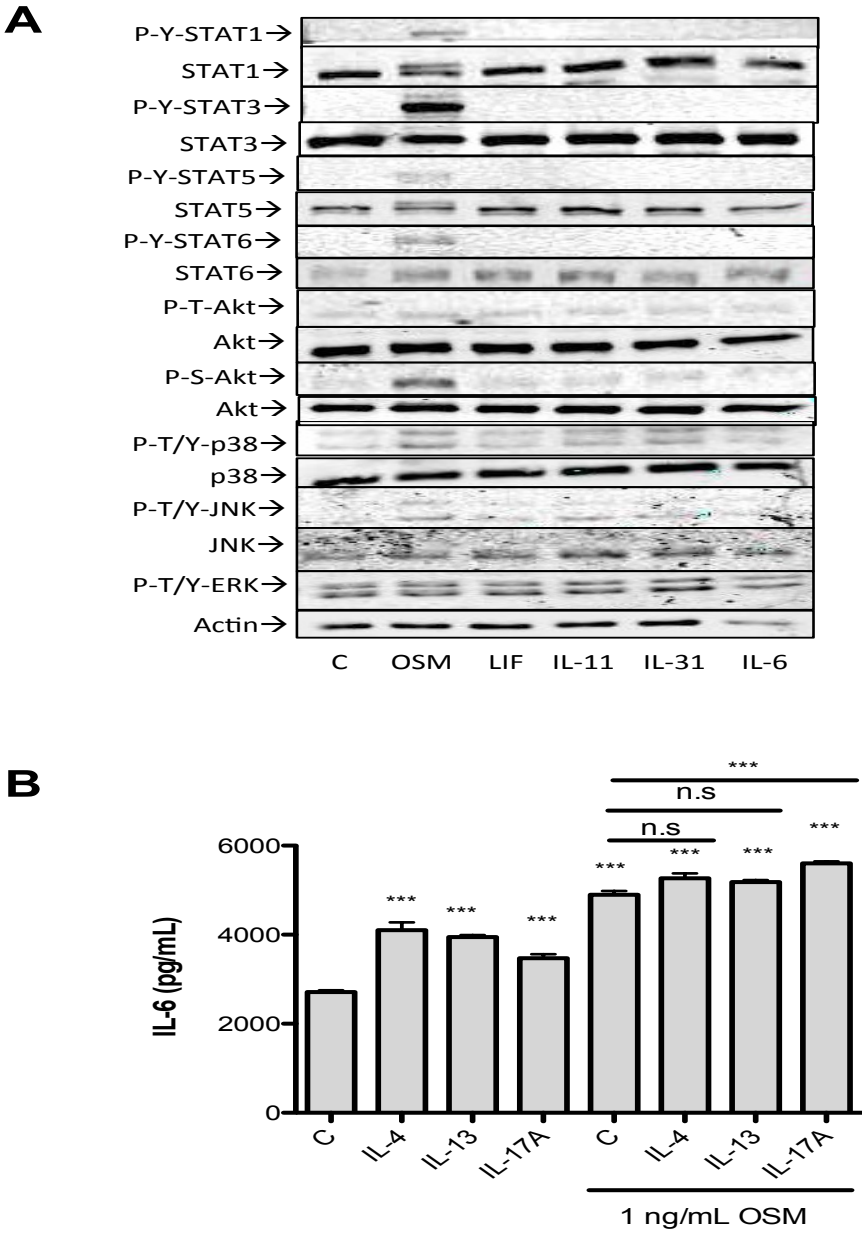
STAT-1→
P-Y-STAT-3→
STAT-3→
P-Y-STAT-5→
STAT-5→
P-Y-STAT-6→
P-Ser Akt→
Akt→
P-p38→
P-ERK→
P-JNK→
Actin→
C OSM LIF IL-11 IL-31 IL-6
B
Molarity: N/A 113.6 127.6 131.6 158.2 123.1 (pM)
P-(Y694)STAT-5/ Total STAT5
P-(Y701)STAT-1/ Total STAT 1
P-(Y705)STAT-3/ STAT-3
P-(T180/Y182)p38/ Actin
P-(T183/Y185)JNK/ Actin
P-(S473) Akt/ Total Akt
Relative Density (a.u.)
C OSM LIF IL-11 IL-31 IL-6

FIGURE 10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P-Y-STAT1→
STAT1→
P-Y-STAT3→
STAT3→
P-Y-STAT5→
STAT5→
P-Y-STAT6→
STAT6→
P-T-Akt→
Akt→
P-S-Akt→
Akt→
P-T/Y-p38→
p38→
P-T/Y-JNK→
JNK→
P-T/Y-ERK→
Actin→
C
OSM
LIF
IL-11
IL-31
IL-6
B
IL-6 (pg/mL)
6000
4000
2000
0
***
n.s
n.s
C
IL-4
IL-13
IL-17A
1 ng/mL OSM

FIGURE 11- STAT-3, Akt and p38 activation in HASMC treated with OSM alone and in combination with IL-4, IL-13 and IL-17A in multiple cell lines. Three HASMC cell lines (HASMC 8N1, HASMC 8N2 and HASMC 6N) were treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media only) with or without 1 ng/ml OSM and incubated for 20 minutes and lysed. Total lysates were probed for P (Y) STAT-3, Total STAT-3, P (Y) STAT-6, Total STAT-6, P (S) Akt, Total Akt, P (T/Y) p38, Total p38 using standard immunoblot protocols (A) Representative western blot (HASMC 6N). (B) Densitometry using ImageJ software was completed to quantify band intensities and presented as graphs illustrating average between 3 cell lines, standard deviation and statistical analysis using GraphPad Prism. *=P<0.05; **=P<0.01 using students unpaired T-test.

FIGURE 12-OSM induces elevated STAT1 and STAT3 mRNA but not STAT4 and STAT6 in multiple HASMC RNA experiments. Multiple HASMC RNA experiments where cells were stimulated with 2 ng/ml OSM or left untreated and mRNA levels were assessed using nanostring for STAT1 (A), STAT3 (B), STAT4 (C) and STAT6 (D). n=3 for cells treated for 6 hours; n=2 for cells treated for 18 hours. Graph illustrates average nanostring count ±SEM. *=P<0.05; **=P<0.01 using an unpaired T test examining 6-hour groups only.

FIGURE 11

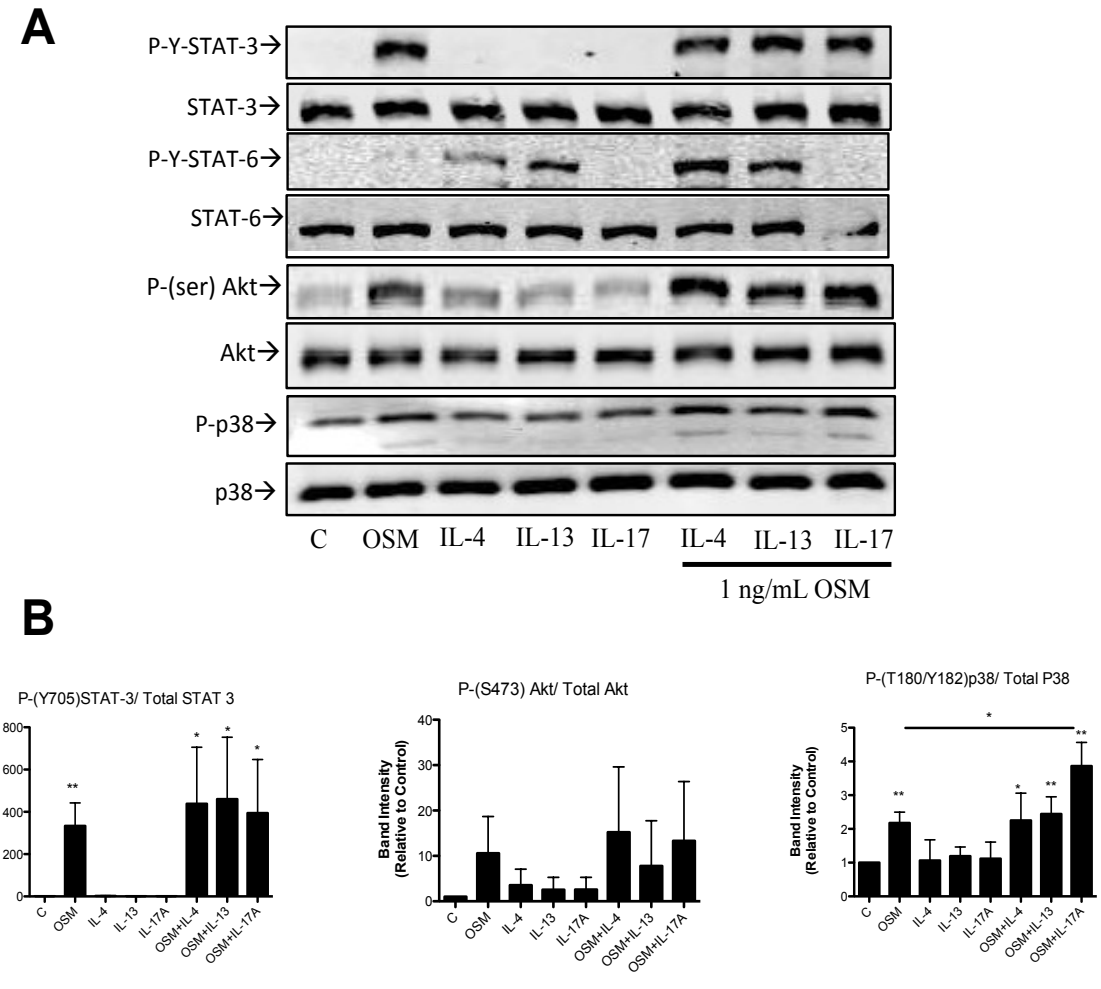

A
P-Y-STAT-3→
STAT-3→
P-Y-STAT-6→
STAT-6→
P-(ser) Akt→
Akt→
P-p38→
p38→
C OSM IL-4 IL-13 IL-17 IL-4 IL-13 IL-17
1 ng/mL OSM
B
P-(Y705)STAT-3/ Total STAT 3
Band Intensity (Relative to Control)
C OSM IL-4 IL-13 IL-17A OSM+IL-4 OSM+IL-13 OSM+IL-17A
P-(S473) Akt/ Total Akt
Band Intensity (Relative to Control)
C OSM IL-4 IL-13 IL-17A OSM+IL-4 OSM+IL-13 OSM+IL-17A
P-(T180/Y182)p38/ Total P38
Band Intensity (Relative to Control)
C OSM IL-4 IL-13 IL-17A OSM+IL-4 OSM+IL-13 OSM+IL-17A

FIGURE 12

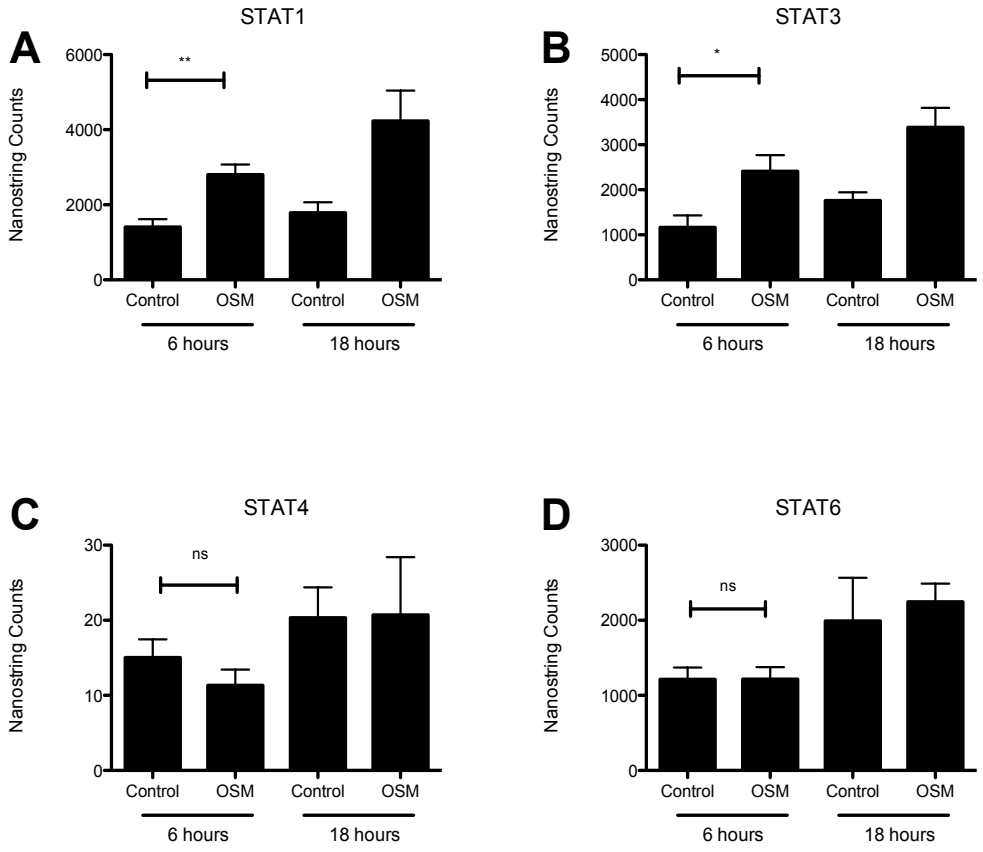

A
STAT1
Nanostring Counts
6000
4000
2000
0
**
Control
OSM
Control
OSM
6 hours
18 hours
B
STAT3
Nanostring Counts
5000
4000
3000
2000
1000
0
*
Control
OSM
Control
OSM
6 hours
18 hours
C
STAT4
Nanostring Counts
30
20
10
0
ns
Control
OSM
Control
OSM
6 hours
18 hours
D
STAT6
Nanostring Counts
3000
2000
1000
0
ns
Control
OSM
Control
OSM
6 hours
18 hours

FIGURE 13- Effects of Rapamycin, mTOR inhibitor, on OSM synergy with IL-4, IL-13 and IL-17A. HASMC 8N2 and HASMC 8N1 (A-B) were cultured and serum deprived as previously described. Cells were pre-incubated with 10 ug/ml Rapamycin for 30 minutes. Cells were then treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml OSM (C) HASMC 8N1 cells were cultured in the same manner as previously described. Cells were pre-incubated with 20 ug/ml Rapamycin for 30 minutes and subsequently treated with 5 ng/ml of recombinant human IL-4, IL-13 and IL-17A with or without 2ng/ml OSM. Supernatants from each well were collected and analyzed for levels of hMCP-1 and hIL-6. ***=$P<0.001$; **=$P<0.01$ using Two-Way ANOVA with Bonferroni post-hoc.

FIGURE 14- Dose response in cultured HASMC stimulated with increasing dosages of Stattic in two primary HASMC cell lines. HASMC 8N2 (A) and HASMC 8N1 (B) were cultured as previously described. Cells were pre-incubated with increasing concentrations (0 uM -10 uM) of static for 30 minutes. Cells were then treated with 1 ng/ml of OSM. Supernatants from each well were collected and analyzed for levels of hMCP-1 and hIL-6. $p<0.001$ using Two-Way ANOVA with Bonferroni post-hoc for all groups.

FIGURE 13

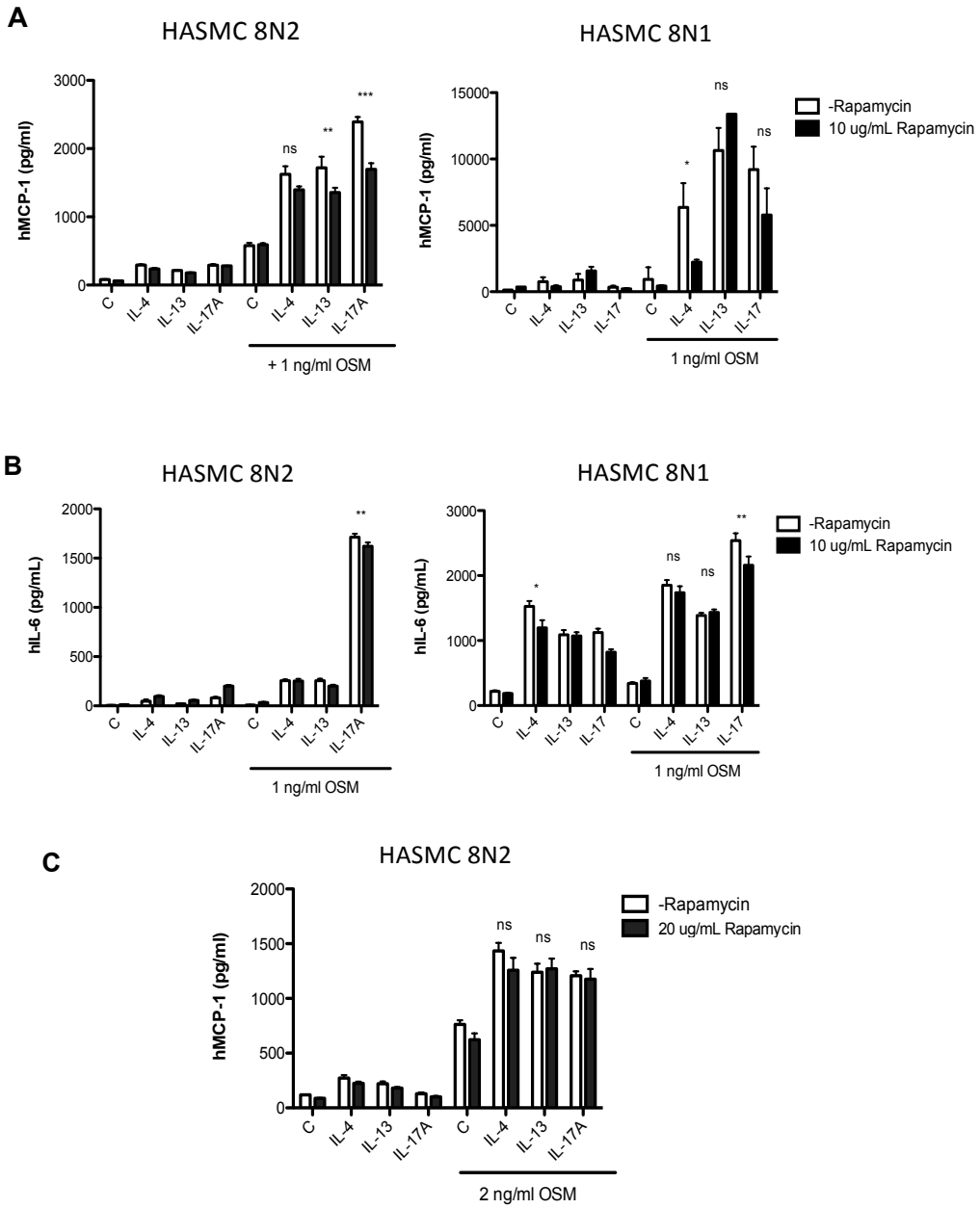

A
HASMC 8N2
HASMC 8N1
hMCP-1 (pg/ml)
3000
2000
1000
0
15000
10000
5000
ns
**
***
*
C
IL-4
IL-13
IL-17A
IL-17
+ 1 ng/ml OSM
1 ng/ml OSM
-Rapamycin
10 ug/mL Rapamycin
B
HASMC 8N2
HASMC 8N1
hIL-6 (pg/mL)
2000
1500
1000
500
0
3000
**
*
ns
1 ng/ml OSM
-Rapamycin
10 ug/mL Rapamycin
C
HASMC 8N2
hMCP-1 (pg/ml)
2000
1500
1000
500
0
ns
2 ng/ml OSM
-Rapamycin
20 ug/mL Rapamycin

FIGURE 14

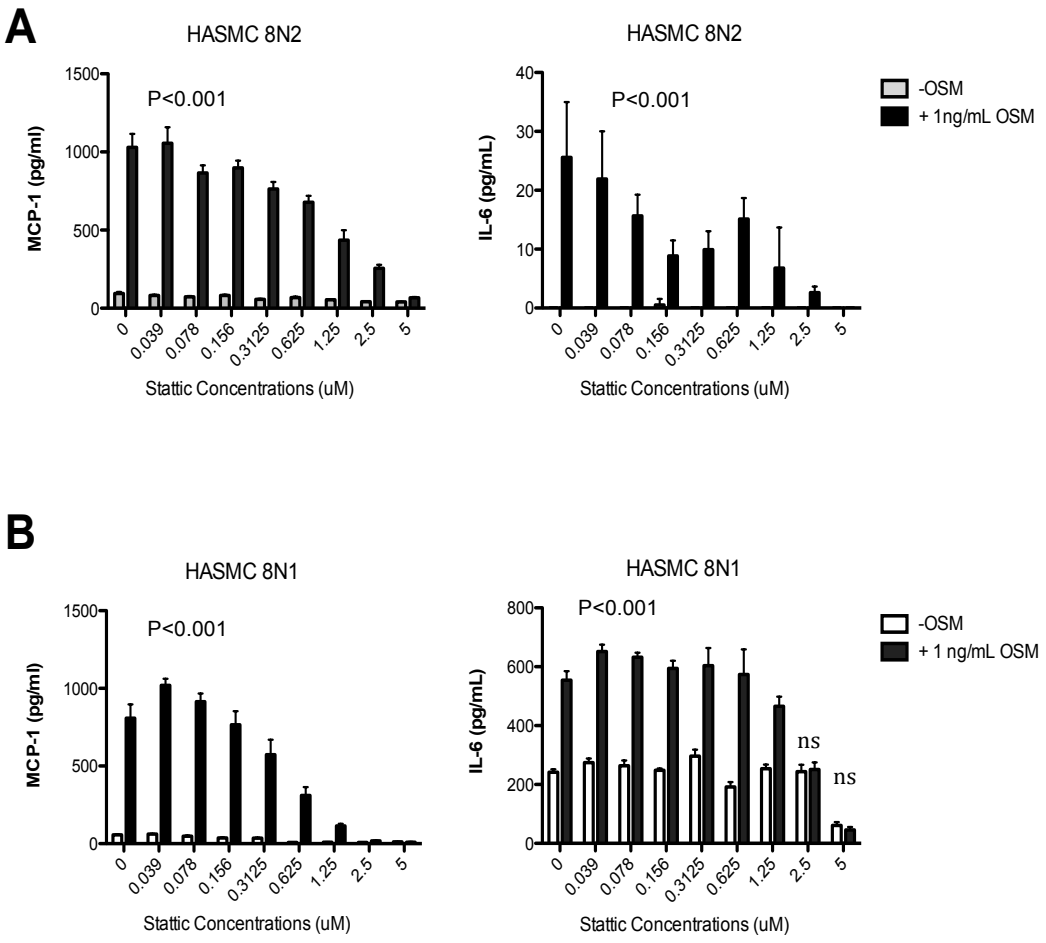
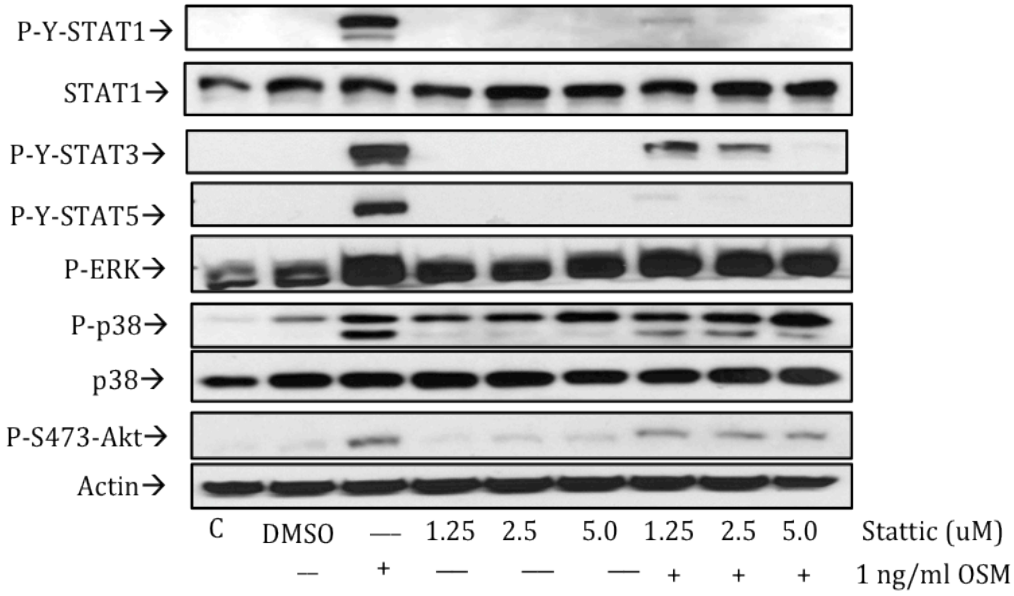

A
HASMC 8N2
P<0.001
MCP-1 (pg/ml)
1500
1000
500
0
0
0.039
0.078
0.156
0.3125
0.625
1.25
2.5
5
Stattic Concentrations (uM)
HASMC 8N2
P<0.001
IL-6 (pg/mL)
40
30
20
10
0
Stattic Concentrations (uM)
-OSM
+ 1ng/mL OSM
B
HASMC 8N1
P<0.001
MCP-1 (pg/ml)
Stattic Concentrations (uM)
HASMC 8N1
P<0.001
IL-6 (pg/mL)
800
600
400
200
ns
ns
Stattic Concentrations (uM)
-OSM
+ 1 ng/mL OSM

FIGURE 15- Stattic diminishes the activation of STAT proteins but does not affect other cell signaling pathways. HASMC 8N1 were treated with 1 ng/ml of recombinant human OSM, DMSO or media alone and incubated for 20 minutes. Total cell lysates were prepared for immunoblots. Blots were probed for phosphorylated STAT1 (Tyr 701), STAT3 (Tyr 705), STAT5 (Tyr 694), ERK, AKT (Serine 473) and total STAT1 and Actin as loading control. (B) Densitometry analysis of PSTAT1, P-STAT-3, PSTAT5, P-ERK, P-Akt using ImageJ described previously

FIGURE 16- The effects of Stattic application on STAT 3 and STAT 1 activation in two primary HASMC cell lines.

HASMC 8N1 and HASMC 8N2 were treated with 1 ng/ml of recombinant human OSM or media alone and incubated for 20 minutes. Cells were lysed to collect total cell lysates. Lysates were denatured in SDS-containing buffer, separated on 8% SDS-PAGE and transferred to nitrocellulose membrane. Membrane blots were probed for phosphorylated STAT1 (Tyr 701), STAT3 (Tyr 705) and total STAT1, STAT3 and Actin as loading control. (B) Densitometry using ImageJ in HASMC 8N1 (C) Densitometry using ImageJ in HASMC 8N2.

FIGURE 15


P-Y-STAT1→
STAT1→
P-Y-STAT3→
P-Y-STAT5→
P-ERK→
P-p38→
p38→
P-S473-Akt→
Actin→
C DMSO — 1.25 2.5 5.0 1.25 2.5 5.0 Stattic (uM)
— + — — — + + + 1 ng/ml OSM

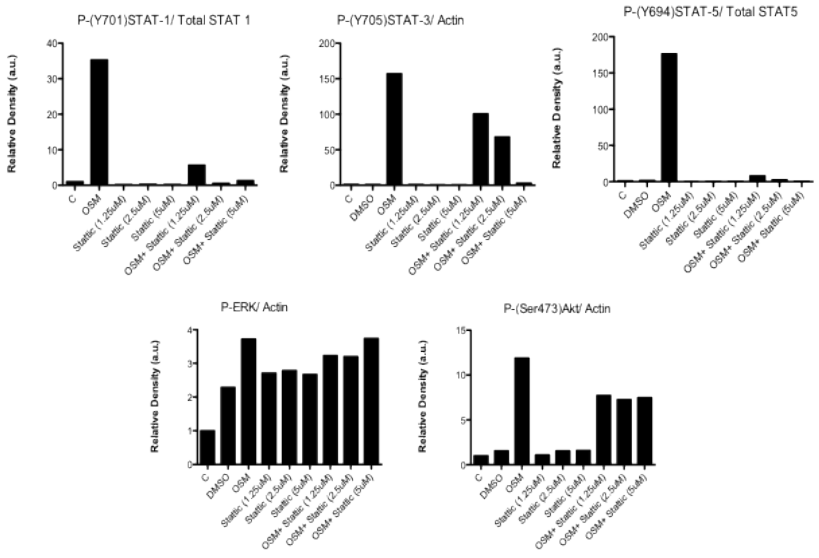

P-(Y701)STAT-1/ Total STAT 1
P-(Y705)STAT-3/ Actin
P-(Y694)STAT-5/ Total STAT5
P-ERK/ Actin
P-(Ser473)Akt/ Actin
Relative Density (a.u.)
C
DMSO
OSM
Stattic (1.25uM)
Stattic (2.5uM)
Stattic (5uM)
OSM+ Stattic (1.25uM)
OSM+ Stattic (2.5uM)
OSM+ Stattic (5uM)

FIGURE 16

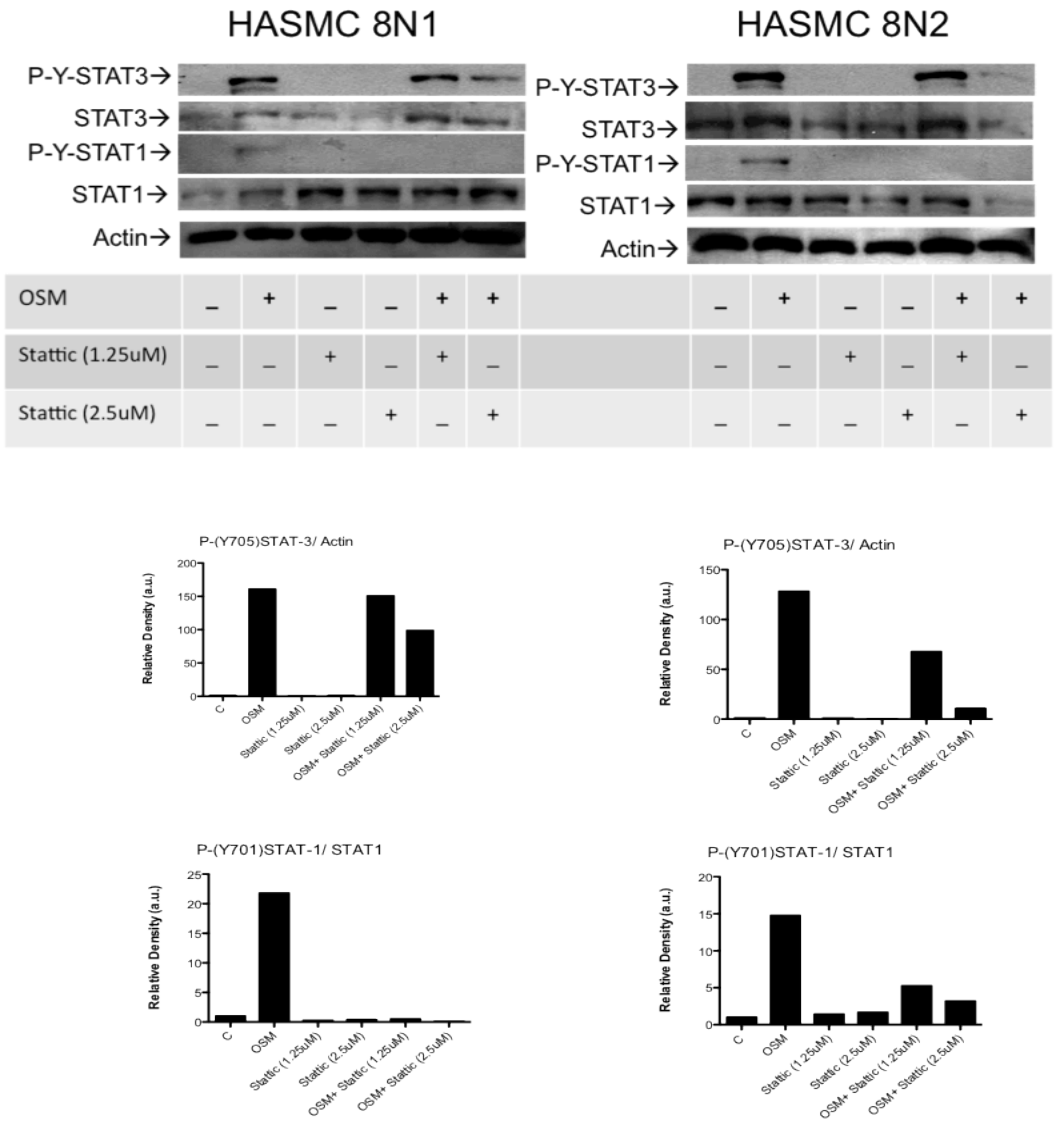

HASMC 8N1
HASMC 8N2
P-Y-STAT3→
STAT3→
P-Y-STAT1→
STAT1→
Actin→
OSM
Stattic (1.25uM)
Stattic (2.5uM)
P-(Y705)STAT-3/ Actin
Relative Density (a.u.)
C
OSM
Stattic (1.25uM)
Stattic (2.5uM)
OSM+ Stattic (1.25uM)
OSM+ Stattic (2.5uM)
P-(Y701)STAT-1/ STAT1

FIGURE 17- STAT inhibitor, Stattic, reduces IL-6 and MCP-1 production in HASMC 8N1. HASMC 8N1 were pre-incubated with either 1.25 uM (A-B), 2.5 uM (C-D) or 5uM (E-F) for 30 minutes. Cells were then treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml OSM. Supernatants from each well were collected and analyzed for levels of hMCP-1 and hIL-6. ***=$P < 0.001$ using two-way ANOVA with Bonferroni post-hoc.

FIGURE 18- Stattic reduced MCP-1 production in HASMC 8N2. HASMC 8N2 were pre-incubated with either 1.25 uM (A) 2.5 uM (B) for 30 minutes. Cells were then treated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml OSM. Supernatants from each well were collected and analyzed for levels of hMCP-1. ***=$P < 0.001$ using two-way ANOVA with Bonferroni post-hoc.

FIGURE 17

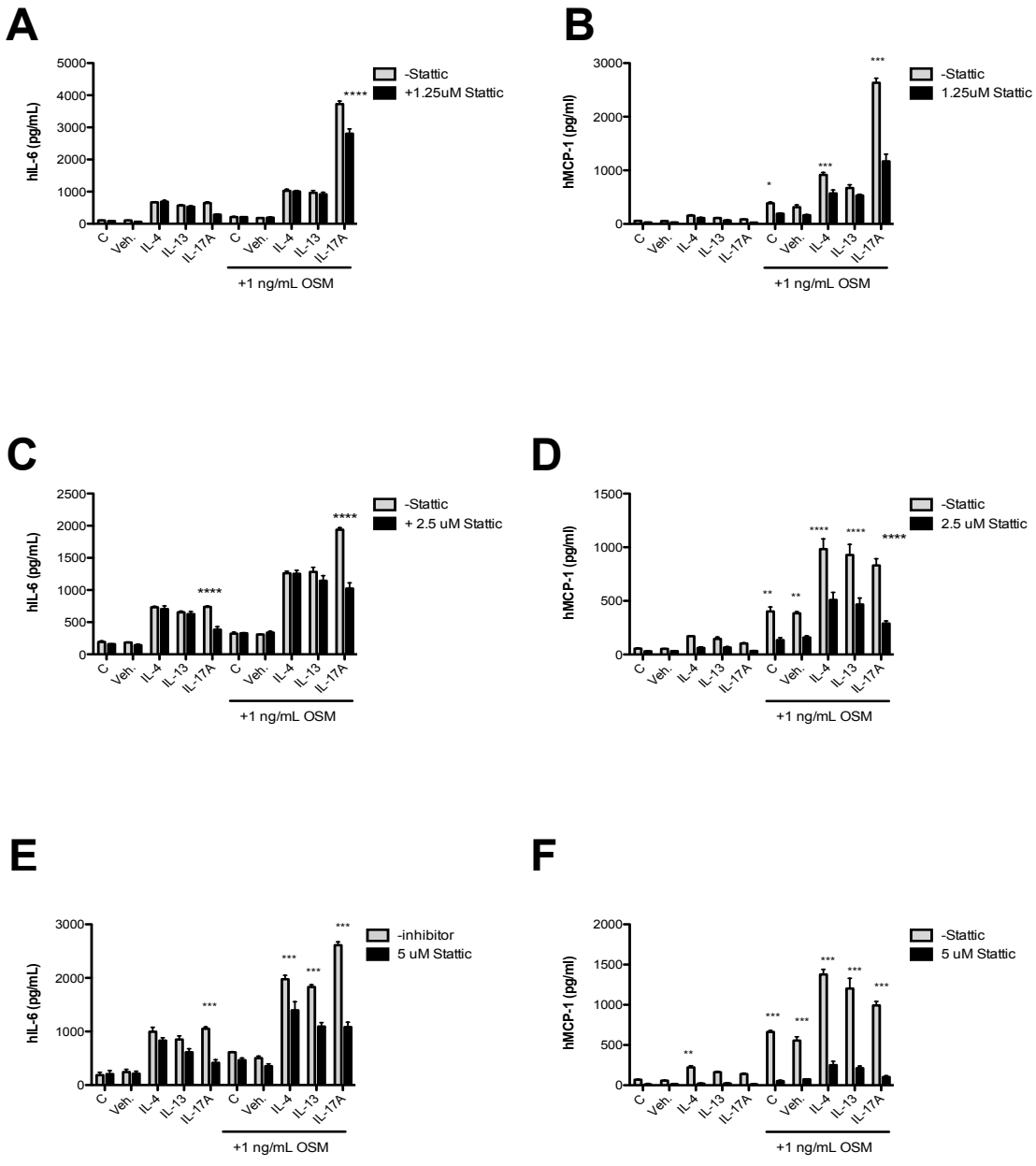

A
hIL-6 (pg/mL)
-Stattic
+1.25uM Stattic
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM
B
hMCP-1 (pg/ml)
-Stattic
1.25uM Stattic
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM
C
hIL-6 (pg/mL)
-Stattic
+ 2.5 uM Stattic
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM
D
hMCP-1 (pg/ml)
-Stattic
2.5 uM Stattic
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM
E
hIL-6 (pg/mL)
-inhibitor
5 uM Stattic
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM
F
hMCP-1 (pg/ml)
-Stattic
5 uM Stattic
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM

FIGURE 18

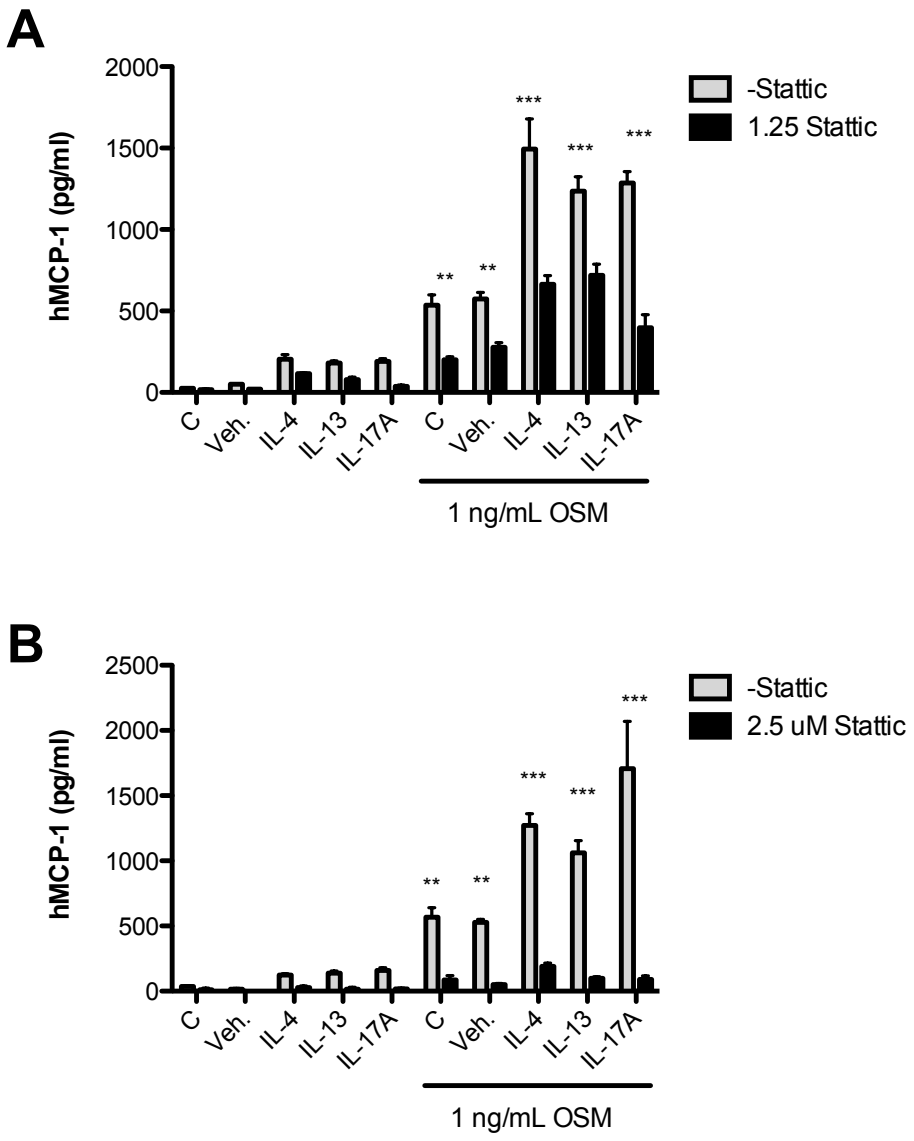

A
hMCP-1 (pg/ml)
2000
1500
1000
500
0
-Stattic
1.25 Stattic
C
Veh.
IL-4
IL-13
IL-17A
C
Veh.
IL-4
IL-13
IL-17A
1 ng/mL OSM
B
hMCP-1 (pg/ml)
2500
2000
1500
1000
500
0
-Stattic
2.5 uM Stattic
C
Veh.
IL-4
IL-13
IL-17A
C
Veh.
IL-4
IL-13
IL-17A
1 ng/mL OSM

FIGURE 19 - The effects of the STAT-3 inhibitor, Stattic, on IL-6 and MCP-1 mRNA expression by HASMC at 24 Hours. HASMC 8N2 were either left untreated (Control) or treated with 5 ng/ml IL-4, IL-13 or IL-17A with or without 1 ng/ml OSM in the presence or absence of Stattic (2.5 uM) in which cells were pre-incubated for 30 minutes prior to stimulation. Cells were incubated for 24 hours. Total RNA was assessed using qRT-PCR (Taqman). Fold changes relative to Control of MCP-1 (A) and IL-6 (B) when the cells were treated with the indicated cytokines and Stattic were graphed. β-Actin was used as endogenous control. (SEM ± TaqMan triplicates). ***=P<0.001 using Two-ANOVA with Bonferroni post-hoc comparing with or without Stattic treatment.

FIGURE 20- Examining the effects of various inhibitors of cell signaling pathways on OSM synergy with IL-4, IL-13 and IL-17A in the stimulation of MCP-1 and IL-6 by cultured HASMC. HASMC 8N1 were cultured as previously described. Cells were then pre-incubated with indicated concentrations of: p38 inhibitor SB 203580 (SB) for 1 hour (A-B), an ERK inhibitor, PD 98059 (PD) for 2 hours (C-D) and Akt inhibitor (Akt X) for 1 hour (E-F), prior to stimulations. Cells were then stimulated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml OSM. Supernatants from each well were collected and analyzed for levels of hMCP-1 and IL-6 *** $P < 0.001$ using two-way ANOVA with Bonferroni post-hoc

FIGURE 19

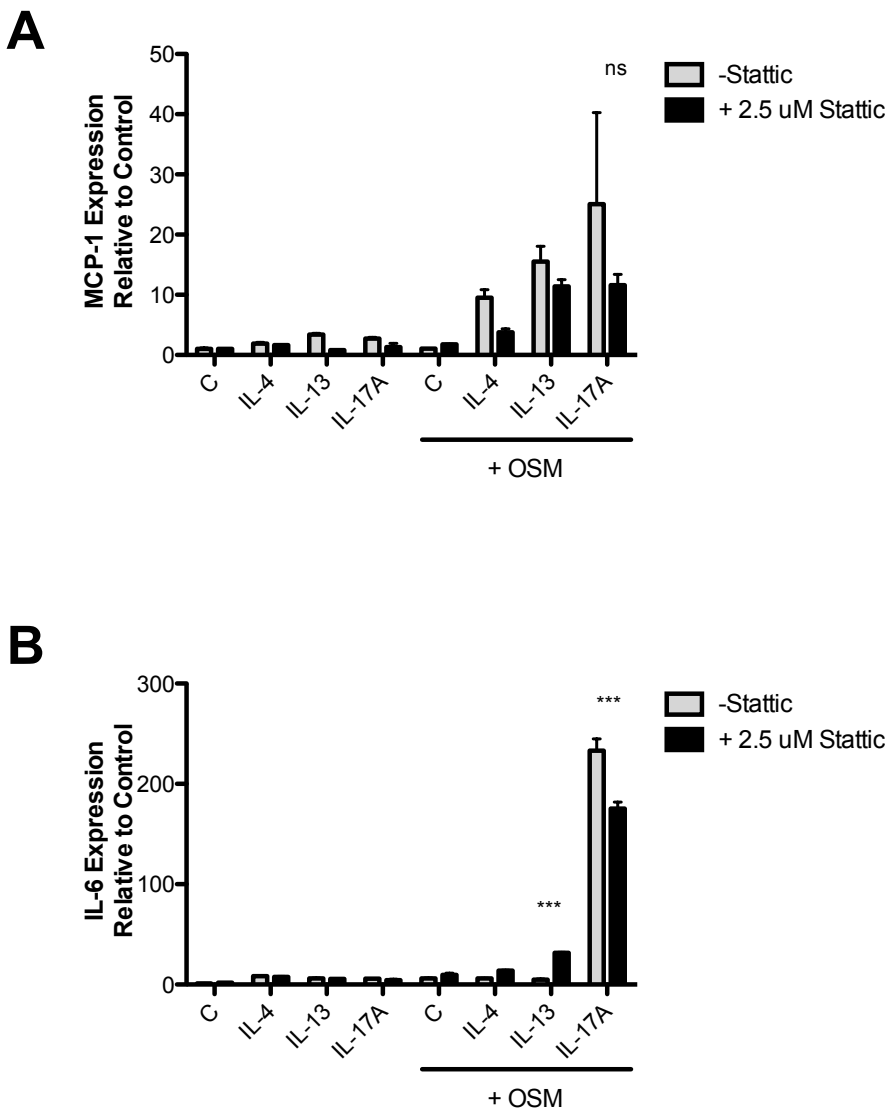

A
MCP-1 Expression Relative to Control
50
40
30
20
10
0
ns
-Stattic
+ 2.5 uM Stattic
C
IL-4
IL-13
IL-17A
C
IL-4
IL-13
IL-17A
+ OSM
B
IL-6 Expression Relative to Control
300
200
100
0
***
***
-Stattic
+ 2.5 uM Stattic
C
IL-4
IL-13
IL-17A
C
IL-4
IL-13
IL-17A
+ OSM

FIGURE 20

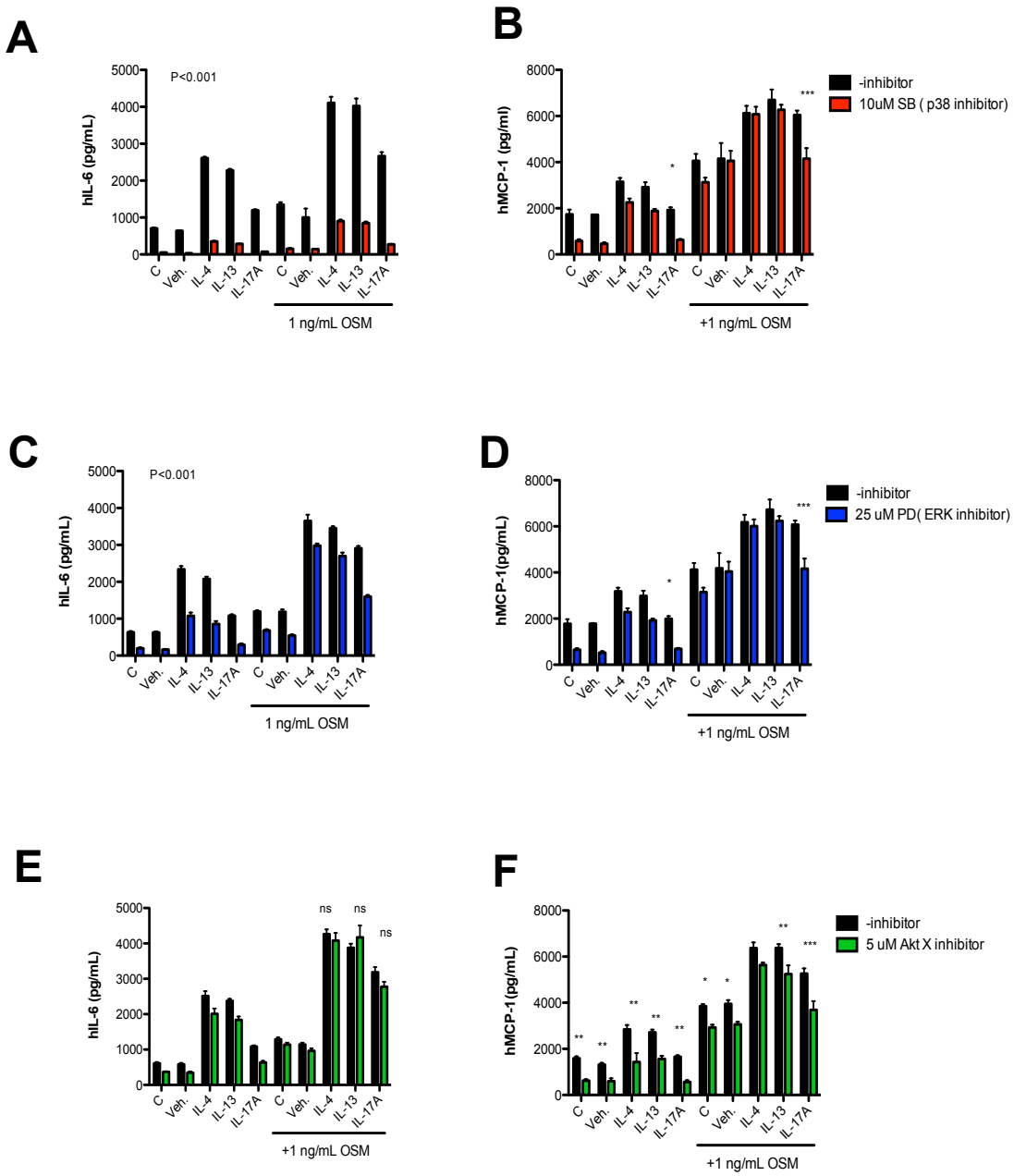

A
hIL-6 (pg/mL)
P<0.001
C
Veh.
IL-4
IL-13
IL-17A
1 ng/mL OSM
B
hMCP-1 (pg/ml)
-inhibitor
10uM SB (p38 inhibitor)
+1 ng/mL OSM
C
hIL-6 (pg/mL)
P<0.001
1 ng/mL OSM
D
hMCP-1(pg/mL)
-inhibitor
25 uM PD(ERK inhibitor)
+1 ng/mL OSM
E
hIL-6 (pg/mL)
ns
+1 ng/mL OSM
F
hMCP-1(pg/mL)
-inhibitor
5 uM Akt X inhibitor
+1 ng/mL OSM

FIGURE 21- The effects of thep38 selective inhibitor (SB 203580) at 3 concentrations on OSM synergy with IL-4, IL-13 and IL-17 in the production of IL-6 by HASMC. HASMC 8N1 were cultured as previously described. Sub-confluent cells were serum deprived for 3 hours using 2% FBS RPMI. Cells were then pre-incubated with p38 inhibitor SB 203580 (SB) for 1 hour at 1 uM (A), 2.5 uM (B) and 5uM (C) prior to stimulations. Cells were then stimulated with 5 ng/ml of recombinant human IL-4, IL-13 or IL-17A (or media alone) with or without 1 ng/ml OSM. Supernatants from each well were collected and analyzed for levels of hIL-6. Mean levels ± SEM and two-way ANOVA with Bonferroni post-hoc analysis showing ***=$P < 0.001$

FIGURE 22- Fold change in mRNA expression of selected receptors requires for IL-4, IL-13, Il-17A and OSM signaling at 6 hours in two cell lines. HASMC 8N1 and HASMC 8N2 were cultured as described previously. Cells were treated with 2 ng/mL of OSM or 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 6 hours. Total RNA were assessed using qRT-PCR using PDAR for: (A) IL-17RA (B) IL-17RC (C), gp130 receptor (D) OSMRβ and (E) IL-4Rα. Levels of receptor mRNA were expressed as fold change relative to control (unstimulated) corrected to human β-Actin as the endogenous control. Error bars represent mean ± SEM of two HASMC cell lines.

FIGURE 21

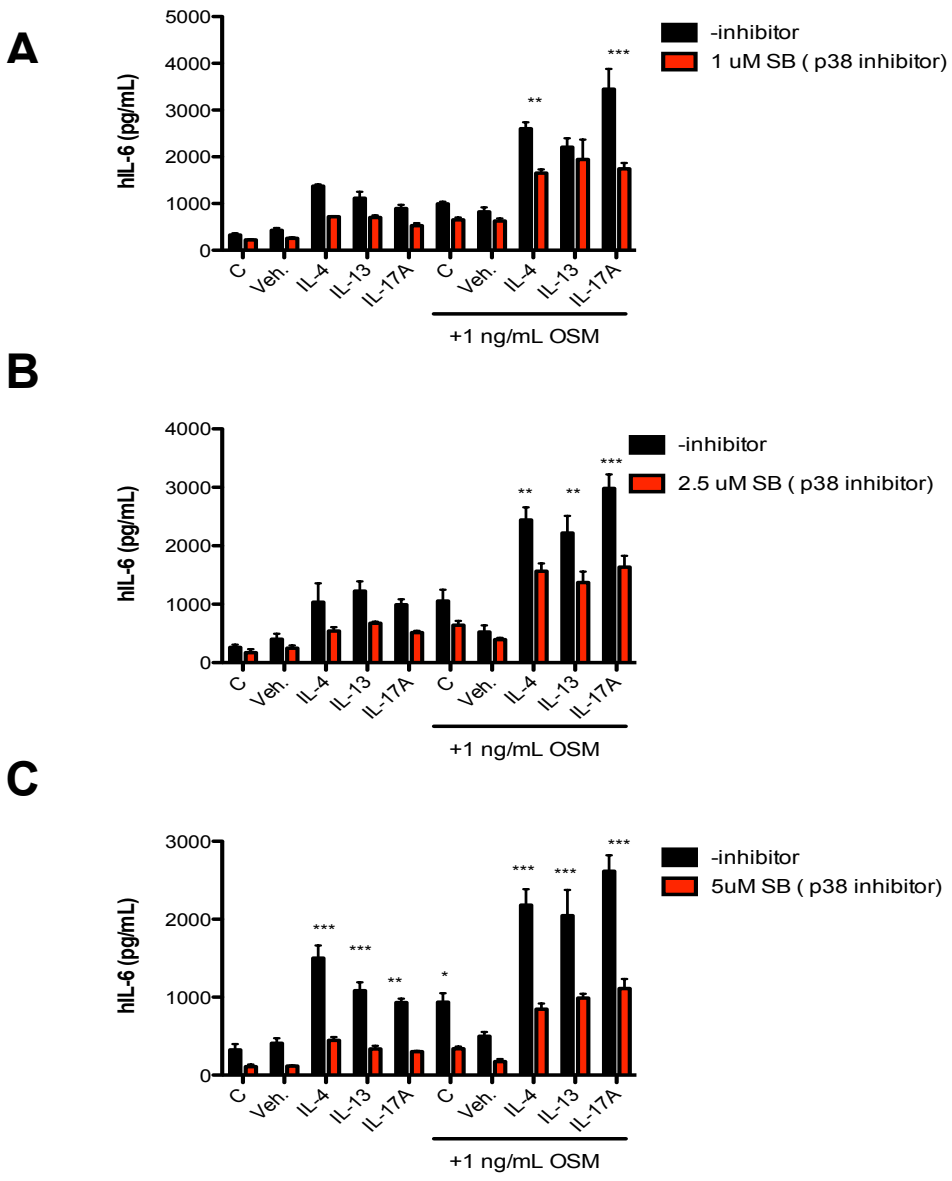

A
hIL-6 (pg/mL)
5000
4000
3000
2000
1000
0
-inhibitor
1 uM SB (p38 inhibitor)
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM
B
hIL-6 (pg/mL)
-inhibitor
2.5 uM SB (p38 inhibitor)
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM
C
hIL-6 (pg/mL)
-inhibitor
5uM SB (p38 inhibitor)
C
Veh.
IL-4
IL-13
IL-17A
+1 ng/mL OSM

FIGURE 22

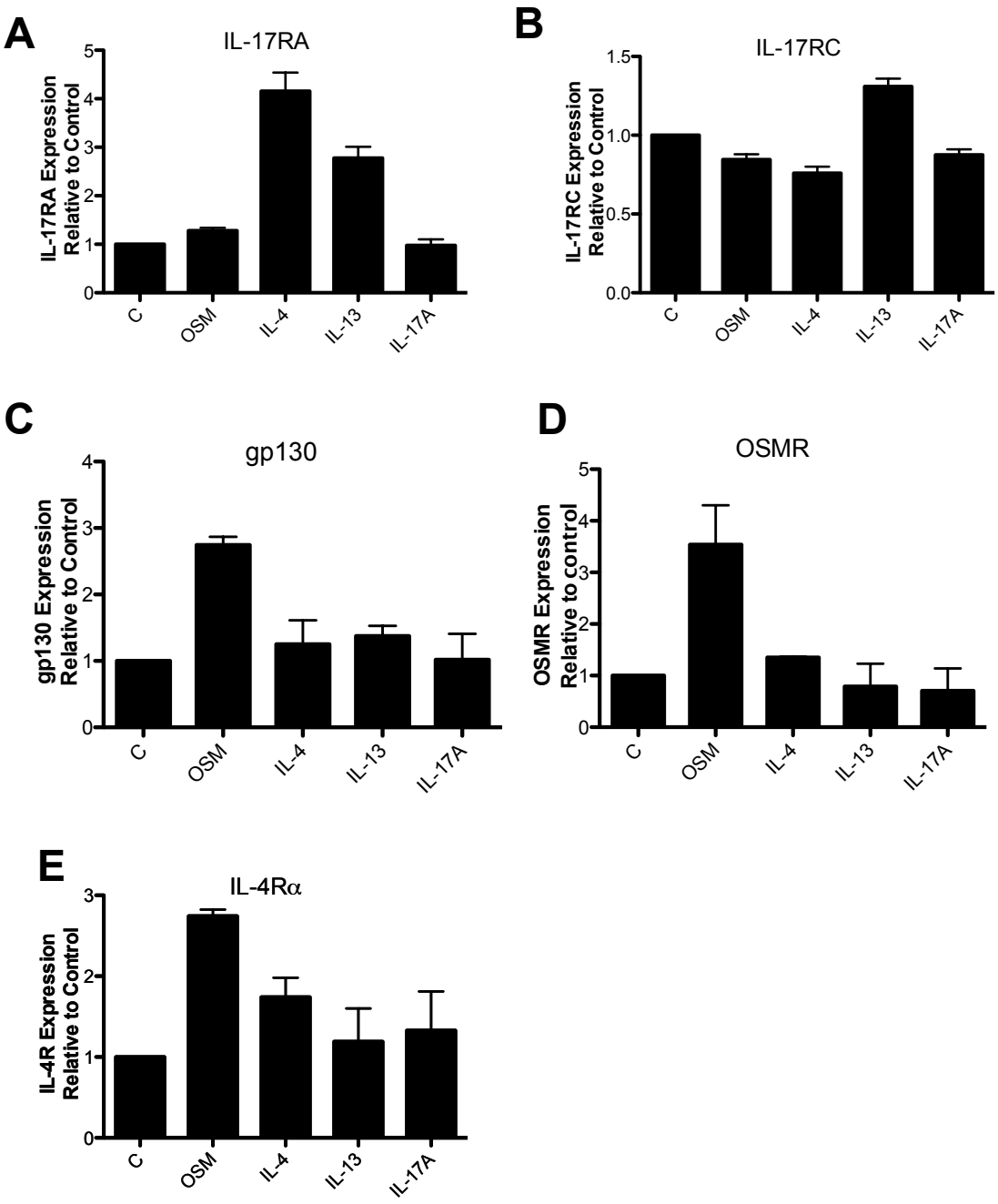

A
IL-17RA
IL-17RA Expression Relative to Control
0
1
2
3
4
5
C
OSM
IL-4
IL-13
IL-17A
B
IL-17RC
IL-17RC Expression Relative to Control
0.0
0.5
1.0
1.5
C
OSM
IL-4
IL-13
IL-17A
C
gp130
gp130 Expression Relative to Control
0
1
2
3
4
C
OSM
IL-4
IL-13
IL-17A
D
OSMR
OSMR Expression Relative to control
0
1
2
3
4
5
C
OSM
IL-4
IL-13
IL-17A
E
IL-4Rα
IL-4R Expression Relative to Control
0
1
2
3
C
OSM
IL-4
IL-13
IL-17A

FIGURE 23- HASMC proliferation after stimulation with IL-4, IL-13 and IL-17A with or without OSM (1 ng/ml or 5 ng/ml). HASMC 8N2 cells was plated at 15,000 cells/cm^2 in 96 well plates and stimulated with 5 ng/ml IL-4, IL-13 or IL-17A or unstimulated (Control) with or without 1 ng/ml or 5 ng/ml OSM and incubated for 48 hours (A) and 7 days (B). MTT assay was performed as described in methods. Cell proliferation/cell numbers is proportional to formazan production as represented by OD value. *=$P<0.05$ using one-way ANOVA with Tukey post-hoc.

FIGURE 23

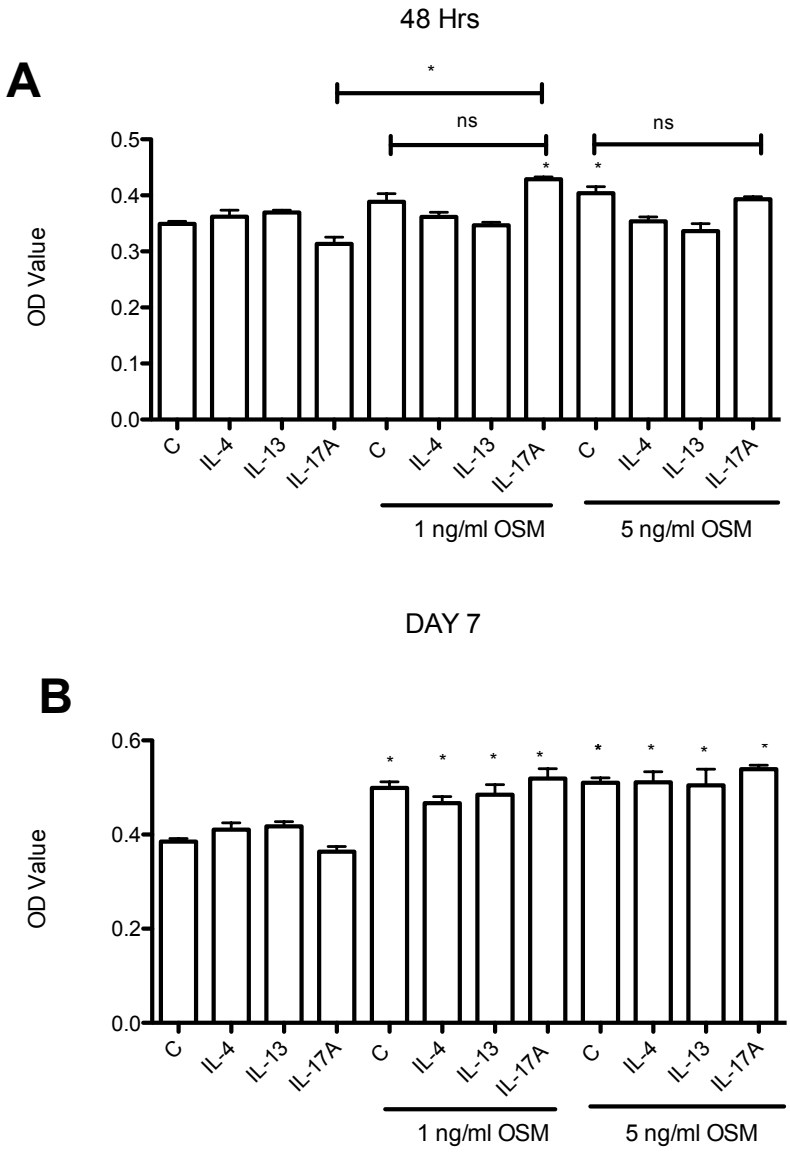

48 Hrs
A
*
ns
ns
OD Value
0.5
0.4
0.3
0.2
0.1
0.0
C
IL-4
IL-13
IL-17A
1 ng/ml OSM
5 ng/ml OSM
DAY 7
B
0.6
0.4
0.2
0.0

FIGURE 24- Extracellular and connective tissue proteins expression by HASMC 8N2 at 6 Hours. Cells were treated with 1 ng/mL of OSM or 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 6 hours. Total RNA assessed using qRT-PCR (Taqman) and probed for Collagen 1A1 (Col1A1) (A), TIMP-1 (B), MMP-1 (C) and MMP-3 (D). mRNA levels are expressed as mean fold change relative to control. Human β-Actin was used as the endogenous control.

FIGURE 25- Extracellular and connective tissue proteins expression by HASMC 8N2 at 18 Hours. HASMC 8N2 RNA was extracted as described above. mRNA levels for Collagen 1A1 (Col1A1) (A), TIMP-1 (B), MMP-1 (C) and MMP-3 (D) were assessed using qRT PCR (Taqman). Graphs illustrate mean ± SEM (of Taqman replicate) and mRNA levels expressed as mean fold change relative to control and corrected to human β-Actin as the endogenous control.

FIGURE 24

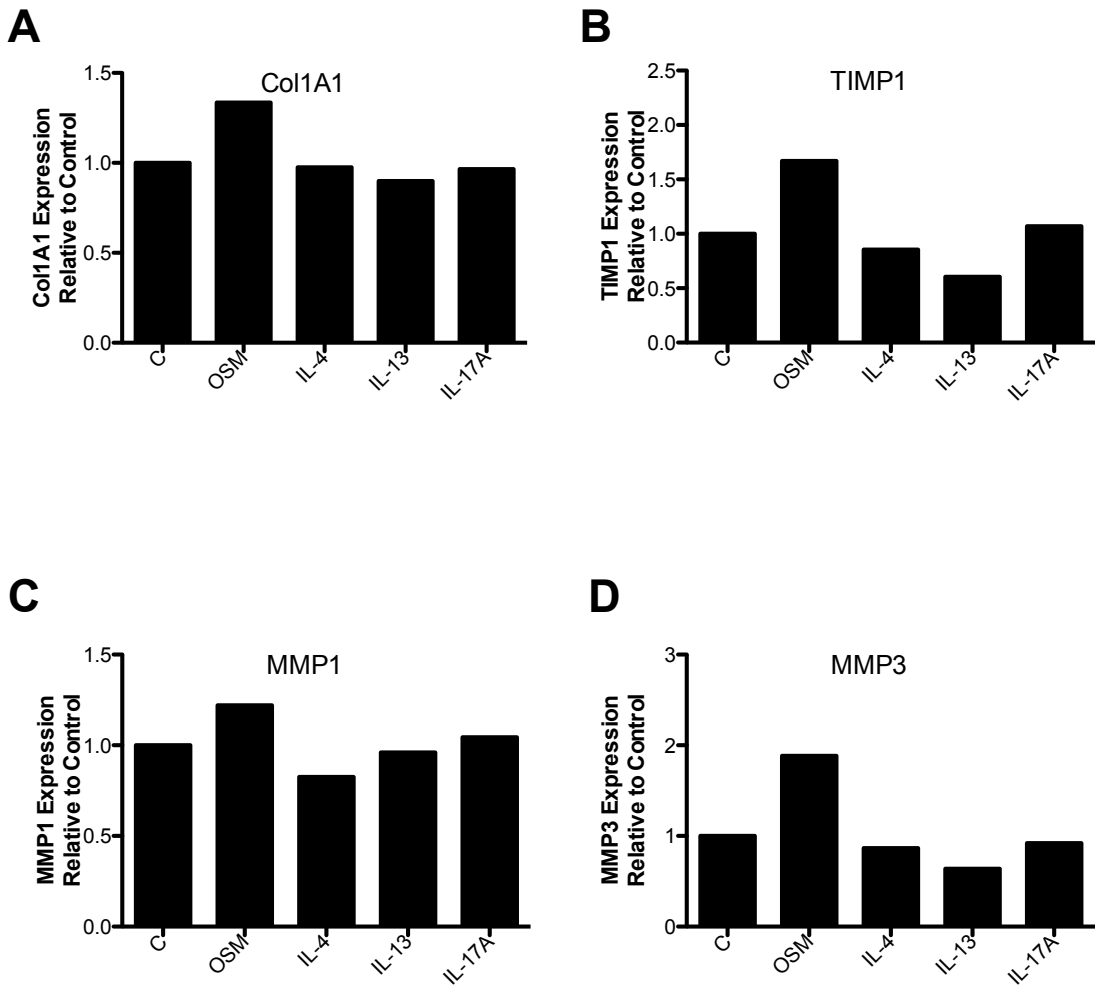

A
Col1A1
Col1A1 Expression Relative to Control
1.5
1.0
0.5
0.0
C
OSM
IL-4
IL-13
IL-17A
B
TIMP1
TIMP1 Expression Relative to Control
2.5
2.0
1.5
1.0
0.5
0.0
C
OSM
IL-4
IL-13
IL-17A
C
MMP1
MMP1 Expression Relative to Control
1.5
1.0
0.5
0.0
C
OSM
IL-4
IL-13
IL-17A
D
MMP3
MMP3 Expression Relative to Control
3
2
1
0
C
OSM
IL-4
IL-13
IL-17A

FIGURE 25

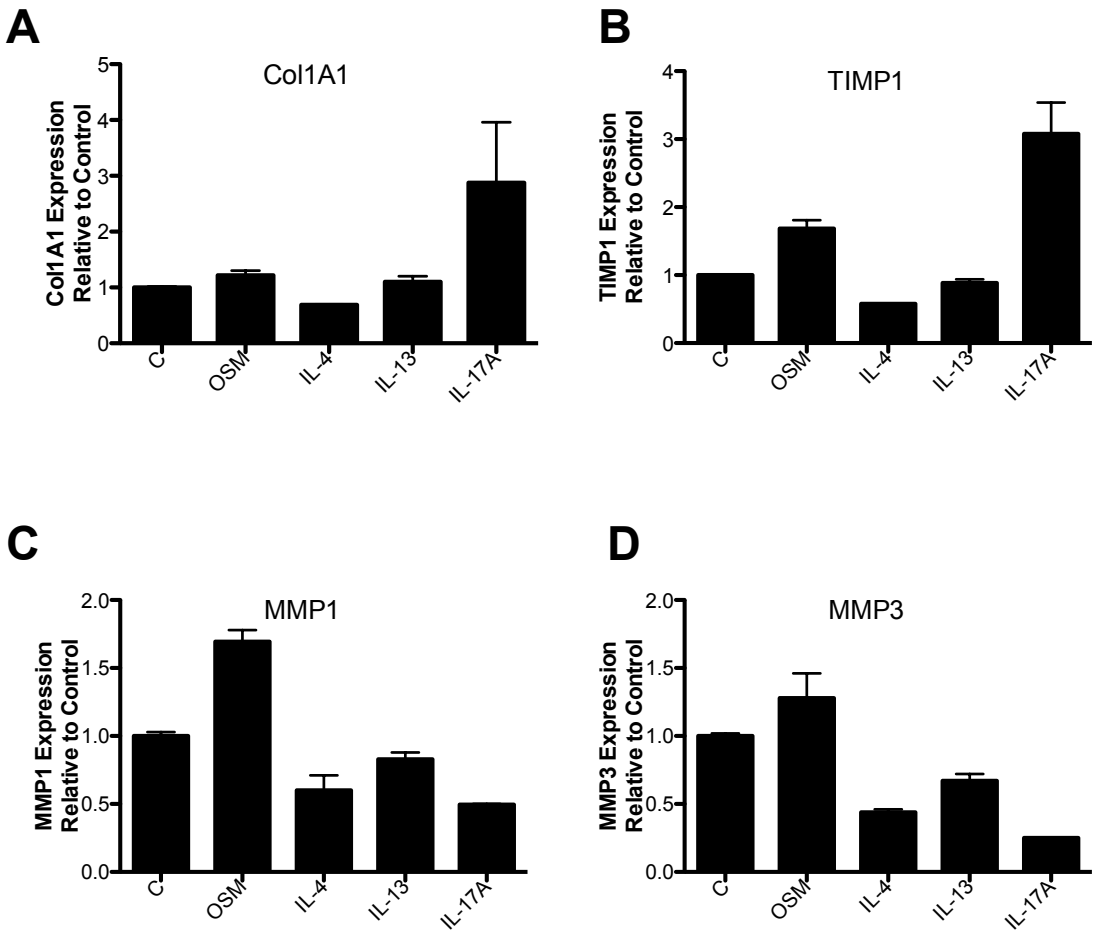
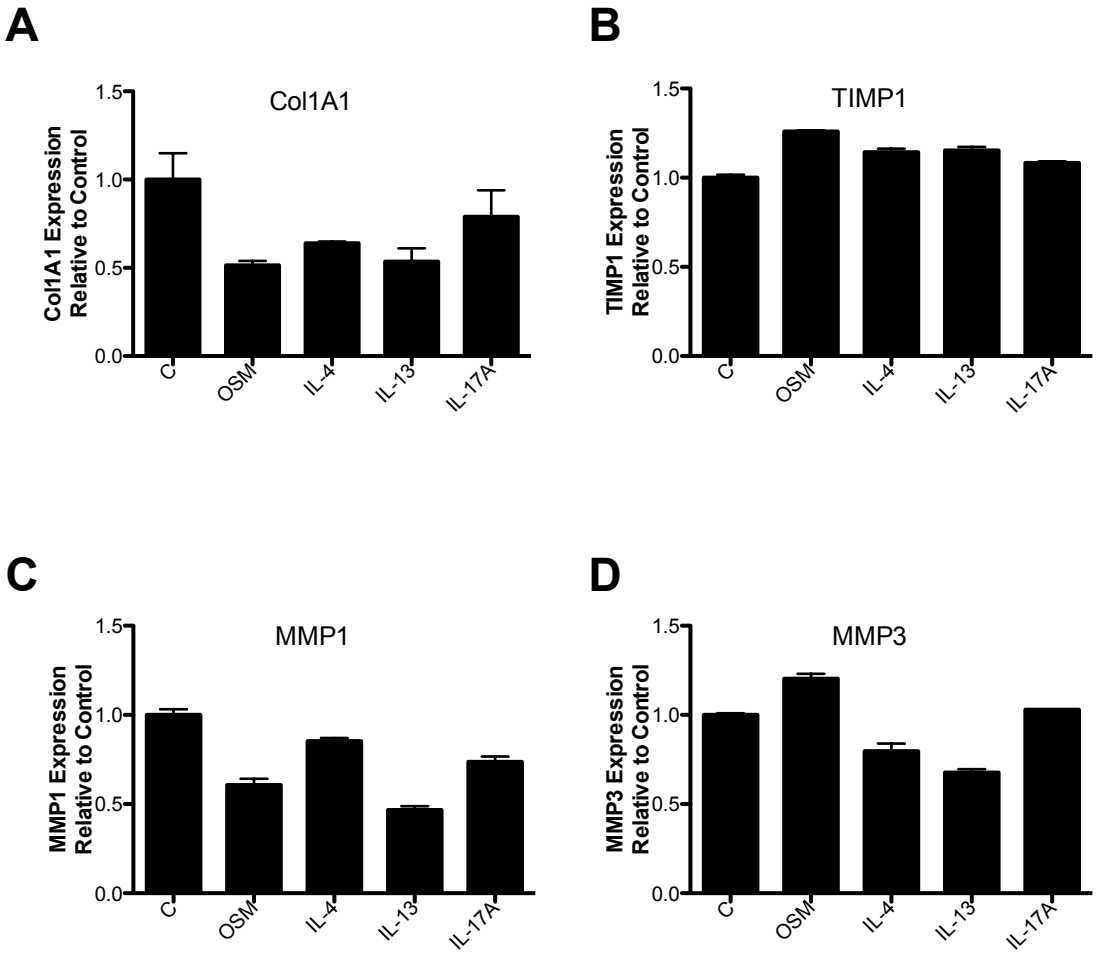

A
Col1A1
Col1A1 Expression Relative to Control
0
1
2
3
4
5
C
OSM
IL-4
IL-13
IL-17A
B
TIMP1
TIMP1 Expression Relative to Control
0
1
2
3
4
C
OSM
IL-4
IL-13
IL-17A
C
MMP1
MMP1 Expression Relative to Control
0.0
0.5
1.0
1.5
2.0
C
OSM
IL-4
IL-13
IL-17A
D
MMP3
MMP3 Expression Relative to Control
0.0
0.5
1.0
1.5
2.0
C
OSM
IL-4
IL-13
IL-17A

FIGURE 26- Extracellular and connective tissue proteins expression by HASMC 8N1 at 6 Hours. HASMC 8N1 were treated with 1 ng/mL of OSM or 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 6 hours. Total RNA assessed using qRT-PCR (Taqman) and probed for Collagen 1A1 (Col1A1) (A), TIMP-1 (B), MMP-1 (C) and MMP-3 (D). Graphs illustrate mean ± SEM (of Taqman replicate) and mRNA levels expressed as mean fold change relative to control and corrected to human β-Actin as the endogenous control.

FIGURE 27- Extracellular and connective tissue proteins expression by HASMC 8N1 at 18 Hours. HASMC 8N1 were treated with 1 ng/mL of OSM or 5 ng/mL of IL-4, IL-13 or IL-17A (or media alone) for 18 hours. Total RNA assessed using qRT-PCR (Taqman) and probed for collagen 1A1 (Col1A1) (A), TIMP-1 (B), MMP-1 (C) and MMP-3 (D). Graphs illustrate mean ± SEM (of Taqman replicate) and mRNA levels expressed as mean fold change relative to control and corrected to human β-Actin as the endogenous control.

FIGURE 28- Extracellular and connective tissue proteins expression by multiple HASMC experiments. Multiple HASMC RNA experiments where cells were stimulated with 2 ng/ml OSM or left untreated and mRNA levels were assessed using nanostring for TIMP-1 (A), MMP-1 (B), MMP-3 (C) and MMP-9 (D). N=3 for cells treated for 6 hours; N=2 for cells treated for 18 hours. Graph illustrates average nanostring count ±SEM. Unpaired T test for 6-hour groups only.

FIGURE 26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Col1A1
Col1A1 Expression Relative to Control
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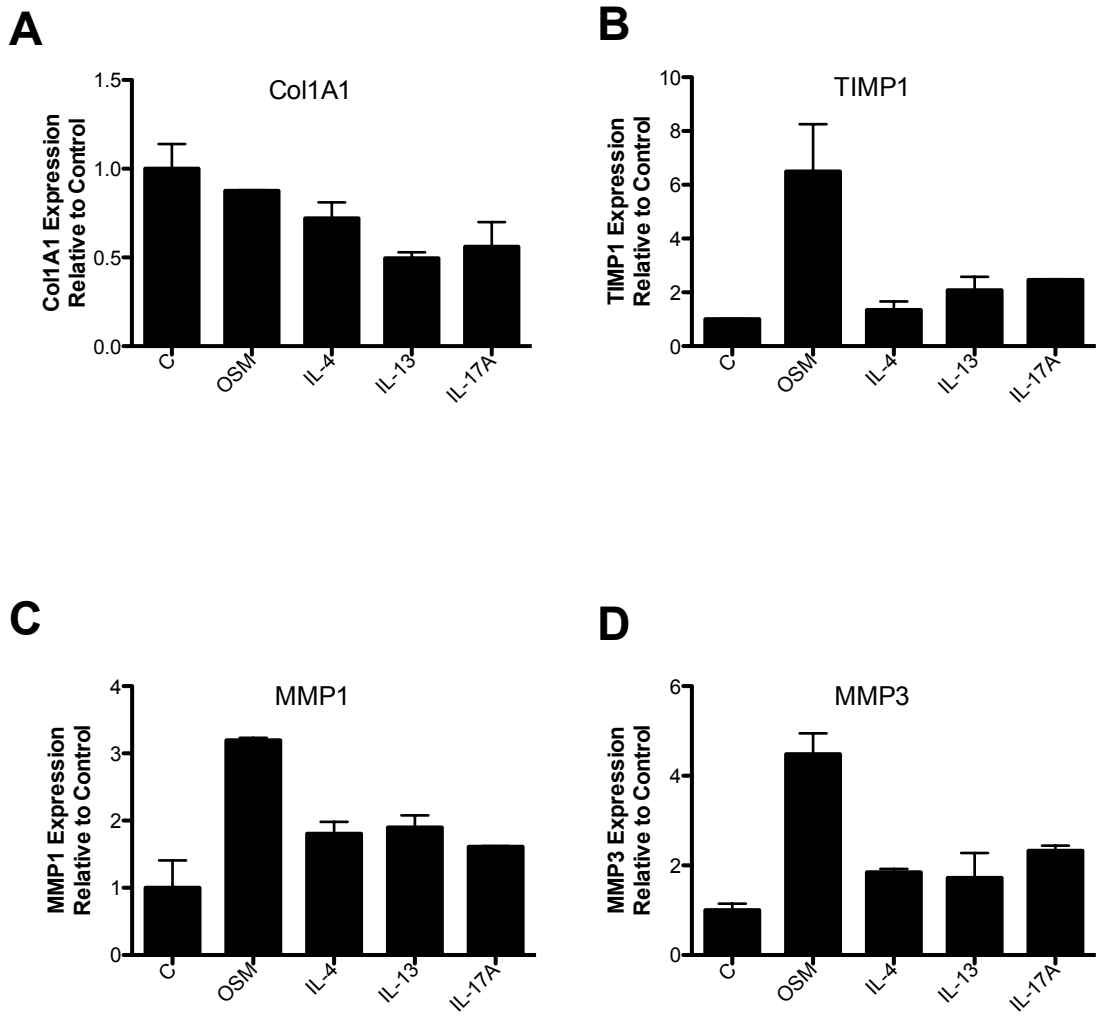
1.0
0.5
0.0
C
OSM
IL-4
IL-13
IL-17A
B
TIMP1
TIMP1 Expression Relative to Control
C
MMP1
MMP1 Expression Relative to Control
D
MMP3
MMP3 Expression Relative to Control

FIGURE 27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Col1A1
Col1A1 Expression Relative to Control
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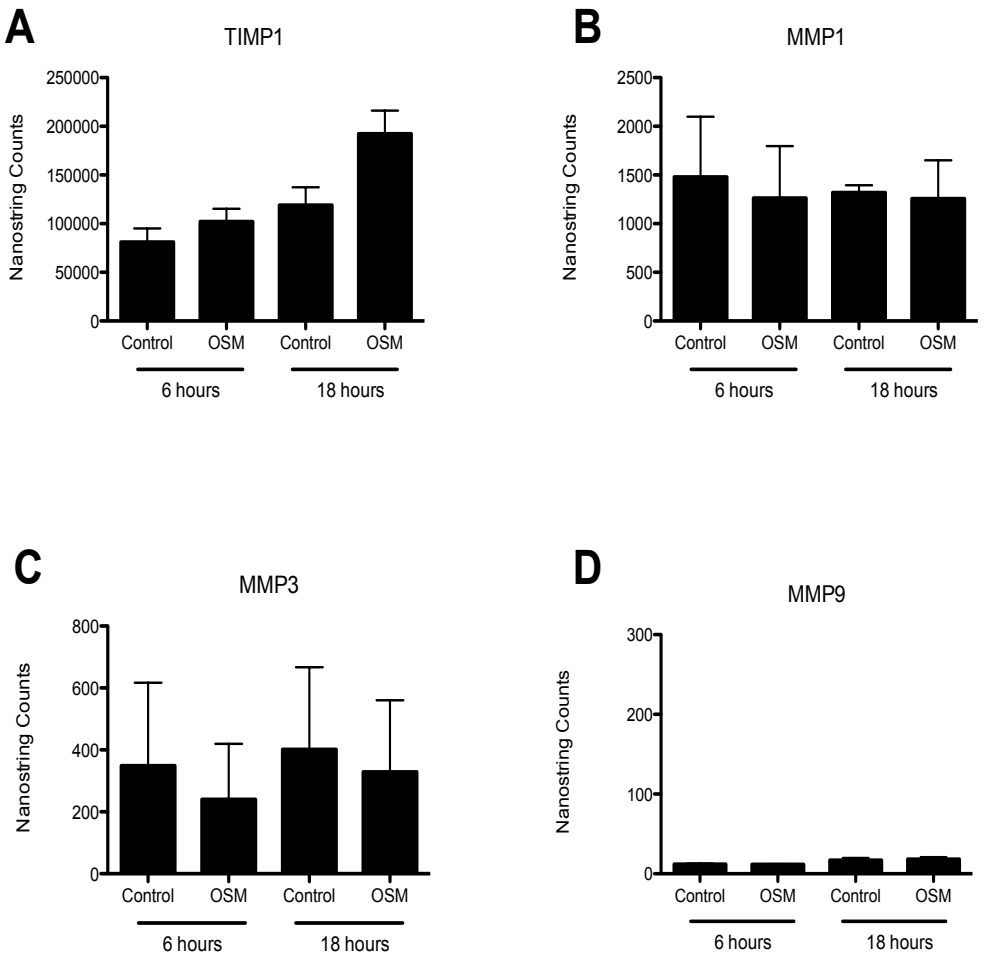
1.0
0.5
0.0
C
OSM
IL-4
IL-13
IL-17A
B
TIMP1
TIMP1 Expression Relative to Control
10
8
6
4
2
0
C
OSM
IL-4
IL-13
IL-17A
C
MMP1
MMP1 Expression Relative to Control
4
3
2
1
0
C
OSM
IL-4
IL-13
IL-17A
D
MMP3
MMP3 Expression Relative to Control
6
4
2
0
C
OSM
IL-4
IL-13
IL-17A

FIGURE 28


A
TIMP1
Nanostring Counts
250000
200000
150000
100000
50000
0
Control
OSM
Control
OSM
6 hours
18 hours
B
MMP1
Nanostring Counts
2500
2000
1500
1000
500
0
Control
OSM
Control
OSM
6 hours
18 hours
C
MMP3
Nanostring Counts
800
600
400
200
0
Control
OSM
Control
OSM
6 hours
18 hours
D
MMP9
Nanostring Counts
300
200
100
0
Control
OSM
Control
OSM
6 hours
18 hours

CHAPTER 4

RESULTS-PART 2

In this chapter, levels of OSM stimulation in human blood mononuclear cells were examined in response to bacterial and viral PAMPs *in vitro*. Specific TLR agonists were used to model products of common pathogens associated with asthma exacerbations.

4.1 Select TLR agonists induce OSM production by cultured human PBMCs

In **Figure 29-30** peripheral blood mononuclear cells (PBMCs) were isolated from two healthy donors (Donor D and Donor E) as previously described in methods. Cells were subsequently treated with the indicated treatments for 24 hours and 48 hours **(Figure 29).** CL075 (a TLR-7/8 agonist) treatment induced elevated levels of OSM protein within the supernatants of cultured total PBMC and CD14+ cells but very much less so, if at all in CD14- cells as detected by ELISA. OSM levels detected in the supernatants of CD14+ cells treated with CL075 were noticeably greater than the levels detected in supernatants of total PBMCs. Total PBMC and CD14+ cells also produced elevated levels of OSM in the supernatants when the cells were treated with 20 ng/ml LPS. When these cultured cells were treated with 10 ng/ml of PHA (phytohemoagglutinin). All three preparations (Total PBMC, CD14- and CD14+ cells) treated with PHA also showed markedly increased in supernatants. Some induction of OSM by Pam2CsK4, a TLR-2 synthetic agonist(127), in total PBMC and CD14+ cells were observed but greatest levels were detected in CD14+ cells (**Fig 29C)**. Cells treated

with poly I:C (a TLR-3 agonist), a long form of the polymer purchased from General Electric (GE) and CpG (aTLR-9 agonist) did not elevate OSM levels as detected in the assay. At 48 hours **(Fig 29, right panel)**, further elevation of OSM was observed within the supernatants of these cells with the same trends that were observed at 24 hours. The same trends in OSM induction by LPS, CL075, and PHA and to a lesser extend Pam2CSK4 were also observed in total PBMC preparation from a third donor (Donor F)

To determine if these TLR-agonists were able to induce a well-known typical TLR responsive gene, TNFα levels were examined within the same supernatants at 24 hours (**Figure 30)**. Elevated levels of TNFα were detected within the supernatants from PBMC treated with 20 ng/ml LPS (between 6,000-9000 pg/ml), CL075 (roughly 9,000 pg/ml), Pam2CSK4 and PHA (approximately 2500-3000 pg/ml) albeit the levels varied between donors from which the PBMC were isolated from. One donor PBMC induced about 1000 pg/ml of TNFα in the supernatants of cells treated with Poly (I:C) whereas donor E PBMC showed approximately 100-300 pg/ml TNFα detected. Both donors showed very little TNFα elevation when cells were stimulated with prostaglandin (**Figure 30)**.

Whether these TLR agonists could induce OSM regulation at the mRNA level was then examined. Total PBMC isolated from four healthy donors were used and treated with LPS, CL075 and a short polymer of Poly (I: C) purchased from Invivogen. In **Figure 31,** it was observed in all four donors that CL075 treated PBMC markedly increased steady state OSM mRNA levels as indicated by the 15-40-fold increase relative to unstimulated control and at least 3-fold greater elevation than LPS or Poly (I:C) although both stimuli

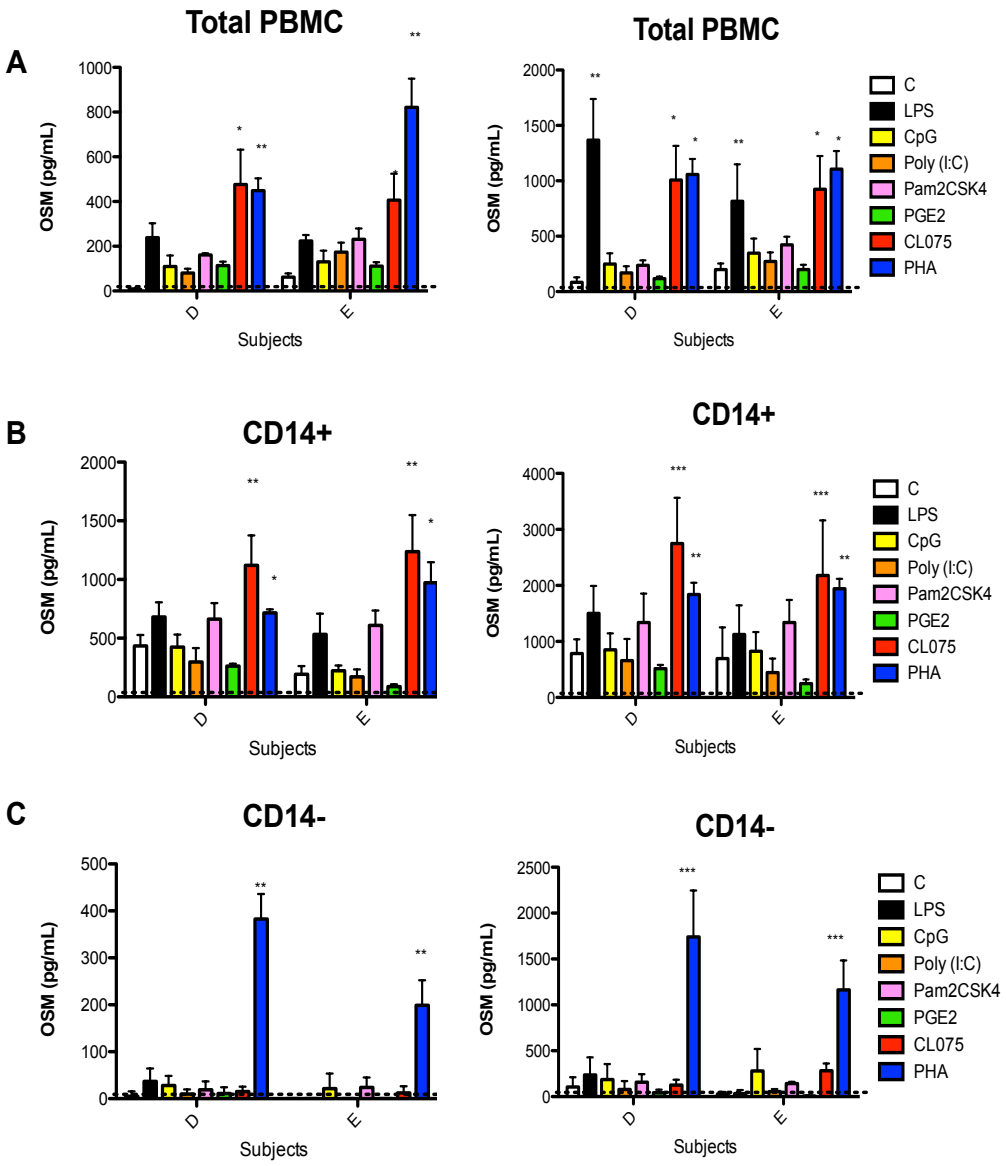
induced up to 10-fold elevation in Donors G, B and D(**Fig 31A-B;D)** and as little as 2.5 fold, as observed in Donor F (**Fig 31C**).

To determine if culturing the separate PBMC preparations in M-CSF was necessary for OSM induction, total PBMC and $CD14^+$ cells were isolated as previously described and cultured in the presence and absence of 10 ng/ml M-CSF for 3 days or 7 days. **Figure 32** illustrates total PBMC cultured for 3 days with or without M-CSF. Here, it was evident that cells cultured in the absence of M-CSF did not show elevated levels of OSM in supernatants and were below the limit of detection of the assay. Whereas cells treated with 10 ng/ml for even only 3 days had OSM levels up to 150 pg/ml for cells treated with CL075. In this experiment, it was observed that Pam2CSK4 and Poly (I:C) from invivogen also induced OSM levels comparable to CL075 treated cells (**Fig 32A and C)** where as in donor D, levels of OSM were just above the limit of detection when treated with Pam2CSK4 or Poly(I:C) (**Fig 32B**). Similar trends were observed in $CD14^+$ cells and PBMC cultured for the full 7 days in that cells cultured without M-CSF had significantly diminished OSM levels produced in response to TLR-ligands.

FIGURE 29 -OSM stimulation by cultured peripheral blood mononuclear cell (PBMC) in response to TLR agonists. Peripheral blood mononuclear cells from two healthy donors were isolated as previously described. Total PBMC (A)**,** CD14+(B) and CD14- (C) cells were then cultured in 10% FBS RPMI containing 10 ug/ml M-CSF for 7 days at 37°C, 5% $C0_2$. Cells were then treated in quadruplicate with LPS (10 ng/ml), CpG (2ug/ml), Poly (I:C) (2 ug/ml), Pam2CSK4 (2 ng/ml), CL075 (2 ug/ml), Prostaglandin E_2 (PGE2) (1uM) or Phytohemagglutinin (PHA) (10ug/ml) and were incubated for 24 hours **(left panel)** and 48 Hours **(right panel)**. Levels of OSM were examined in the collected supernatants from each well using an ELISA. The graph illustrates mean OSM levels ± SEM where *=P<0.05; **=P<0.01 using one-way ANOVA with Tukey post hoc analysis.

FIGURE 30- TNFα stimulation by cultured PBMC in response to TLR agonists. PBMC from two healthy donors were isolated. Total PBMC were cultured as described previously. Cells were then treated with LPS (20 ng/ml), CpG (2ug/ml), Poly (I:C) (2 ug/ml), Pam2CSK4 (2 ng/ml), CL075 (2 ug/ml), prostaglandin E_2 (PGE2) (1uM) or phytohemagglutinin (PHA) (10ug/ml) and were incubated for 24 hours. Levels of TNFα were examined in the collected supernatants from each well using an ELISA. The graph illustrates mean TNFα levels ± SEM where ***=P<0.001 using one-way ANOVA with Tukey post hoc analysis.

FIGURE 29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Total PBMC
OSM (pg/mL)
Subjects
D
E
C
LPS
CpG
Poly (I:C)
Pam2CSK4
PGE2
CL075
PHA
B
CD14+
C
CD14-

FIGURE 30

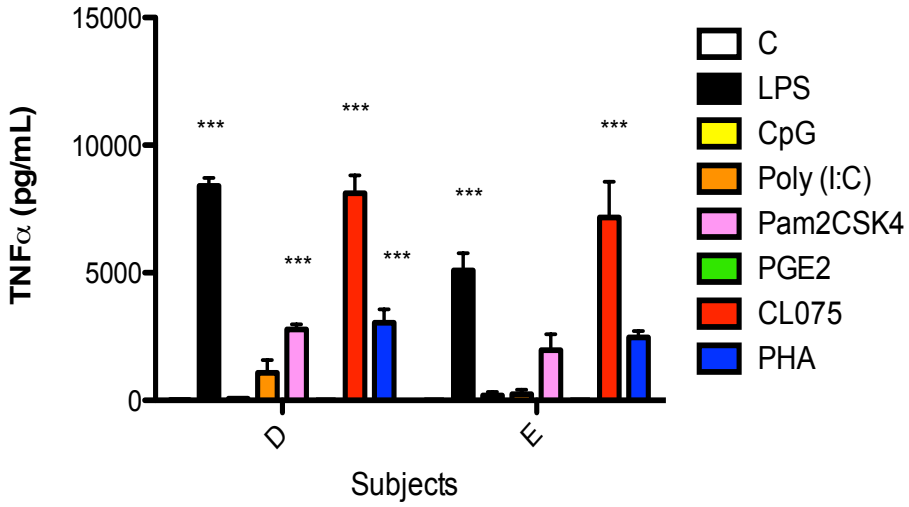
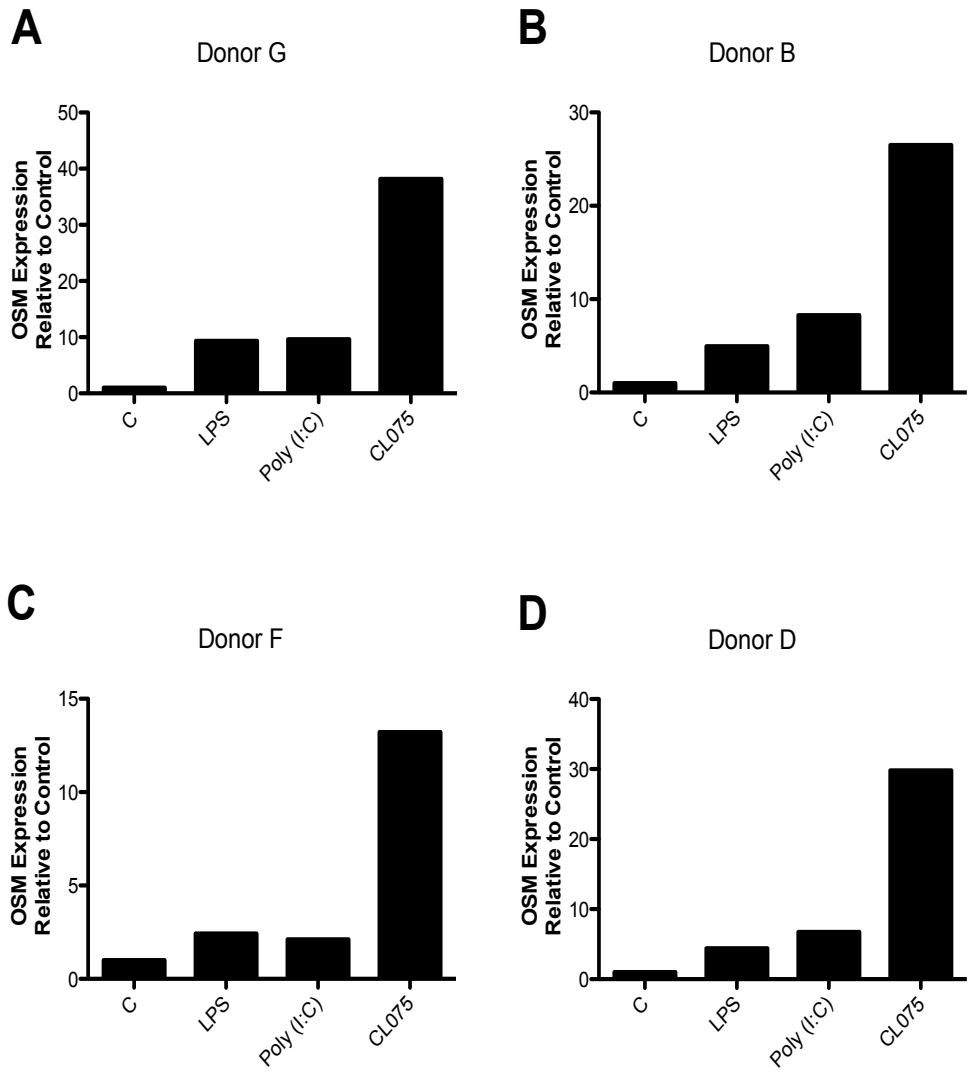

15000
10000
5000
0
TNFα (pg/mL)
***
***
***
***
***
***
D
E
Subjects
C
LPS
CpG
Poly (I:C)
Pam2CSK4
PGE2
CL075
PHA

FIGURE 31- OSM mRNA expression by cultured PBMC in response to select TLR agonists. PBMC from four healthy donors were isolated and cultured as previously described in methods. Cells were then treated with LPS (20 ng/ml), Poly (I:C) (2 ug/ml), CL075 (2 ug/ml) or left untreated (Control) and incubated for 24 hours. Total RNA was isolated as described in methods. Steady state mRNA levels of OSM expression were analyzed by qRT-PCR (Taqman) and expressed as mean fold change relative to control using β-Actin as endogenous control gene.

FIGURE 32- OSM stimulation by TLR agonists is optimal when cells are treated with M-CSF. PBMC from three healthy donors were isolated as described in methods. Total PBMC were cultured in 10% FBS RPMI containing 10 ng/ml M-CSF for 3 days at 37°C, 5% CO_2. Cells were then treated LPS (20 ng/ml), CpG (2ug/ml), Poly (I:C) (2 ug/ml), Pam2CSK4 (2 ng/ml), CL075 (2 ug/ml), or left untreated (Control) and incubated for 24 hours. Levels of OSM were examined in the collected supernatants from each well using an ELISA. Graph illustrated mean OSM levels ± SEM where **=$P<0.01$; ***=$P<0.001$ using two-way ANOVA with Bonferroni post hoc comparing M-CSF treatment to no M-CSF treatment.

FIGURE 31


A
Donor G
OSM Expression Relative to Control
50
40
30
20
10
0
C
LPS
Poly (I:C)
CL075
B
Donor B
OSM Expression Relative to Control
30
20
10
0
C
LPS
Poly (I:C)
CL075
C
Donor F
OSM Expression Relative to Control
15
10
5
0
C
LPS
Poly (I:C)
CL075
D
Donor D
OSM Expression Relative to Control
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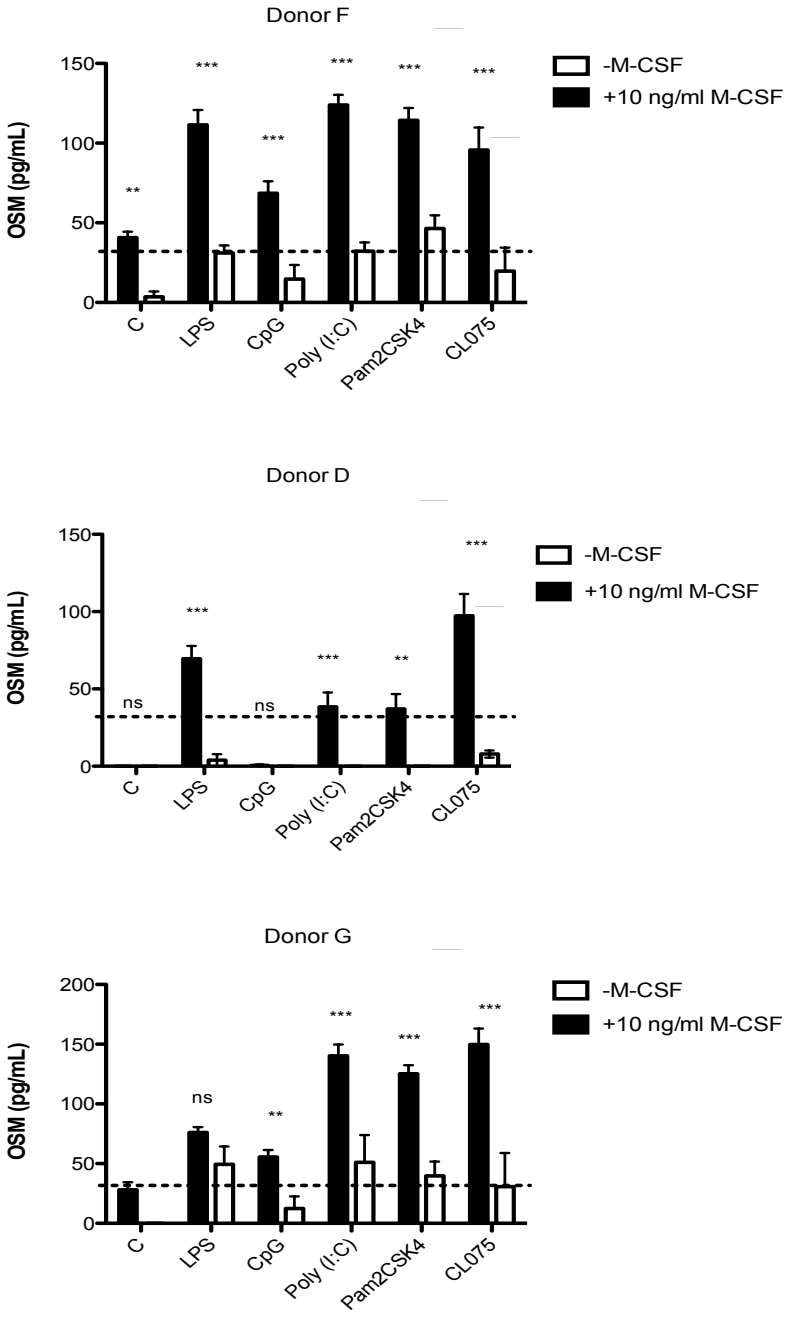
30
20
10
0
C
LPS
Poly (I:C)
CL075

FIGURE 32


Donor F
OSM (pg/mL)
150
100
50
0
**
***
***
***
***
***
C
LPS
CpG
Poly (I:C)
Pam2CSK4
CL075
-M-CSF
+10 ng/ml M-CSF
Donor D
OSM (pg/mL)
150
100
50
0
ns
***
ns
***
**
***
C
LPS
CpG
Poly (I:C)
Pam2CSK4
CL075
-M-CSF
+10 ng/ml M-CSF
Donor G
OSM (pg/mL)
200
150
100
50
0
ns
**
***
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***
C
LPS
CpG
Poly (I:C)
Pam2CSK4
CL075
-M-CSF
+10 ng/ml M-CSF

-CHAPTER 5-

DISCUSSSION

5.1 Summary of Thesis Findings

Collectively, the data from *in vitro* experiments using primary human airway smooth muscle cells demonstrated that stimulation of these cells with recombinant human Oncostatin M (OSM) could elevate expression of multiple cytokines and chemokines. Synergistic expression of select mediators (described below) was also observed when HASMC cultures were stimulated with OSM in combination with selected cytokines elevated in asthma: IL-4, IL-13 and IL-17A (summarized in **Table 2)**. These synergistic responses were observed both in levels of protein production and in steady state mRNA expression **(Fig.1-8).** When analyzing SMC cell signaling in response to OSM and select members of gp130 cytokines, the results demonstrated that OSM induced marked activation in STAT1, STAT3, STAT5, Akt p38 and JNK, whereas the other gp130 cytokines tested showed minimal or no activation of these pathways **(Fig. 9-10).** When HASMC responses to OSM, IL-4, IL-13, IL-17A and the combinations were examined for cell signaling activation, similar activations in cell signaling to individual cytokine stimulation were observed **(Fig.11).**

To examine plausible mechanisms associated with OSM synergy, pharmacological inhibitors were used to examine the requirement of various cell-signaling pathways. Inhibition of mTOR, STAT3, p38, Akt and ERK pathways using specific inhibiting agents demonstrated that mTOR had no consistent affects whereas p38, ERK and STAT3 inhibition altered HASMC responses **(Fig.13- 21)**. Modulation of cell

surface receptor expression was also examined as a possible mechanism contributing to synergic responses in HASMC. The data demonstrated that stimulation of HASMC with OSM induced IL-4Rα and OSMRβ, but not IL-17RA or IL-17RC in a manner consistent between two different HASMC cell lines **(Fig. 22)**.

To examine the potential affects of OSM on airway remodeling through HASMC proliferation or expression of ECM proteins, MTT proliferation assays and mRNA analysis of select ECM remodeling proteins were completed. The data demonstrated that OSM had minimal affect on cell proliferation **(Fig. 23)** and consistent (MMP-3, MMP-9 and TIMP-1) or inconsistent effects (Collagen 1A1 and MMP-1) on expression of select ECM remodeling proteins in different HASMC cell lines **(Fig. 24-27).**

In subsequent experiments, expression of OSM was examined in PBMC, cultured in M-CSF for 7 days in response to stimulation by various TLR- agonists. Marked elevation in OSM and TNFα protein levels were detected in supernatants of total PBMC and $CD14^+$ PBMC that were stimulated with agonists for TLR 7/8, TLR-4, TLR-2 and PHA, whereas $CD14^-$ PBMC OSM elevation was only evident in response to PHA stimulation **(Fig. 29-30, 32)**. When examining OSM mRNA expression, CL075 (a TLR7/8 agonist), LPS (a TLR-4 ligand) and poly (I: C) (a TLR-3 agonist) markedly elevated OSM levels in all of the PBMC from 4 human donors (**Fig. 31)**. Below is a more detailed discussion of the current findings and the implications that these results may have for pathogenesis of asthma.

5.2 HASMC pro-inflammatory responses to OSM and OSM synergy with IL-4/IL-13/IL-17

Several pieces of data have demonstrated that OSM can induce pro-inflammatory responses by cultured HASMC, indicating that these cells have sufficiently expressed levels of OSMR complexes (OSMRβ and gp130). Work previously demonstrated in this laboratory has illustrated that OSM can to induce various pro-inflammatory responses in HASMC including: IL-6, TARC, Eoxtain-1, MCP-1 and IP-10 (149). In addition, OSM has been shown to act in a synergistic manner with other cytokines resulting in amplified pro-inflammatory responses. For instance, in 2005, one group published that OSM could synergize with T_H2-type cytokines, IL-4 and IL-13, in the production of eotaxin-1 (41); and synergize with IL-1β in VEGF expression(112) by airway smooth muscle cells. The synergistic responses to OSM and T_H2-type cytokines in eotaxin-1 expression was also demonstrated in fibroblasts, another cell type found in lungs that are also capable of immune modulation through cytokine/chemokine release (113, 141). The findings of this thesis have further demonstrated OSM capability to act in a synergistic manner specifically with IL-4, IL-13 and IL-17A and are discussed below. The concentrations of cytokine used in this thesis were deemed to be physiologically relevant and within the range of that observed clinically. For instance, Simpson *et al*(9) demonstrated that patients with severe asthma have detectable levels between 1-10 ng/ml OSM as measured in their sputum. As reviewed previously, multiple studies have demonstrated that IL-4, IL-13 and IL-17A, amongst many others, appear to be elevated in the tissues(12), sputum and BAL fluid of asthmatics (25, 26, 77).

5.2.1 MCP-1 Expression

MCP-1, a chemotactic factor for monocytes, was elevated in the cell supernatants of HASMC treated with OSM and synergistically augmented MCP-1 levels when combined with either IL-4, IL-13 or IL-17A **(Fig. 1)**. Synergistic MCP-1 expression in response to IL-17/OSM combination was also observed in a dose-dependent manner **(Fig. 3A)** suggesting that OSM could lower the threshold to IL-17A responses with OSM concentrations as low as 0.1pg/ml. MCP-1 has been shown to be highly elevated in asthmatics (51, 106, 152), indicating a potential role of OSM in contributing to MCP-1 levels through HASMC *in vivo*. In addition, this synergistic response to OSM and IL-4/IL-13/IL-17 in the stimulation of MCP-1 was also observed at 6 hours **(Fig.4A and Fig.6A)** and 18 hour-steady state mRNA levels (**Fig. 5A and Fig 7A)** in two differing HASMC patient cell lines. This is indicative of induction of *de novo* transcription, translation and secretion of MCP-1 by these cells. However, whether post-translational modifications are involved (such as changes in mRNA half-life) is unknown at this time. These results are novel and have been incorporated into a submitted manuscript. In addition, data generated by nanostring technology supported the results observed using qRT-PCR (TaqMan) and previously published work generated within the laboratory that OSM induced stimulation of MCP-1 mRNA in HASMC **(Figure 8A)** (149) and in human aortic smooth muscle cells (HAoSMC)(129).

These results may provide a potential mechanism for the increased macrophage numbers observed in the lungs of patients with asthma, particularly in severe asthma. Previously published data suggests that the increased release of pro-inflammatory

mediators by macrophages may contribute to increased pulmonary injury and enhanced airway remodeling in response to viral and bacterial pathogens commonly detected in lungs of atopic asthmatics(107). Furthermore, MCP-1 has been demonstrated to be a chemotactic factor for fibrocytes through their expression of CCR2 (chemokine receptor for MCP-1)(152, 153). This could potentially result in increased airway remodeling such as that observed in severe asthma(154).

5.2.2 MCP-3 Expression

Nanostring analysis of multiple HASMC RNA experiments showed the novel observation that MCP-3 steady state mRNA was also highly induced by OSM **(Figure 8E).** However, it has not yet been determined if MCP-3 protein is also expressed in response to OSM. Furthermore whether OSM synergizes with IL-4, IL-13 or IL-17A in the induction of MCP-3 awaits further analysis. MCP-3 is another monocyte chemotactic factor that attracts CCR2 expressing cells (50, 152, 153). Should the highly elevated mRNA translate to protein release, expression of MCP-3 by HASMC may also attract fibrocytes and possibly contribute to asthma pathology. Furthermore, MCP-3 can also be recognized by CCR3 chemokine receptor, which is expressed on eosinophils, as reviewed in Holgate (4). Thus, OSM-induced expression of MCP-3 **(Figure 8E)** may contribute to increased eosinophils, monocyte/macrophage and fibrocyte migration to the lungs potentially contributing to asthma severity. Whether other cell types (such as lung epithelial cells or lung fibroblasts) also respond to OSM with MCP-3 expression is not yet known and is the subject of future experiments.

5.2.3 IL-6 Expression

OSM was able to induce IL-6 mRNA expression in cultured HASMC as observed by qRT-PCR (**Fig4B-7B)** or nanostring technology **(Figure 8B)** and as previously published(149). OSM has also been shown to induce IL-6 expression in other cell types including fibroblasts(114) and HAoSMC **(Figure 10B)** (129). The findings of this thesis also demonstrated that OSM further augments IL-6 expression in HASMC through synergy with IL-4, IL-13 and IL-17 as observed in mRNA expression **(Figure 4B-7B)** and in protein detection, which, like MCP-1 expression, was in a dose-dependent manner **(Figure 3B).** In contrast, when cultured HAoSMC were stimulated with OSM in combination with IL-4, IL-13 or IL-17A, there was no synergistic expression of IL-6 protein **(Figure 10B)** despite both cell types (HASMC and HAoSMC) responding very similarly in cell signaling activations through the activation of STAT, Akt and MAPK proteins **(Figure 9 & Figure 10).** This may suggest that the synergistic response observed in HASMC to OSM in combination with IL-4/IL-13/IL-17A occurs in selected cell-types. Whether the marked increases of mRNA involved changes in IL-6 mRNA half-life could also be the subject of future experimentation with OSM and IL-17A. Since IL-6 levels were found to be elevated in the sputum of allergen challenged asthmatics when compared to healthy controls (77, 155) and this elevation was associated with reduced lung function(155), the ability of OSM to elevate IL-6 expression in combination with a variety of cytokines may contribute to asthma alterations in lungs.

5.2.4 Eotaxin-1 Expression

Eotaxin-1/CCL11 is a chemotactic factor for eosinophils and other CCR3 expressing cells(113, 156). OSM has been identified as a cytokine capable of inducing eotaxin-1 expression in previous studies (41, 113, 115, 141, 157). The results of this thesis have further confirmed these observations using qRT-PCR at both 6 **(Figure 4D& Figure 6D)** and 18 hours **(Figure 5D& Figure 7D** and nanostring analysis **(Figure 8D).** The results of qRT-PCR analysis also demonstrated synergistic elevation of eotaxin-1 mRNA when HASMC were treated with IL-4/OSM or IL-13/OSM but a lesser degree with IL-17A/OSM combinations **(Figure 4D-7D).** This synergistic induction of eotaxin-1 in HASMC in response to OSM and the T_H2 cytokines has been previous described(41), however the observation that OSM could also synergize with IL-17A but to a very lower degree is novel and indicates selectivity in responses. In fact, the results suggest that IL-17A had negligible effects in HASMC expression of eotain-1 compared to IL-4 or IL-13-induced eotaxin-1 expression **(Figure 4D-7D)** in two HASMC cell lines. Previous work from a separate group demonstrated that IL-17A could induce marked expression of eotaxin-1(158, 159). The discrepancy between these observations may be due to differences in cell culture conditions such as use of DMEM growth medium and complete serum deprivation in Ham's F-12 medium prior to IL-17A stimulation by Rahman, *et al* (158) and Saleh, *et al* (159) . Whereas the culture conditions in this experiment described here include RPMI growth medium and 2% FBS instead of serum free.

5.2.5 Eotaxin-3 Expression

Similar to eotaxin-1, eotaxin-3 was also shown to be elevated by the T_H2 cytokines: IL-4 and IL-13 **(Figure 4F-7F)** and synergistic expression when HASMC (both cell lines) were stimulated with IL-4/OSM combinations **(Figure 4F-7F)**. Induction of eotaxin-3 by IL-4 or IL-13 in HASMC has been previously published(61) however, these results demonstrate novel results that OSM may act synergistically with IL-4 in the expression of eotaxin-3 by HASMC. Although IL-13 induced expression of eotaxin-3 mRNA, as demonstrated in publications (61), IL-13/OSM combination did not consistently show synergistic expression of eotaxin-3, suggesting OSM selectively synergizes with IL-4 in the expression of eotaxin-3, although one cell line (HASMC 8N1) did show augmented expresison of eotaxin-3 mRNA when the cells were treated with IL-13/OSM combination at 18 hours **(Figure 7F).** This indicates that there is slight differences in cell responses between the HASMC cell lines. This may be due to a number of reasons generated by differences in genetics or the fact that the HASMC 8N2 patient also suffered from COPD while HASMC 8N1 did not **(Table 1).** Changes in environment of the lungs due to disease may result in differing cell phenotypes or variations in cell responses *in vitro*.

Consistent between the two cell lines was the observation that there was no elevated expression of eotaxin-3 steady state mRNA (at either time point) when cells were treated with IL-17A or OSM/IL-17A combination. This observation that OSM could synergize with IL-4 in eotaxin-3 expression but not IL-17A has not been demonstrated before and provides a possiblity that elevated levels of OSM may worsen eosinophilia in

the lungs of asthmatics through selective synergistic action with IL-4 of both eotaxin-1 and eotaxin-3. Interestingly, recent evidence has shown that eotaxin-3 is a more effective eosinophilic chemoatttractant than eotaxin-1 or eotaxin-2 (156) and this suggests that eotaxin-1, eotaxin-3 and eosinophil chemotaxis are not as effectively activated by IL-17A or OSM/IL-17A presence *in vivo*.

5.2.6 IL-8 Expression

The results of this thesis further illustrates OSM selective synergistic action through the induction of IL-8/CXCL8, a potent neutrophil chemo-attractant (160). The findings demonstrated that OSM synergized with IL-17A in the expression of IL-8 as detected by protein content of cell supernatants **(Figure 2B)** and steady state mRNA levels **(Figure 5C and Figure 7C).** OSM alone did not stimulate IL-8 expression, however combination of IL-17A with 1 ng/ml or 5ng/ml OSM elevated IL-8 protein **(Figure 2B)** and steady state mRNA levels **(Figure 4C).** Interestingly, IL-4 or IL-13 did not have any detectable affect on IL-8 expression on cultured HASMC, nor was there a synergistic expression of IL-8 observed when these cytokines were combined with OSM either in protein levels **(Figure 2B)** or steady state mRNA levels **(Figure 4C-7C).** These findings demonstrate that OSM can synergize with IL-17A selectively and in a time-dependent manner and suggest that OSM/IL-17A synergy may be attributed to the elevated levels of IL-8 in the lungs of airway inflammation, as reviewed in Mukaida (2003) (76). Interestingly, data generated previously in the laboratory showed that OSM reduced LPS-induced IL-8 levels in cultured HAoAF (Human Aortic Adventital Fibroblasts) and HAoSMC, yet OSM alone did not modulate IL-8 expression (129, 161).

A similar result was documented in synovial and lung fibroblasts, in which OSM reduced IL-1-induced IL-8 expression but did not alter IL-8 expression when cells were treated with OSM alone(162). Here, OSM alone did not modulate IL-8 expression, however OSM increased IL-17A-induced levels of IL-8. If such action occurs *in vivo*, this suggests that OSM/IL-17A effects may selectively recruit neutrophils whereas OSM/IL-4/IL-13 selectively recruits eosinophils. Clinically, infections with *haemophilus influenzae* are involved in acute asthma exacerbations (12, 27, 31) and have been shown to induce neutrophilia through a IL-17A-mediated mechanism(39). The findings of this thesis suggest the investigation of OSM in such a paradigm, which may be elevated due to infections, to increased IL-8 production by HASMC. Such increased IL-8 likely influences increased neutrophilia, possibly contributing to increased severity and steroid-resistance that is often observed with neutrophilic severe asthma (31).

5.2.7 VEGF Expression

Previous work generated in the laboratory showed that VEGF expression could be induced by OSM in fibroblasts (129, 138). Here, the results demonstrate consistent data both at the mRNA level **(Figure 4D-7D)** and the VEGF proteins detected in the supernatants **(Figure 2A)**. Most interesting was the induction of VEGF protein with IL-17A/OSM combinations **(Figure 2A).** The results illustrate a synergistic relationship between OSM and IL-17A in the expression of VEGF when cells were treated with 1 ng/ml OSM and further VEGF expression was observed when using 5 ng/ml OSM **(Figure 2A)** or at the mRNA levels at 18 hours analysis in HASMC 8N2 **(Figure 5D)** and synergistic in analysis of HASMC 8N1 responses **(Figure 7D).** Synergistic induction of

VEGF was not observed when cells were treated with IL-4/OSM or IL-13/OSM combinations indicating selectivity in VEGF regulation. Others have indicated that OSM could synergize with IL-1β in VEGF expression in HASMC(138) however, the results that OSM can synergize with IL-17A and not IL-4 or IL-13 has not been demonstrated previously. VEGF induces endothelial proliferation and angiogenesis(163), a process that is observed in some asthmatics (164). In addition, elevated levels of VEGF expression in tissues of asthmatics have been correlated with decreased lung function and airway remodeling(151, 164, 165). Collectively, results suggest that OSM may contribute significantly to VEGF levels *in vivo* and thus influence asthma pathology.

5.2.8 TSLP Expression

When examining mRNA expression in multiple HASMC RNA experiments, there was a slight elevation in TSLP at 6 hours and 18-hour stimulations with OSM and not statistically significant **(Figure 8F)**. As mentioned previously, TSLP has been demonstrated to be involved in sensitization of allergic responses and progression of allergic responses in the airways (47). Similar to the cytokines/chemokines described previously, TSLP has also been shown to be elevated in the BAL of allergic airway subjects compared to healthy controls and was inversely correlated to lung function (46, 47, 132). TSLP expression in HASMC (previously published) showed that IL-1 and TNF-alpha stimulated TSLP mRNA expression in cultured HASMC from asthmatics(166). TSLP has established roles in asthma and allergen sensitization through dendritic cell-mediated Th2 responses(46, 47) but also potentially mediating Th17 responses, therefore influencing the progression of airway inflammation(132). It is not clear whether this

slight elevation in TSLP mRNA by OSM-stimulated HASMC cultures translates to elevated protein TSLP secretion, however regulation of the mRNA expression of TSLP by OSM in combination with IL-4, IL-13 or IL-17A may be of interest as no published work (currently) has indicated that OSM can modulate TSLP levels.

The results described and discussed above argue that OSM is capable of selectively stimulating various cytokines and chemokine expression in HASMC. In addition, OSM selectively synergizes with IL-4, IL-13 and/or IL-17A for several of the chemokines and cytokines described. Most importantly, several OSM-regulated products may have significant implications in asthma and/or airway remodeling.

5.3 HASMC Responses and Cell Signaling Pathways

Levels of protein phosphorylation were also examined to identify cell-signaling activation. OSM, along with other members of the gp130 family, has been shown to activate various cell signaling pathways including JAK-STAT pathway, PI'3K/AKT/mTOR pathway and MAPK pathways in other cell types (82, 86, 88, 139).

In this present study, HASMC responses were examined 20 minutes after stimulation with 10 ng/ml of OSM and other gp130 cytokines (LIF, IL-11, IL-31 and IL-6). OSM treatment induced clearly marked levels of: tyrosine phosphorylation of STAT-1 (Y701), STAT3 (Y705), STAT5 (Y694) and slightly elevated levels of tyrosine phosphorylated STAT6 (Y641); serine phosphorylated Akt at residue 473; tyrosine/threonine phosphorylation of p38 MAPK (T180/Y182) and JNK MAPK (T183/Y185) in both HASMC **(Figure 9)** and HAoSMC **(Figure 10)**. Other gp130 cytokines tested showed undetectable phosphorylation levels in these cell signaling

pathways, with the exception of STAT3 and JNK which show slightly elevated phosphorylation levels when HASMC were treated IL-6 or IL-31 **(Figure 9)**. It is clear that OSM amongst the other gp130 cytokines tested uniquely induced HASMC cell signaling responses. The concentration in picomolar (pM) was calculated for each of the cytokines based on molar mass provided by R&D systems and indicated similar molarity of cytokine concentrations used. Therefore, the lack of LIF, IL-11, IL-31 and IL-6 induced cell signaling activation cannot be attributed to differences in molarity, alternatively, the results observed suggests that there is a lack of (or low expression of) cell surface receptors on these cell that are required for the signaling of the other gp130 cytokines (129). This was also observed in HAoSMC, as published in Schnittker *et al* (2013)(129) and also indicated in **Figure 10.** For instance, expression of cell surface IL-6Rα is highly regulated and many cell types do not express membrane bound IL-6Rα. However, IL-6 can potentially signal through soluble forms of IL6Rα since gp130 is expressed broadly (167, 168). In addition, in human system, OSM can signal through the LIFR complex (LIFRα and gp130) and OSMR complex (OSMRβ and gp130)(87) however, the findings demonstrated here indicate minimal HASMC responses to LIF, suggesting that the HASMC response are likely attributed to signaling through the OSMR complex (type II) as opposed to the type I complex (LIFRα and gp130).

STAT-3 activation, as indicated by elevated levels of tyrosine phosphorylation at residue 705, was observed when HASMC were treated with OSM alone or in combination with IL-4, IL-13 and IL-17A. This was observed in three different HASMC cell lines (HASMC 8N1, HASMC 8N2 and HASMC 6N) (**Figure 11A).** STAT-3

activation and its nuclear activity have been implicated in eotaxin-1 expression(41, 159), VEGF expression(138) and IL-6 expression(169), all of which have been previously described. STAT6 phosphorylation was observed when HASMC were treated with the T_H2 cytokines alone or in combination with OSM, however, at the concentration used; OSM did not induced STAT6 activation. T_H2 cytokines: IL-4 and IL-13 have been previously established to effectively activate STAT6(113, 134). Akt phosphorylation at serine 473 and p38 activation were also observed. Interestingly, p38 activation was statistically significant in cells treated with IL-17A/OSM combination when compared to either cytokine alone **(Figure 11B)**. Recent research examining human lung fibroblast responses to OSM indicated that fibroblast function including chemotaxis and αSMA expression were mediated by STAT3 and p38 activation (110). Whether other cell types including smooth muscle cells respond identically or differently is not yet known. These findings provide new information about the cell signaling pathways that may be involved in HASMC synergistic responses to OSM, which will be further discussed in the next section.

5.4 Mechanisms of OSM synergy in HASMC

The results of the present study have demonstrated that OSM can act in a synergistic manner with IL-4, IL-13 and IL-17A in the expression of select cytokines/chemokine *in vitro*. Potential mechanisms implicated in how these synergistic responses might involve OSM were examined.

5.4.1 HASMC responses to pharmacological inhibition of select cell signaling pathways

Based on previously published data generated within the laboratory(129, 161) and the finding of this thesis that demonstrated that OSM could activate Akt pathway(143, 170, 171) as indicated by serine 473 phosphorylation **(Figure 11),** mTOR involvement, a downstream target of Akt (171), was examined using rapamycin. Rapamycin has been demonstrated to be an effective inhibitor of mTOR activity(172, 173) and previously published data have shown affects of mTOR inhibition by rapamycin on immune related responses including T cell activation (147, 148, 174). More recently, there have been emerging data that suggests mTOR inhibition may a valuable target in asthma and allergic airway inflammation (175). For instance, one group demonstrated that mTOR inhibition using an rapamycin analogue (SAR 943), reduced bronchial smooth muscle proliferation and cytokine release, therefore potentially reducing airway remodeling and inflammation (176). Another group demonstrated that rapamycin reduced IgE levels, goblet cell activation and T cell activation however also elevated IL-4 levels, eotaxin-1 levels and no change in overall AHR (177). The findings of this thesis show inconsistent alteration by rapamycin in modulation of the synergistic expression of IL-6 and MCP-1 *in vitro*. In fact, when rapamycin concentrations were doubled, there was no change in MCP-1 protein levels **(Figure 13C).** It is possible that 30 minute pre-incubation or the concentrations of rapamycin used were not sufficient to observe consistent affects, however such concentrations were sufficient to induce affects in other systems that examined rapamycin inhibition of IL-4-induced DC maturation *in vitro* (148). Thus, it was concluded that mTOR inhibition did not modulate OSM synergistic responses by

HASMC and therefore other cell signaling pathways and mTOR upstream pathways (Akt pathway) were subsequently examined.

Since significant elevation in STAT3 activation when HASMC were stimulated with OSM and that STAT-3 mRNA was elevated by OSM (**Fig 12B),** STAT-3 inhibition was next examined. A small molecule termed Stattic was used to inhibit STAT3 and designed to selectively bind the SH2 domain of STAT-3 molecules, therefore preventing phosphorylation upon docking to JAK tyrosine proteins, dimerization, which occurs through SH2 domain and therefore preventing nuclear translocation(178). Preliminary results using the recommended concentration of Stattic resulted in obliterated HASMC responses, changes in HASMC morphology and compromised cell viability (data not shown). Therefore, a non-toxic yet effective dosage was determined using a dose response in two HASMC experiments **(Figure 14).** Here, it was observed that concentration of 1.25-5 uM was effective at reducing HASMC production of MCP-1 and IL-6 protein levels while maintaining cell viability and morphology of HASMC.

Publications examining Stattic suggested that Stattic is a STAT-3 specific inhibitor(178). The western blot analysis demonstrated that this concentration could reduce STAT3 activation, however, surprisingly, STAT1 and STAT5 phosphorylations levels were also diminished upon Stattic treatments (**Figure 15)**. Inhibition of STAT-1 or STAT-5 was not observed when the agent was originally characterized in a breast cancer cell line(178, 179). OSM-induced STAT3 activation also appeared less sensitive to Stattic application quite possibly due to the substantial level of STAT3 activation that is induced by OSM treatment in HASMC. Fortunately, the affects on the other cell signaling

pathways including Akt, p38 MAPK and ERK were not substantially or dose-dependently reduced by Stattic treatments up to 5 uM **(Figure 15)**. To test difference between cell lines, a similar study was done simultaneously in two HASMC cell lines, HASMC 8N1 and HASMC 8N2. Here, the results showed that HASMC 8N2 was slightly more sensitive to Stattic treatment that HASMC 8N1. However, both cell lines responded as expected with decreased detection of phosphor- STAT3 and phosphor-STAT1 **(Figure 16).** From these studies, it was concluded that Stattic was selective for STAT activation but not specific for STAT3.

When Stattic was applied prior to stimulation with OSM or combination with IL-4, IL-13 or IL-17A, there was clear reduction in MCP-1 and IL-6 levels expressed (**Fig 17-18)**. It appeared that MCP-1 levels were more sensitive to Stattic than IL-6, in that lower concentrations resulted in significant suppression of MCP-1 but not IL-6. In addition, HASMC 8N2 was shown to be more sensitive to Stattic, as exemplified in **figure 18** as MCP-1 induction by OSM treated cells was reduced significantly with only 1.25 uM of Stattic. As mentioned previously, differences between HASMC cell lines may be attributed to patient health and preexisting conditions such as COPD **(Table 1).** COPD may generate populations of altered HASMC that sustain an altered phenotype *in vitro.* These results suggest that STAT proteins, likely STAT3, STAT-5, and STAT1 are important in MCP-1 or IL-6**.** These results demonstrate novel findings that implicate STAT signaling in OSM-induced synergy in MCP-1 or IL-6 production by HASMC. Inhibition of STAT-3 as a therapeutic approach may be possible and may function in inflammation based on the inhibition of such cytokines.

When the effects of Stattic treatments on HASMC mRNA expression of IL-6 and MCP-1 were examined, the results were similar in trend but not as clear. Twenty-four hour stimulation showed that Stattic could reduce IL-17A/OSM induced MCP-1 steady state mRNA levels, similar to what was observed in protein. There was also significant reduction in IL-17A/OSM induced IL-6 steady state mRNA levels **(Figure 19).** These results may suggest that Stattic treatment could inhibit *de novo* production of MCP-1 and IL-6 through mRNA reduction, albeit the time-points chosen for steady state mRNA may not reflect optimal activity of Stattic and further studies should expand time points examined. Further complicating the issue of time course alternations, are the results of analysis through nanostring technology, which demonstrated that OSM stimulation for 6 hour and 18 hours could significantly induce STAT1 **(Figure 12A)** and STAT3 **(Figure 12B)** mRNA levels but did not elevate STAT4 **(Figure 12C)** and STAT6 **(Figure 12D).** This implies that OSM induction of total STAT proteins may modify responses in cells overtime. Whether the induction of STAT mRNA is dependent on STAT-1, 3, or 5 activation is not known. Thus, further examination on Stattic effects on HASMC mRNA responses must be completed in order to confidently make conclusions about the use of this inhibitor.

Inhibition of p38 and ERK pathways were examined using the established selective inhibitors, SB 203580 and PD 98059, respectively. Here, the result demonstrated that p38 and to a lesser degree, (although statistically significant) ERK inhibition could reduce IL-6 protein levels, regardless of the stimulation used in HASMC cultures **(Figure 20A and C, Fig. 21**). These results are consistent with data previously

published that airway smooth muscle cells can secrete pro-inflammatory cytokines including IL-6 and IL-8, which are highly sensitive to p38 and ERK inhibition whereas IL-1β was only sensitive to p38 inhibition (180). However, Akt inhibition (shown in green bars) demonstrated no affect on IL-6 levels **(Figure 22C, left panel)**. These finding suggest that maximal expression of IL-6 and likely IL-8 as well (in context of Hedges, *et al* (2000) (180)) require MAPK activation and signaling. When MCP-1 levels were examined using these inhibitors, there was a more selective reduction by the MAPK inhibitors SB 203580 and PD 98059. IL-17A and IL-17A/OSM-induced MCP-1 levels were significantly reduced by p38 and ERK inhibition, whereas other combinations or cytokines alone were not significantly altered **(Figure 22 A & B, right panel)**. These results demonstrate that IL-17A/OSM synergistic expression and IL-17A induced responses in HASMC require MAPK and differ mechanistically from IL-4/IL-13 synergy with OSM and this idea is further discussed in the subsequent section. However, Akt inhibition non-selectively inhibited MCP-1 levels regardless of the stimulation used on HASMC **(Figure 22C, right panel)**. Collectively, the data suggest that STAT signaling is important for maximal MCP-1 expression, whereas p38 is critical for maximal IL-6 expression in HASMC.

5.4.2 Modulation of HASMC Receptor Expression in response to OSM and IL-4/IL-13/IL-17A

Previously published data indicated that synergistic expression of eotaxin-1 was closely associated with OSM-induced up-regulation of IL-4Rα and IL-13Rα1 protein and mRNA (113). This up-regulation primed fibroblasts, making the cells more sensitive to

IL-4 and IL-13 treatment, therefore resulting in greater expression of eotaxin-1 when OSM is present when compared to the cytokines alone(113). When two HASMC cell lines were used to examine such potential changes in receptor subunit expression, a similar result was observed for IL-4Rα expression **(Figure 22E).** The up-regulation of IL-4Rα is a plausible mechanism for the synergistic expression of both eotaxin-1 and eotaxin-3 mRNA in HASMC **(Figure 4F-7F).** OSM also up-regulated its own receptor complexes, gp130 **(Figure 22C)** and OSMR **(Figure 22D)** and has been previously observed in the laboratory (149). When IL-17A receptor complex subunits were examined, there was no elevation in either receptor subunit IL-17RA or IL-17RC by OSM stimulation at 6 hours. This suggests that OSM does not sensitize the cells by up-regulating IL-17 receptors similar to that observed in IL-4 and IL-13 responses previously(113). It is possible that OSM may modulate IL-17A receptor subunits at other time points, than the 6-hour and 18-hour stimulation assessed. IL-17A itself does not modulate its own receptor complexes at mRNA level similar to that described in Liang *et al* (21) in HASMC. Surprisingly, IL-4 and IL-13 induced up-regulation of IL-17RA mRNA at 6 hours as well as IL-17RC mRNA although this was more pronounced upon IL-13 treatment. These findings appear novel and suggest that IL-4 or IL-13 may enhance IL-17A-induced responses in HASMC. Further experimentation is needed to test this possibility.

5.5 HASMC expression of Extracellular Matrix/Remodeling Proteins and Proliferation in response to OSM

As previously described, airway smooth muscle have been demonstrated to have immune-modulatory properties through their secretion of various cytokines and chemokines (44, 112, 130, 152, 158, 181). Airway smooth muscle cells also express extracellular matrix (ECM) proteins and proteins involved in matrix remodeling (181, 182). Several reports have demonstrated that changes in ECM or airway remodeling to be contribute to asthma severity(138, 183, 184). These include increased levels of collagen, smooth muscle hyperplasia, (182, 184, 185) increased levels of growth factors (138, 151, 164) and changes in expression of MMPs and TIMPs (186). This thesis study also examined OSM-induced changes in ECM proteins and remodeling mechanisms in HASMC to further examine potential contributions of OSM in acute asthma exacerbations and severe asthma.

A common feature of asthma is increased smooth muscle mass through hyperplasia or hypertrophy that contribute to airway remodeling and decreased lung function(151, 165, 183). Airway smooth muscle hyperplasia may occur in response to elevated levels of various cytokines and growth factors. The presented study examined changes in HASMC proliferation in response to OSM and whether there were modulations in proliferation in response to OSM combined with IL-4, IL-13 or IL-17A.

In **Figure 23**, HASMC stimulated with the indicated cytokines for 48 hours did not show substantial elevation in proliferation as determined by OD value of soluble formazan crystals **(Figure 23A),** however at 7 days stimulation there was a small but

significant elevation in cells stimulated with OSM **(Figure 23B).** Modulation in proliferation was not altered in a synergistic manner such as that observed in select chemokine/ cytokine expression. Although statistical analysis demonstrated that these results were significant, it has been established that the MTT assay overestimates HASMC cell numbers (187, 188) In fact, serum levels of 10% alters the enzymatic activity responsible for generating formazan crystals and thus may overestimate cell number up to 20% (187). Thus, the findings of this study did not conclusively demonstrate that OSM could alter HASMC proliferation as a possible mechanism implicating OSM in severe asthma. The demonstration of OSM up-regulation of MCP-1 and MCP-3 and synergy with IL-4, IL-13 and IL-17A in chemokine expression may be the more relevant of its activities, at least assessed *in vitro*. MCP-1 and MCP-3 chemokines have also been shown to recruit fibrocytes, cells that are capable of becoming myofibroblasts and therefore contributing to airway remodeling, as previously discussed (153, 189).

When examining HASMC expression of ECM proteins and matrix remodeling proteins, inconsistent results were observed between the two HASMC cell lines tested (HASMC 8N1 and HASMC 8N2) and between the two time points chosen **(Figures 24-28).** When select ECM remodeling proteins were examined in multiple HASMC experiments using nanostring technology, highly variable results were observed resulting in no statistical significance in MMP-1 and MMP3 (**Figure 28**). MMP9 expression was consistently undetectable in both HASMC cell lines using qRT-PCR (data not shown) and nanostring analysis **(Figure 28D)**. This implies that MMP9 is not expressed in this cell

type although MMP9 has been shown to be elevated in sputum of asthmatics and further elevated upon allergen challenge(186). TIMP-1 expression consistently was elevated by OSM **(Figure 24B-28B**, consistent with data in house and previously published (102, 149). Collagen mRNA levels was highly variable between the cell lines tested **(Figure 24-27).** Previous works generated in the laboratory showed overexpression of OSM through endotracheally administered adenovirus in mice resulted in increased collagen and alpha smooth actin(190, 191). This difference between cell lines tested in regards to ECM and ECM remodeling proteins were also observed previously within the laboratory(149). As previously mentioned, this is likely through pre-existing diseases of the HASMC patient, and genetic variability contributing to altered cell phenotypes and cellular responses *in vitro*. In order to confidently examine OSM-induced changes in ECM proteins, a larger sample size of number of HASMC cell lines should be examined.

5.6 PBMC modulation of OSM through TLR-agonist(s) stimulation

OSM levels have been shown to be elevated in allergic dermatitis (192) and in the sputum of patients with asthma (9) and after allergen challenge (unpublished data). Previous reports have demonstrated that OSM can be stimulated by cultured human PBMC *in vitro* (92–96, 193). In this thesis, OSM expression was examined using cultured PBMC stimulated with various stimuli including TLR- agonists, prostaglandin and PHA. The results demonstrated that total PBMC and $CD14^+$ PBMC up regulated OSM protein when stimulated with CL075, LPS, Pam_2CSK4 and PHA at both 24 hours and 48-hour stimulation. In addition, CL075, LPS and Poly I:C (short) were able to increase steady state mRNA levels of OSM **(Figure 29 and 31)**

CL075 is identified as a synthetic thiazoloquinoline immune-stimulatory molecule that activates human TLR-7/8 (131). TLR-7 and TLR-8 recognize ssRNA and, similar to other nucleic acid sensing TLRs, are located intracellularly within endolysosomal compartments(125, 131, 194). Here, activation of TLR-7/8, through the use of CL075, elevated OSM production, implying that human PBMC can up-regulate OSM protein and mRNA expression in response to intracellular detection of ssRNA. Therefore, based on this data, ssRNA viruses such as HRV and other viruses implicated in virally induced asthma exacerbations may be sensed by infiltrating immune cells, such as monocytes, through TLR-7/8 and induce significant OSM production. These finding are novel and demonstrate a potential contribution to the increased OSM levels in lung, possibly reflected in the increased levels in sputum of individuals with severe asthma. Since MCP-

1 is elevated in asthma, this OSM-induced action may further facilitate recruitment of monocytes to the airways, therefore further increasing cells potential OSM expression and a potential positive feed loop.

As mentioned previously, total PBMC stimulated with CL075 clearly elevated **(Figure 29)** suggesting that OSM production was synthesized *de novo*. The observed increase in mRNA levels may also have been due to changes in mRNA half-life as opposed to elevated transcriptional activity in response to this agonist. To confirm that these results were not due to changes in mRNA half-life, time-course experimentation using CL075 stimulation could be conducted. In addition, methods that assess mRNA decay could be used such as transcriptional inhibition using actinomycin D which inhibits transcription by intercalating DNA or kinetic mRNA labeling (195). Assessing the mRNA half-life could be the subject of future work.

LPS stimulation also significantly increased OSM protein within the cell culture supernatants **(Figure 29, black bars)**. These results has been observed previously within the laboratory previously (149, 161). In addition, previously published data demonstrated that LPS and fixed *Staphylococcus aureus* induced large amounts of OSM mRNA expression and OSM protein within the supernatants of cultured dendritic cells. Those results demonstrated for the first time that dendritic cells could produce OSM in response to bacterial products (94). Whether monocyte-derived dendritic cells can produce OSM in response to TLR-7/8 agonists such as CL075 will the subject of future work, as no work to date has demonstrated DC production of OSM in response to ssRNA analogs. Since

neutrophils have also been shown to produce OSM in response to GM-CSF and LPS(99, 100), it would be interesting to examine whether neutrophils can secrete OSM in response to CL075 as well. Indeed, neutrophils have been reported to be recruited in high numbers to the lungs of non-eosinophilic asthmatics (74). In addition, the findings of this thesis have demonstrated elevated IL-8 expression in response to OSM/IL-17A by HASMC and if reflected *in vivo*, may contribute to the recruitment of more neutrophils and generating a positive feedback loop of chemokine and inflammatory cell infiltration. Examining neutrophil capacity to produce OSM in response to CL075 and other viral products would be a subject of future work.

At the mRNA level, LPS induced elevated steady state OSM levels **(Figure 31**), however not as elevated as changes induced by CL075. These results were intriguing since LPS stimulated protein levels similar to levels induced by CL075. In fact, in some cases, LPS-induced mRNA levels were less than the mRNA levels induced by Poly (I:C) stimulation, which at the protein level, showed far less protein. These differences may reflect alterations in time course of steady state mRNA levels. Since changes in the mRNA steady state levels could also be modulated by alterations in mRNA half-life or transcriptional activity, such factors may influence observations. Therefore, further studies such as time course mRNA analysis or kinetic labeling techniques or transcriptional inhibition as described in Chen, *et al* (2008) (195) would need to be conducted in order to determine reasons for differences between mRNA steady state levels.

Poly (I:C) is a synthetic dsRNA analog recognized by TLR-3 and potentially reflects the detection of viral infections *in vivo*, since dsRNA is the molecular pattern observed in many viruses during the replication cycles (125, 194). The results in **figure 29 (orange bars)** showed low OSM levels when PBMC were stimulated with Poly (I:C). These results were interesting, since TLR-7/8 activation, using CL075, markedly elevated OSM levels. A plausible explanation for these results is the length of the Poly (I:C) polymer used in these experiments. This preparation of Poly (I:C) was generated by General Electric (GE) producing a polymer of 8kbp in size. Recently, a study demonstrated that the length of the Poly (I:C) used markedly affects immune responses in differing cell types. Longer polymers (greater than 5 kbp) preferentially induced elevated cytokines and anti-viral responses in stromal cells such as fibroblasts, but lower responses by myeloid-derived cells such as macrophages (196). This was observed within the laboratory using HASMC (149). When using poly (I:C) that are shorter in length (below 2 kbp), greater cytokine production and anti-viral responses were observed in myeloid cells(196). Hence, the preparation purchased from GE, which was a long polymer, may alter how these PBMC respond. To examine this further, TNFα levels were tested in the same cell supernatants of total PBMC **(Figure 30, orange bars)**. Again, poly (I:C) from GE (the longer polymer) induced minimal TNFα levels, confirming that these cells were less responsive to this preparation. Poly (I:C) purchased from Sigma-Aldrich, which provides a preparation that is approximately 2 kbp in length (considered short). When this preparation of poly (I:C) was used there was induction of OSM mRNA steady state levels comparable to LPS stimulated PBMC. These results although not yet confirmed by

protein analysis, demonstrate novel data indicating viral products, as well as bacterial products; can induce OSM production in cultured PBMC (differentiated with M-CSF). Further experimentation and comparative analysis using the short Poly (I:C) and long Poly (I:C) should be completed in the future to confirm the resulted observed.

Pam_2CSK4, a synthetic diacylated lipoprotein, is an agonist of TLR-2 which recognizes bacterial lipoproteins(125, 127). The results of this thesis showed that total PBMC stimulated with this agonist did not stimulate significant levels of OSM protein as detected in the supernatants **(Figure 29A, pink bars)**. $CD14^+$ PBMC stimulated with pam_2CSK4 showed some elevation in OSM levels, but these were not statistically significant. Since minimal induction of OSM was observed using Pam_2CSK4, the other TLR-2 heterodimer (TLR-2/TLR-1) should be examined as well. Pam3CSK4 activates TLR2/TLR1 heterodimers. Examining this agonist would confidently indicate whether TLR-2 activation can or cannot induce OSM production in cultured PBMC. Pam_2CSK4 traditionally activates the heterodimer of TLR-2/TLR-6, but published data suggests that this synthetic diacylated lipopeptide may signal through a TLR-6 independent mechanisms and may signal using TLR2/TLR1 heterodimer(197).

The TLR-9 agonist, unmethylated CpG was also used to stimulate cultured PBMC. CpG did not elevate OSM protein levels in total PBMC **(Figure 29, yellow bars)**. When TNFα levels were examined to test cell responsiveness to the agonist, no TNFα levels were detected **(Figure 30, yellow bars)**. These results may suggest that, like poly (I:C) from GE, these cells did not recognize the compound. One reason for this may be

degradation of the nucleotide polymer by nucleases, therefore resulting in no TLR-9 activation. To alleviate this problem, a nuclease-resistant CpG, purchased from invivogen was used for a different experiment examining M-CSF affects **(Figure 32).** Here, CpG did not induce substantial levels of OSM, albeit levels were above limit of detection in two donors. Should additional experiments allow confident exclusion of CpG as a significant stimulant for OSM production in PBMC, this would suggest that these cells do not express sufficient TLR-9 in order to respond to the agonist and thus, OSM production by PBMC is in response to select bacterial products, such as LPS and not others (unmethylated CpG).

Prostaglandin E_2 did not have any affect on OSM protein levels **(Figure 29, green bars)**. These results were not expected as Prostaglandin E_2 has been published inducer of OSM in cultured macrophages previously (97, 198). Despite similar concentrations used, the published result could not be reproduced in the thesis experiments. Possible explanations include stability of prostaglandin E_2 or differences in cell types examined. Chronic wound macrophages, microglia (89) and THP-1 cells stimulated with prostaglandin E_2 in the published works(97, 198), whereas blood monocyte derived macrophages were assessed in the presented work.

The findings of this study showed that total PBMC and $CD14^+$ PBMC cultures produced elevated levels of OSM in response to select TLR agonist as detected by ELISA in the supernatants **(Figure 29B)** whereas $CD14^-$ PBMC did not **(Figure 29C**). Lack of response to LPS was expected of the $CD14^-$ cells since CD14 is a required component of

LPS receptors, however soluble forms of CD14 can induce LPS recognition in $CD14^-$ cells but this was not assessed in these cultures, therefore it is unclear if LPS recognition was occurring at this time. One possibility explaining why $CD14^-$ did not respond similar to the $CD14^+$ and total PBMC cultures to other TLR-ligands was the use of M-CSF in differentiating these cells. Treatment of these cells with M-CSF may result in alterations of cytokine expression. It was previously determined that only $CD14^+$ cells of blood mononuclear cells express CD115, the receptor for M-CSF(48, 50). Therefore, these $CD14^-$ cells likely did not respond to M-CSF. In addition, $CD14^-$ cells which include T cells, B cells and NK cells all express the TLRs(199) that were tested in this thesis. The likely reason for $CD14^-$ cells not responding is that these cell types (specially T and B cells) need activation in order to fully respond. For example, Caron *et al* (2005)(200) demonstrated that $CD4^+$ T cells isolated from human PBMC could respond to the TLR-7/8 agonist, R-848 and TLR-5 ligand, flagellin in the stimulation of IFNγ however, this was only observed when these cells were activated using anti-CD3, anti-CD2, anti-CD28 or IL-2. When these cells were not stimulated with these activating antibodies, IFNγ production was minimal(200). This may also explain why PHA was the only stimuli observed to induce OSM in this population of cells. PHA is a mitogen that has been used to activate PBMC specifically T cells *in vitro* (201). Therefore, $CD14^-$ PBMC OSM expression in response to CL075 and the other TLR agonists would need to be done in combination with agents that effectively activate these cells and would be studied in future work.

PBMC collected from healthy donors were cultured for 7 days in recombinant M-CSF (**Figure 29-31**). M-CSF was included to differentiate blood monocytes into tissue macrophages as described previously(202), however these culturing conditions were also used for $CD14^-$ cells (non-monocyte cells) to maintain consistency between culture conditions. To examine whether culturing these cells in M-CSF had an effect on OSM protein levels, PBMC were cultured in the presence or absence of M-CSF for 3 and 7 days. **Figure 32** showed that cells stimulated in the absence of M-CSF had significantly lower levels of OSM than compared to cells treated with M-CSF for 3 days. This was also observed after 7 days (data not shown). These results demonstrate that blood monocytes cultured *in vitro* require M-CSF for optimal production of OSM. Whether M-CSF levels are elevated in the airways of asthmatics or during acute exacerbations is not clear.

Differentiating PBMC in M-CSF towards macrophage phenotype may be a limitation of this study. This differentiation *in vitro* was used due the relative technical difficulty in obtaining monocytes/macrophage population from patient lungs in sufficient quantities for analysis as well as the time taken for ethics approval. The use of M-CSF has been used by multiple groups as an established method in differentiating blood monocytes into macrophages (95, 198, 202). However whether the phenotype induced by this method of differentiation accurately reflects the phenotypes of monocytes/macrophages that are present in normal or diseased lungs is not clear. Despite this limitation, the results show the potential of OSM induction in response to TLR-ligands.

-CHAPTER 6-

CONCLUSION

Asthma is characterized as a chronic inflammatory condition of the airways. The airway smooth muscle cells have been shown to be an important cell type in asthma due to its roles in the structural integrity of the lungs and its immune-modulatory capacity. Therefore, in addition to contributing to bronchoconstriction and airway narrowing as a result of AHR, these cells are also involved in the secretion of various inflammatory mediators involved in asthma. Viral and bacterial infections have been shown to be associated with asthma exacerbations that result in increased AHR, cell infiltration and severity in asthma symptoms resulting in increased hospitalizations and morbidity. In this thesis, it was postulated that OSM, a gp130 cytokine, is involved in asthma exacerbations and severity by inducing HASMC pro-inflammatory responses. It was also postulated that OSM could be secreted upon recognition of various TLR-ligands such as viral and bacterial products.

The findings demonstrated that *in vitro* HASMC stimulation with recombinant OSM could regulate HASMC responses including cell signaling activation, cytokine/chemokine expression, receptor mRNA modulation and alterations in ECM remodeling proteins. In addition, stimulation with OSM acts synergistically with IL-4, IL-13 or IL-17A in HASMC expression of select chemokines and cytokines. Several of these results demonstrate novel findings that can contribute to the fields of HASMC biology and airway inflammation. In addition, OSM stimulation of HASMC did not alter or consistently modulate ECM remodeling proteins and HASMC proliferation (under the

conditions of the *in vitro* studies completed here). These results suggest that OSM, in this *in vitro* model of cell responses contributes to asthma acute exacerbations by augmenting pro-inflammatory responses in HASMC through STAT and p38 signaling, resulting in increased inflammation and cellular infiltrations. In contrast, it is not clear whether OSM contributes to asthma severity through direct actions on smooth muscle participation in extracellular matrix remodeling.

In addition, the findings of this thesis demonstrate that blood mononuclear cells differentiated to macrophages upon recognition of ssRNA/ select viral products or LPS can markedly up-regulate OSM expression. Recognition of such viral or bacterial pathogens by infiltrating cells or resident cells in the airway results in increased expression of various pro-inflammatory responses. Here, OSM has been demonstrated to be elevated in macrophages in response to certain viral and bacterial products suggesting that OSM may be elevated in the airways of severe asthmatics in responses to these pathogens *in vivo*.

Taken together, the data generated *in vitro* supports the hypothesis that OSM stimulation upon viral and bacterial pathogen recognition by resident or infiltrating immune cells may be elevated during these infections. This elevation of OSM may synergize with the milieu of cytokines and inflammatory mediators of asthmatic/allergic airways including IL-4, IL-13 and IL-17A. Synergistic expression of various chemokines and cytokines including: MCP-1, IL-6, Eotaxin-1 and IL-8 would further amplify lung inflammation resulting in acute asthma exacerbations and possible increases in asthma severity through indirect mechanisms.

-CHAPTER 7-

APPENDIX

TABLE 1

Cell Line	Date of Surgery	Gender	Age	Smoker	Other Conditions	Comments
HASMC 8N1	Nov 8, 2012	Female	75	40 pack years, quit 10 years	No	
HASMC 8N2	Nov 8, 2012	Female	75	35 pack year, quit 5 years	COPD	Produced little IL-6
HASMC 6N	Nov 6, 2012	Male	65	40 pack years, quit 1 month	No	Grew slowly
HASMC-O	Oct 16, 2012	Male	50	60 pack year	COPD/Asthma	Cells died
HASMC-A	April 5/2005	N/A	82	Current Smoker	Mild COPD Emphysema Stroke Squamous cell Carcinoma	

Table 1- Human Airway Smooth Muscle cell patient information.

TABLE 2

Cell Line	HASMC 8N1					HASMC 8N2				
	OSM Alone	Synergy With (Y/N):			Time Point (hrs)	OSM Alone	Synergy with:			Time Point (hrs)
		IL-4	**IL-13**	**IL-17A**			**IL-4**	**IL-13**	**IL-17A**	
MCP-1										
Protein	Incr.	Y	Y	Y	18	Incr.	Y	Y	Y	18
mRNA	Incr.	Y	Y	Y	6 &18	Incr.	Y	Y*	Y	6 &18
MCP-3										
Protein	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C
mRNA	Incr.	N.C	N.C	N.C	6 &18	Incr.	N.C	N.C	N.C	6 &18
Eotaxin-1										
Protein	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C
mRNA	Incr.	Y	Y	Y*	6 &18	Incr.	Y	Y	Y	6 &18
Eotaxin-3										
Protein	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C
mRNA	N	Y	N	N	6 &18	N	Y	Y	N	6 &18
IL-8										
Protein	N	N	N	Y	18	N	N	N	Y	18
mRNA	N	N	N	Y	18	Incr.	N	N	Y	18
IL-6										
Protein	Incr.	Y	Y	Y	18	Incr.	Y	Y	Y	18
mRNA	Incr.	Y	Y	Y	6 &18	Incr.	Y	Y	Y	6 &18
VEGF										
Protein	Incr.	N	N	Y	18	Incr.	N	N	Y	18
mRNA	Incr.	N	N	Y	18	Incr.	N	N	Y	18
TSLP										
Protein	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C	N.C
mRNA	N	N.C	N.C	N.C	6 &18	N	N.C	N.C	N.C	6 &18

N.C= Not yet completed; Incr.= Increased; Y= Yes; N= No

* Synergy only observed at **6 hours**

Table 2- HASMC pro-inflammatory responses to OSM and OSM-synergy with IL-4, IL-13 or IL-17A

-CHAPTER 8-

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