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MCP-1 Release Modulation Through Interaction of Pulmonary Endothelial Cells and Mesenchymal Stromal Cells

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**MCP-1 RELEASE MODULATION THROUGH INTERACTION OF PULMONARY
ENDOTHELIAL CELLS AND MESENCHYMAL STROMAL CELLS**

by

Kaavya Giridhar

Approved:

Sean I. Savitz, M.D.
Advisory Professor

Jaroslav Aronowski, M.D., Ph.D

David W. Marshak, Ph.D

Scott D. Olson, Ph.D

Harry Karmouty-Quintana, Ph.D

Matthew T. Harting, M.D., M.S.

Approved:

Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

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ENDOTHELIAL CELLS AND MESENCHYMAL STROMAL CELLS**

A

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Presented to the Faculty of

The University of Texas

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in Partial Fulfillment

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for the Degree of

MASTER OF SCIENCE

By

Kaavya Giridhar, B.S.

Houston, Texas

May 2020

Dedicated to my dearest Amma, Appa & Paatti

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MCP-1 RELEASE MODULATION THROUGH INTERACTION OF PULMONARY ENDOTHELIAL CELLS AND MESENCHYMAL STROMAL CELLS

Kaavya Giridhar, B.S.

Advisory Professor: Sean I. Savitz, M.D.

Background: Ischemic stroke is a leading cause of death and long-term disability around the world. Current treatment options are limited to the administration of tissue plasminogen activator (tPA) and/or endovascular therapy, administered within a limited time window. However, cell-based therapies such as mesenchymal stromal cells (MSCs) have increasingly shown great promise for ischemic stroke recovery with some therapies already in various stages of clinical trials. Intravenous (IV) administration of the MSCs leads to the entrapment of these MSCs in the lungs. These entrapped MSCs interact with the pulmonary endothelial cells (PECs) and could modulate the immune response through the release of cytokines and chemokines. Monocyte Chemoattractant Protein - 1 (MCP-1), is an important chemokine involved in the recruitment of monocytes and macrophages. In our study, we wanted to explore the interactions between MSCs with PECs and how this interaction changes the expression levels of MCP-1 and other cytokines after an inflammatory event such as stroke. We also wanted to see if MCP-1 released through the interaction between MSCs and PECs under inflammatory conditions, modulates the immune response through the modification of monocytes.

Methods: Cultured murine PECs were grown either alone or in combination of murine MSCs, and were exposed to 1) a combination of IFN- γ and TNF- α inflammatory stimuli or 2) Anti MCP-1 antibody to neutralize any secreted MCP-1. The secretome release of IL-1 β , IL-1ra, IL-6, MCP-1, and VEGF were analyzed

using ELISA (BD Biosciences and R&D Systems). To further understand the immunomodulatory response, the collected media from the previous step was added to splenic immune cells (CD11b+) and splenic monocytes (CD115+). The secretome release was analyzed from these cells using ELISA.

Results: MCP-1 secretion levels were increased from PECs as well as co-cultures of PECs and MSCs when they were exposed to inflammatory stimuli. When co-cultures of PECs and MSCs were exposed to recombinant MCP-1 or MCP-1 neutralizing antibody, VEGF secretion levels decreased. In the presence of inflammatory stimuli, co-cultures of PECs and MSCs secreted elevated levels of VEGF. While under inflammation, we also observed that IL-6 levels were elevated and they remain elevated even when MCP-1 was neutralized. We did not observe any difference in secretome release from neither the splenic immune cells (CD11b+) nor the splenic monocytes (CD115+).

Conclusion: Our data show that MCP-1 release under stroke like conditions is modulated through the interaction of PECs and MSCs. However, our study was unable to elucidate MCP-1's role in the modification of monocytes.

TABLE OF CONTENTS

Dedication.....	iii
Acknowledgment.....	iv
Abstract.....	vi
Table of Contents.....	viii
List of Tables.....	xi
List of Figures.....	xii
Abbreviations.....	xiii
Chapter 1: Introduction.....	1
1.1 Stroke.....	2
1.1.1 Ischemic Pathophysiology.....	2
1.1.2 Current Stroke Treatments.....	3
1.1.2.1 tPA.....	3
1.1.2.2 Endovascular Therapy.....	4
1.2 Cell Therapy.....	4
1.2.1 MSC Therapy.....	6
1.2.2 Intravenous Administration and Pulmonary Passage.....	7
1.3 Immunomodulatory Role of MSCs.....	7
1.4 Monocytes and Macrophages.....	8
1.5 Monocyte Chemoattractant Protein – 1.....	9
1.5.1 Role of MCP-1 in Pathologies Involving Neuro-inflammation.....	9
1.5.2 Role of MCP-1 in Ischemia.....	10
1.5.3 Role of MCP-1 in Cell Therapy.....	11

1.6 Objectives and Hypothesis.....	12
Chapter 2: Materials and Methods.....	14
2.1 Isolation and culture of primary murine mesenchymal stromal cells.....	15
2.2 Primary murine pulmonary endothelial cells.....	15
2.3 Isolation and verification of murine splenic monocytes.....	16
2.4 Viability Assessment	17
2.5 Immunofluorescence Staining	18
2.6 Cytokine Chemokine Analysis using Enzyme Linked Immunosorbent Assay (ELISA).....	18
2.7 Statistical Analysis.....	19
Chapter 3: Results.....	20
3.1 Primary Murine Pulmonary Endothelial Cell Characterization.....	21
3.2 Cell Viability.....	22
3.3 MCP-1 Secretion Levels Increase Under Inflammatory Conditions.....	23
3.4 MCP-1 Released by Inflammation Modulates Secretome Release.....	25
3.5 Secretome Release Profile of mPECs and co-cultures of mPECs and mMSCs changes after neutralization of MCP-1 in inflammation.....	30
3.6 MCP-1's Modulation of the Secretome Release Response of Splenic Immune Cells and Splenic Monocytes.....	36

Chapter 4: Discussion and Future Directions.....	43
Chapter 5: Bibliography.....	49
Vitae.....	63

LIST OF TABLES

Table 1: Types of Cells Currently or Previously Under Clinical Stroke Trial

Investigations.....5

LIST OF FIGURES

FIGURE 1. A schematic representation of the hypothesis.....	13
FIGURE 2. Primary murine pulmonary endothelial cell characterization.....	21
FIGURE 3. Cell Viability of primary mMSCs and mPECs.....	22
FIGURE 4. Inflammation Increases MCP-1 secretion.....	24
FIGURE 5. MCP-1 modulates secretome release profile.....	27
FIGURE 6. Changes in Secretome Release of mPECs and co-cultures of mPECs and mMSCs Previously Exposed to Inflammatory Stimuli.....	31
FIGURE 7. Changes in Secretome Release of CD11b ⁺ Splenic immune cells.....	38
FIGURE 8. Changes in Secretome Release of CD115 ⁺ splenic monocytes.....	41

ABBREVIATIONS

AD – Alzheimer's disease

AD MSCs – Adipose derived mesenchymal stem/stromal cells

BM MNCs – Bone marrow derived mononuclear cells

BM MSCs – Bone marrow derived mesenchymal stem/stromal cells

BSA – Bovine serum albumin

CD105 – Cluster of differentiation 105

CD115 – Cluster of differentiation 115

CD11b – Cluster of differentiation 11b

CD45 – Cluster of differentiation 45

CD73 – Cluster of differentiation 73

CD90 – Cluster of differentiation 90

CD206 – Cluster of differentiation 206

CM – Conditioned media

cDNA – Complementary deoxyribonucleic acid

DAMP – Damage associated molecular pattern

DAPI – 4,6'-diamidino -2-phenylindole

DMEM – Dulbecco's modified eagle's medium

EDTA – Ethylenediaminetetraacetic acid

ED-Siglec 9 – Secreted ectodomain of sialic acid-binding Ig-like lectin-9

ELISA – Enzyme-linked immunosorbent assay

EPCs – Endothelial progenitor cells

ESCs – Embryonic stem cells

FBS – Fetal bovine serum

FDA – Food and drug administration

HBSS – Hank's balanced salt solution

HGF – Hepatocyte growth factor

HLA – Human leukocyte antigen

IA – Intra-arterial

IC – Intracranial

ICb – Intracerebral

IDO – Indoleamine 2,3-dioxygenase

IFN- γ – Interferon gamma

IL-1 β – Interleukin 1 beta

IL-1ra – Interleukin 1 receptor antagonist

IL-6 – Interleukin 6

IL-12 – Interleukin 12

IL-23 – Interleukin 23

IN – Intranasal

iPSCs – Induced pluripotent stem cells

IT – Intrathecal

IV – Intravenous

LPS – Lipopolysaccharide

MACS – Magnetic-activated cell sorting

MAPCs – Multipotent adult progenitor cells

MCAo – Middle cerebral artery occlusion

MCP-1 – Monocyte chemoattractant protein 1

MS – Multiple Sclerosis

mMSCs – Murine mesenchymal stromal cells

MNC – Mononuclear cells

mPECs – Murine pulmonary endothelial cells

MSCs – Mesenchymal stem/stromal cells

NO – Nitrogen monoxide

NPCs – Neural precursor cells

NSCs – Neural stem cells

PBS – Phosphate-buffered saline

PFA – Paraformaldehyde

PGE-2 – Prostaglandin E₂

SE – Standard error

TGF- β – Transforming growth factor beta

TNF- α – Tumor necrosis factor alpha

tPA – Tissue plasminogen activator

Tregs – Regulatory T-cells

TSG-6 – Tumor necrosis factor alpha induced protein 6

VEGF – Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.1 Stroke

Stroke is a leading cause of death and long-term disability in the United States and around the world. According to CDC reports, the cost burden in the US brought on by stroke has been estimated to be around \$34 billion per year towards medications, health care services, and missed workdays ¹. There are two major types of strokes: ischemic and hemorrhagic stroke, where ischemic stroke constitutes about 87% of all stroke cases ^{1,2}. Research over the years has identified many risk factors and have categorized them as non-modifiable, i.e., age, sex, race/ethnicity, etc., and modifiable, i.e., hypertension, diet, physical activity, smoking, etc., risk factors ³.

1.1.1 Ischemic Stroke Pathophysiology

The majority of the stroke cases comprises of ischemic stroke, especially amongst the developed countries ³. Ischemic stroke is caused due to the occlusion of the blood vessel(s) in the brain, which disrupts the supply of oxygen and glucose to the affected regions in the brain ^{4,5}. The infarct core is the central region affected directly by the loss of blood flow, and the region surrounding this core is called the ischemic penumbra, where there's some residual perfusion present from the neighboring vessels ⁶. The interruption of blood flow to these regions leads to irreversible damage to the brain tissue through a series of events called the ischemic cascade. The cellular and molecular events triggered by the cascade involve the formation of reactive oxygen species, accrual of intracellular calcium, the release of glutamate, mitochondrial damage, initiation of apoptosis and necrosis, excitotoxic

neuronal death, and induction of inflammatory processes both within the brain and peripheral tissues^{4, 7, 8}.

The inflammatory process within the brain is initiated through the release of damage-associated molecular pattern (DAMP) signaling released by the dying cells in the infarct core and ischemic penumbra regions⁶. Subsequently, these signals lead to the production of pro-inflammatory cytokines and chemokines that triggers the immune response cycle both centrally within the brain and peripherally through the entire system⁶. The immune response followed after stroke seems to be emerging as a key player in the development of stroke severity and a good target to obtain therapeutic benefits.

1.1.2 Current Stroke Treatments

1.1.2.1 tPA

Tissue plasminogen activator (tPA) is the only noninvasive treatment approved by the Food and Drug Administration (FDA) for ischemic stroke in the US. It is a factor that aids in the breakdown of blood clots that's causing the occlusion and restoring blood supply to the brain. The administration of tPA is, however, restricted to a narrow time window within 4.5 hours after the onset of stroke symptoms. This time constraint severely limits the number of patients who are eligible to receive it as a treatment.

1.1.2.2 Endovascular Therapy

Endovascular therapy or mechanical thrombectomy is a recently added standard of care treatment for ischemic stroke patients with large vessel occlusion. The clot is removed using a stent retriever. The stent is inserted into the blocked vessel; the stent then opens and grabs the clot for removal. The therapy can only be performed on patients with large vessel occlusion within 24 hours of the onset of acute stroke symptoms.

1.2 Cell Therapy

Cell-based therapies are another promising treatment option that has been explored for ischemic stroke. The main focus of current ischemic stroke treatments is to swiftly restore blood flow to the brain, to prevent any further damage. However, cell-based therapies aim to improve and restore functional recovery in acute and chronic stroke cases. Several pre-clinical animal studies have demonstrated over the years that cell therapy can achieve functional recovery ^{9, 10, 11}. Studies have looked at a variety of cell sources to be used as a therapy; including stem cell types like bone-marrow derived mesenchymal stem/stromal cells (BM MSCs), bone-marrow derived mononuclear cells (BM MNCs), adipose-derived stem/stromal cells (AD MSCs), neural stem cells (NSCs), embryonic stem cells (ESCs), umbilical cord blood-derived stem cells, induced pluripotent stem cells (iPSCs), dental pulp derived stem cells and precursor/progenitor cells like endothelial progenitor cells (EPCs), neural precursor cells (NPCs) and multipotent adult progenitor cells (MAPCs) ^{12, 13}. Pluripotent stem cells such as the ESCs and iPSCs have the potential to differentiate into any cell type from the three germ layers with an ability to self renew

indefinitely ¹³⁻¹⁵. The same property could lead them into forming tumors after engraftment and are tagged with certain ethical concerns ¹³⁻¹⁵. Multipotent cells such as MSCs, NSCs, EPCs, etc., on the other hand, have the potential to differentiate into only certain types of specialized cells but have fewer health risks and ethical issues attached to it ¹³.

Initially, cell therapy aimed to repair the tissue damage through the recruitment of cells for tissue grafting to repair the infarct region ¹⁶. However, growing studies have demonstrated that engraftment was not a factor in seeing the treatment effects; instead, the exogenous cells created an endogenous restorative effect within the brain. It was observed that these cells modulated the changes by providing trophic support and by modifying cell-signaling pathways that downstream enhanced the endogenous restorative process by promoting neurogenesis, angiogenesis, synaptogenesis, immune modulation, etc., ^{9, 13, 16}. Such compelling pre-clinical data has led to the testing of these cell-based therapies on human stroke patients, with certain cell types reaching phase 3 of clinical trial testing. Table 1 gives a brief picture of different cell types that are currently or have been previously under investigation for clinical stroke trials using data from ClinicalTrial.gov, Krause, M. et al., 2019 and, Trounson, A. and McDonald, C., 2015 ^{9, 17}.

Cell Type	Routes of Administration	Clinical Trial Phase
BM MSCs	IA, IV, IN, IT	Phase 1, 2, 3
BM MNCs	IA	Phase 1, 2
AD MSCs	IV, ICb,	Phase 1, 2
Hematopoietic Stem Cells	IV	Phase 1, 2
NSCs	IC, ICb	Phase 1, 2
Umbilical Cord Blood	IV	Phase 1, 2

Table 1 Types of Cells Currently or Previously Under Clinical Stroke Trial Investigations. *IA – Intra-Arterial, IC – Intracranial, ICb – Intracerebral, IN – Intranasal, IT – Intrathecal, IV – Intravenous.*

1.2.1 MSC Therapy

Mesenchymal stem/stromal cells (MSCs) are one of the most widely studied cell types as a cell-based therapeutic for stroke. MSCs, as mentioned previously, are multipotent cells that can be easily isolated and expanded from different adult tissue sources such as bone marrow, adipose tissue, umbilical cord blood, etc.,¹³. According to the International Society for Cellular Therapy, MSCs are defined using certain standards: it's plastic adherence, the expression of CD90, CD73, CD105 and the lack thereof CD45, human leukocyte antigen (HLA) Class II, CD11b, etc., and the ability to be multipotent¹⁸. The lack of HLA Class II markers enables the MSCs to have a relatively low or no immune reaction and also reduces the risk of immune rejection when the cells are allogeneic^{13, 18}. Intravenous (IV) administration of MSCs is one of the safer routes of administration amongst patients, as it's much less invasive compared to other routes such as intracerebral (ICb) and intra-arterial (IA). A growing number of studies have demonstrated that MSCs play an essential role in facilitating neuroprotection, modulating the immune response by acting of various immune cells, suppressing inflammation through the modulation of peripheral organs such as lungs and spleen where MSCs get lodged after an IV administration, etc.,^{8, 19}. Preclinical studies have also widely shown enhanced functional recovery in animals following treatment with MSCs^{20, 21}.

1.2.2 Intravenous Administration and Pulmonary Passage

IV administration of MSCs has been studied in many preclinical and clinical studies. A majority of the cells after an IV infusion get trapped in the lungs, which is referred to as 'pulmonary first-pass' and only a small percentage reaching the spleen, liver, and the actual ischemic brain penumbra ²²⁻²⁵. Studies showed that cultured MSCs overtime changed their morphology from small round shaped cells to long spindle shaped cells and have an average diameter of 20 μm . The large cell diameter made the MSCs too big to pass through the pulmonary microvasculature, which usually ranged between 5-10 μm in diameter ^{23, 26, 27}. Cell surface adhesion molecules expressed by MSCs and cells like endothelial cells were also later found to be a reason for the entrapment by various studies ^{25, 28, 29}. Initially, this entrapment was considered to be a hindrance to the treatment effects of MSCs. However, studies showed that despite a majority of the cells being trapped in the peripheral organs such as the lungs, therapeutic effects were still observed ³⁰. The exact mechanism behind this is still unknown, and many studies are pointing towards an immunomodulatory role played by MSCs ⁸.

1.3 Immunomodulatory Role of MSCs

Over the years, a growing number of studies have suggested that MSCs play a significant role in modulating the immune response after an inflammatory event such as stroke. Exposure to an environment that is inflammatory can influence the immunomodulatory function of the MSCs ³¹. Studies have shown that exposure of MSCs to inflammatory cytokines such as TNF- α and IL-1 β augmented their regenerative potential ³². Other studies have shown that secretory factors such as

TSG-6, TGF- β , HGF, PGE-2 demonstrated a reduced inflammatory immune response, which also includes inhibition of T-cell proliferation and promotion of regulatory T-cells (Tregs) expression^{30, 33-36}. Indoleamine 2,3-dioxygenase (IDO) has also been identified as a factor secreted by the MSCs that play a role in the generation of Tregs²⁷. Studies have also shown that MSCs trapped in peripheral organs such as the spleen and lungs interact with surrounding cell types such as splenocytes or pulmonary endothelial cells to release various secretomes that have systemic effects^{8, 37}.

1.4 Monocytes and Macrophages

Monocytes/macrophages are an important subset of immune cells, through which MSCs carry out their immunomodulatory effects. Under inflammatory conditions, monocytes/macrophages are driven towards the M1 type, a pro-inflammatory subset. Pro-inflammatory factors such as lipopolysaccharide (LPS), TNF- α , and IFN- γ were shown to drive the cells towards the M1 phenotype. These cells also produced more pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-12, IL-23, and nitrogen monoxide (NO)³⁸. MSCs have shown to favor the polarization of monocytes/macrophages towards an M2 anti-inflammatory phenotype, while also reducing the M1 type activity³⁵. The M2 phenotype cells secreted anti-inflammatory cytokines such as IL-10 and reduced pro-inflammatory cytokines such as TNF- α , IL-12^{39, 40}. Studies have also shown M2 phenotype monocyte/macrophages expressed high levels of cytokines/chemokines such as IL-8, IL-6, MCP-1, EGF^{41, 42}. The immunomodulatory effect exerted by MSCs through their modulation of the

monocytes/macrophages could be a key factor in understanding their therapeutic effects in disease conditions.

1.5 Monocyte Chemoattractant Protein – 1

Monocyte chemoattractant protein – 1 (MCP-1), a C-C family chemokine, is primarily involved in the recruitment of monocytes/macrophages. Many cells, including the endothelial cells, monocytes, macrophages, microglia, astrocytes, neurons, etc., produce MCP-1. The role of MCP-1 has been mainly associated with the migration and infiltration of the mononuclear cells, such as monocytes to areas of inflammation and infection. Recent studies have also pointed out their critical involvement in routine immune surveillance and immune modulation ⁴³. Studies have shown that MCP-1 activity is mediated primarily through C-C chemokine receptor 2 (CCR2) ⁴³. There are two isoforms of the receptor, CCR2A, and CCR2B, which possibly activate different pathways and leading to different actions ⁴³.

1.5.1 Role of MCP-1 in Pathologies Involving Neuro-inflammation

Extensive work has been done on the position of MCP-1, primarily being a pro-inflammatory molecule in various pathologies. Some of the widely studied disease models linked to the role of MCP-1 include atherosclerosis, multiple sclerosis, Alzheimer's disease, rheumatoid arthritis, brain ischemia, HIV, etc., ^{43, 44}. Many of these diseases have an inflammatory component associated with them, and studies have shown that MCP-1 directly mediates the recruitment of monocytes to the foci of active inflammation ^{43, 45}. In Multiple Sclerosis (MS), a neuroinflammatory demyelinating disease, the lesions have presented with the infiltration activated

macrophages and lymphocytes. Studies have shown elevated MCP-1 expression in MS plaques ⁴⁴. This would indicate that MCP-1 plays a major role in the inflammation progression in MS. Studies have also indicated that mice who lack the CCR2 receptor have shown no signs of neuroinflammation ⁴⁴. Studies on Alzheimer's disease (AD), another neurodegenerative disease, have also increasingly shown that there is a possible involvement of the MCP-1/CCR2 axis in the development of the pathology. Apart from its detrimental effects, MCP-1 has also been implicated in studies to be involved in the clearance of senile plaques ⁴⁴. Studies are slowly uncovering the fact that MCP-1 could have both positive and negative roles in disease pathologies.

1.5.2 Role of MCP-1 in Ischemia

As mentioned earlier, inflammation plays a major role in the progression and outcome of ischemic injury. The main inflammatory event in post-ischemia is the recruitment of leukocytes, neutrophils, and mononuclear cells like monocytes, which, as mentioned before, are recruited through the actions of MCP-1 ⁴⁶. In the study by Arakelyan et al., 2005, ⁴⁷ patients who suffered from ischemic stroke had about a 2-fold increase in MCP-1 levels in their serum compared to the control groups ⁴⁷. Other animal studies have also indicated that animals, when void of MCP-1 have smaller infarct volumes compared to that of control mice, post an ischemic event ⁴⁸. While on the other hand, studies have also shown that post-ischemia (hind-limb), local infusion of MCP-1 protein, improved homing of monocytes and could also be involved in the regulation of angiogenesis in the ischemic region ⁴⁹.

1.5.3 Role of MCP-1 in Cell Therapy

The role of MCP-1 is seldom looked at in such therapeutic settings. Studies have looked into the migration pattern of MSCs after extracts from brain tissue post a middle cerebral artery occlusion (MCAo) versus controls. They noticed that MCP-1 levels influenced migration patterns of the MSC in these groups ^{50, 51}. A previous study conducted by our group also has shown that autologous post-stroke mononuclear cell (MNC) from rats when administered post an MCAo, the outcomes were much better compared to the saline control group ¹¹. The infarct size was significantly reduced in these animals compared to the saline control group while also showing an enhanced recovery compared to the other groups ¹¹. One significant point to be noted here is that serum levels of MCP-1 were increased in animals treated with post-stroke MNCs compared to the other groups ¹¹. Andres et al., 2011 ⁵² demonstrated that intra-arterially delivered NSCs had better homing in the ischemic region in animals with MCP-1 expression compared to the animals with no MCP-1 expression ⁵². In another study, rats with spinal cord injuries when treated with MCP-1/ED-Siglec-9 secreted by MSC derived from human deciduous dental pulp showed improved hind-limb movement and also demonstrated the recruitment of M2 type monocytes/macrophages to the region of injury ⁵³. The study also showed that when the animals were treated with a combination of MCP-1/EDSiglec-9 the treated animals had increased recruitment of M2 like cells, which was indicated by the cell surface marker CD206, compared to the control group ⁵³.

The function of MCP-1 is altered based on the situation they are presented, and this is clear from the previously described studies. In disease condition, MCP-1 is primarily portrayed as being a molecule that aggravates inflammation, while under

treatment conditions such as cell therapy, they have shown to be anti-inflammatory and more beneficial. The secondary role of MCP-1 is rarely looked at, and it could potentially be involved in protective and regenerative features like neurogenesis, angiogenesis, neuro-protection, etc., which we need to explore more deeply.

1.6 Objectives and Hypothesis

We hypothesize that MSCs directly and/or indirectly modulate the release of MCP-1 through their interaction with pulmonary endothelial cells, and this immunomodulatory effect is via, modification of the monocytes. The following aims were designed to test this hypothesis:

Specific Aim 1: Under inflammatory conditions, the interaction between pulmonary endothelial cells exposed to bone marrow-derived MSCs would modify the release of trophic factors such as MCP-1.

Specific Aim 2: Interaction of bone marrow-derived MSCs and pulmonary ECs after under inflammatory conditions modulates the monocyte mediated immune response through MCP-1.

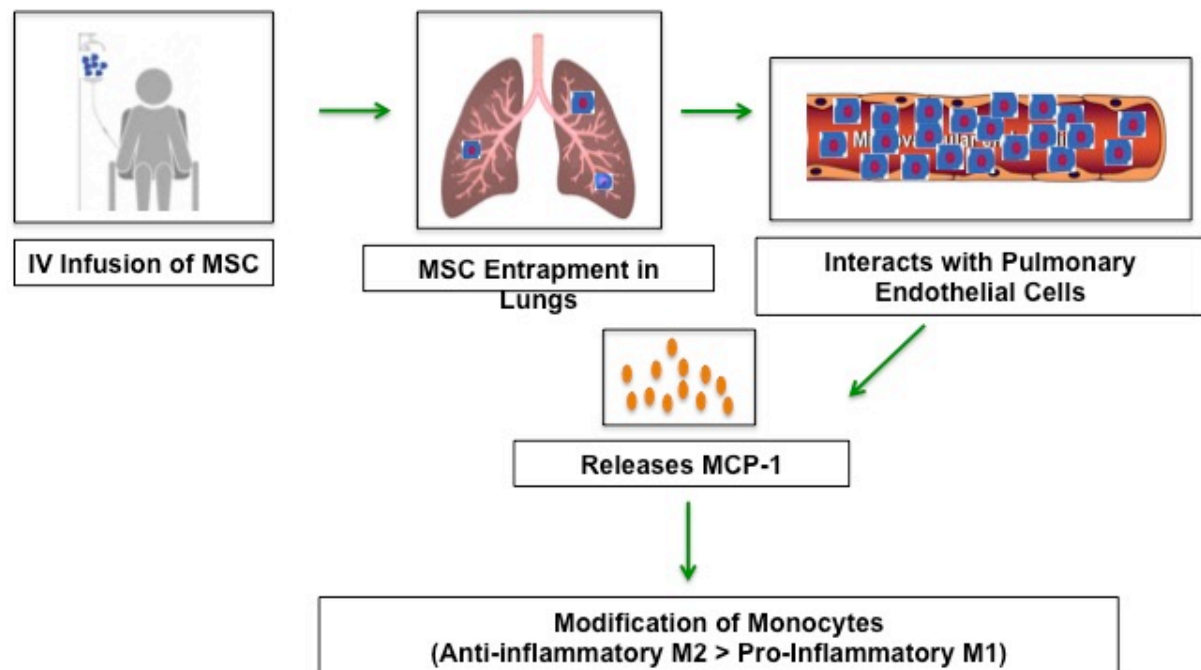


FIGURE 1. A schematic representation of the hypothesis. We hypothesize that after an IV infusion of MSCs, a majority of the cells get trapped in the lungs. One of the first cell type MSCs come in contact and interact with are the pulmonary endothelial cells. This interaction modulates the release of the chemokine MCP-1. This downstream then leads to the modulation of the immune response through the modification of monocytes.

CHAPTER 2: MATERIALS AND METHODS

2.1 Isolation and culture of primary murine mesenchymal stromal cells

MSCs from fresh C57/BL6 mice (mMSCs) bone marrow aspirates were prepared using their preferential attachment to tissue culture plastic as previously described⁵⁴. Briefly, bone marrow aspirate was obtained from the tibia and femoral compartments. The aspirated marrow was then suspended in HBSS (Life Technologies) and was filtered using a 70uM filter (Corning Life Sciences). The filtered suspension was centrifuged at 1500 x g for 5 minutes. The pellet obtained from this was immediately resuspended in complete culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and GlutaMAX Supplement Pyruvate (Gibco), along with 20% fetal bovine serum (Atlanta Biologics), 100 units/ml penicillin (Gibco) and 100ug/ml streptomycin (Gibco). Non-adherent cells were removed after 2 days. The adherent colonies were expanded further till passage 3 to obtain pure colonies, and frozen at passage 3. The cells were checked for typical spindle shape morphology and growth kinetics. The cells were then thawed further and plated at 1×10^6 cells/ml and expanded further by changing media every other day. When the cells reached 80% confluence, the medium was discarded, and the cells were washed with phosphate-buffered saline (PBS) (Life Technologies), and the adherent cells were harvested with 0.25% trypsin/EDTA (Life Technologies) for 3 minutes at 37°C. Cells were used at passages 4-5.

2.2 Primary murine pulmonary endothelial cells

Primary murine pulmonary endothelial cells (mPECs) were purchased from Cell Biologics (Chicago, IL) at the third passage. The cells were expanded and

grown in cell culture plates pre-coated with 0.2% gelatin (Sigma-Aldrich) in complete mouse endothelial cell media with growth factors (Cell Biologics, IL; M1166). Cells were used at passage 4-5 for the subsequent experiment.

2.3 Isolation and verification of murine splenic immune cells and monocytes

Spleen tissue was isolated from C57/BL6 mice. The tissue was first dissociated mechanically and then further enzymatically using 0.25% trypsin/EDTA (Life Technologies) for 20-25 minutes at 37°C. The obtained splenocyte suspension was filtered using a 70uM filter (Corning Life Sciences) with Hank's balanced salt solution (HBSS) (Life Technologies) and centrifuged at 1500 x g for 5 minutes. The obtained cell pellet was used downstream for magnetic beads based isolation of two cell population splenic immune cells CD11b⁺ cells and splenic monocytes CD115⁺ cells. CD11b⁺ cells were first isolated using the positive selection technique where CD11b⁺ cells were magnetically labeled with CD11b MicroBeads conjugated to monoclonal antibodies (Miltenyi Biotec, CA). The labeled cell suspension was passed through MS Column (Miltenyi Biotec, CA), which was placed in a magnetic field of a MACS Separator (Miltenyi Biotec, CA). The positively labeled CD11b⁺ cells were retained in the column, the fraction of the unlabeled cells passed through the column. The positively selected CD11b⁺ cells were flushed out after removing the column from the magnetic field. The unlabeled cell fraction from the above step was used to isolate CD115⁺ cells further. CD115⁺ cells were indirectly labeled with CD115-biotin monoclonal antibodies and anti-biotin MicroBeads (Miltenyi Biotec, CA). The downstream processing was similar to the one described above. The cell fractions obtained from the above isolation processes yielded CD11b⁺ and CD115⁺

cells, respectively. Both CD11b⁺ and CD115⁺ cells were plated and used for subsequent experiments.

2.4 Viability Assessment

mPECs, as well as mMSCs, were exposed to different concentrations of recombinant mouse TNF- α and IFN- γ (R&D Systems) ranging from 500 nM to 0.5 nM. The cells were also exposed to recombinant mouse MCP-1 (R&D Systems) at 10ng/ml and mouse MCP-1 neutralizing antibody (R&D Systems) at 20ug/ml. At 24 hours of incubation, the viability of both mPECs and mMSCs was measured using the colorimetric MTT assay (Promega Life Sciences). The viability was calculated as a ratio based on the vehicle well, which was then converted to a percentage of viable cells in comparison to the vehicle.

The viability of the primary mPECs and mMSCs was also measured every time before the start of an experiment using the trypan blue staining assay. 0.4% trypan blue solution (Gibco) was added at a 1:1 ratio to the cells, and the mixture was loaded to the Countess Cell Counting Chamber slides (Invitrogen). These slides were then read using the Countess II machine (Invitrogen), where the viability of the cells was determined using the ratio between stained cells and the unstained cells. The stained cells are the dead cells that are capable of taking up the trypan blue dye, whereas the unstained cells are viable cells with intact cell membranes. The viability of both the primary cells mPECs and mMSCs used in the experiments remained > 90%.

2.5 Immunofluorescence Staining

Immunofluorescence analysis was performed on the cultured mPECs to confirm their endothelial origin. Briefly, the cells were plated in chamber slides at a density of 60,000 cells. Once the cells were adherent, it was washed gently with PBS and fixed with 4% paraformaldehyde solution (PFA) (Thermo Scientific) in PBS for 20 minutes at room temperature. The fixed cells were then washed with PBS three times for 5 minutes each. The samples were then blocked and permeabilized with a solution of 0.1% Triton X-100 (Sigma-Aldrich) and 3% bovine serum albumin (BSA) (Fisher Scientific) for 1 hour. The samples were rewashed with PBS three times for 5 minutes, followed by their incubation with the Fluorescein labeled *Griffonia Simplicifolia* Lectin I Isolectin B4 antibody (Vector Labs, Burlingame, CA; FL-1201) in 3% BSA and 0.1% Triton-X solution overnight at 4°C. The next morning the samples were washed three times with PBS, 5 minutes each followed by DAPI (Invitrogen) counter-stain for 5 minutes at room temperature. The samples were rewashed with PBS three times, 5 minutes each, and stored in PBS at 4°C until imaging was done. The immunofluorescence images were acquired using the Zeiss Axio Observer (Zeiss) microscope and ZEN Blue Edition Software (Zeiss).

2.6 Cytokine Chemokine Analysis using Enzyme-Linked Immunosorbent Assay (ELISA)

Media of the various cell types used in these experiments, including mPECs, mMSCs, co-cultures of mPECs and mMSCs, CD11b⁺ splenic immune cells, and CD115⁺ splenic monocytes were analyzed for their secretome release using the ELISA assays (BD Biosciences and R&D Systems). In this study, we analyzed the

secretion levels of IL-1 β , IL-1ra, VEGF (R&D Systems), MCP-1, and IL-6 (BD Biosciences). Briefly, 96-well ELISA plates were coated with the appropriate capture antibody and incubated overnight at room temperature (R&D Systems) or 4°C (BD Biosciences). The following morning the plates were washed three times using the appropriate wash buffer and blocked using a solution of PBS with either 1% BSA (R&D Systems) or 10% FBS (BD Biosciences) for an hour at room temperature. Following this, the plates were washed three times and were now ready for the addition of the appropriate samples and standards. The samples and standards were incubated for two hours at room temperature. After two hours, the plates were washed 3-5 times and were ready to be incubated with the detection antibody for 1 hour (BD Biosciences) or 2 hours (R&D Systems) at room temperature. Following this, the plates were washed 3-7 times and were finally incubated with the appropriate HRP conjugated enzyme substrate for 20 minutes. The plates were washed three times, and the substrate solution mixture containing tetramethylbenzidine and hydrogen peroxide, at a 1:1 ratio, were added to the plates. Plates were let to develop at room temperature in the dark for 20-30 minutes, and the reaction was stopped using a stop solution of 2N sulfuric acid. The concentrations of the different cytokines were read at an absorbance of 450 nm and were obtained using the FLUOstar optima microplate reader. .

2.7 Statistical Analysis

Significance between control and test groups was determined using the paired two-tailed t-test. Data were considered to be statistically significant if *P*-value < 0.05. Mean values were reported along with the standard error of the mean (SEM).

CHAPTER 3: RESULTS

3.1 Primary Murine Pulmonary Endothelial Cell Characterization

Murine pulmonary endothelial cells express α -galactose sugar residues that are present on the cell surface. *Griffonia Simplicifolia* Lectin I Isolectin B4 is a useful marker for non-primate endothelial cells that recognizes these residues. We confirmed the biomarker phenotype of our primary murine endothelial cells by staining with *Griffonia Simplicifolia* Lectin I Isolectin B4 antibody and fluorescence analysis (Figure 2). The staining patterns were consistent with that of a pure murine endothelial cell population.

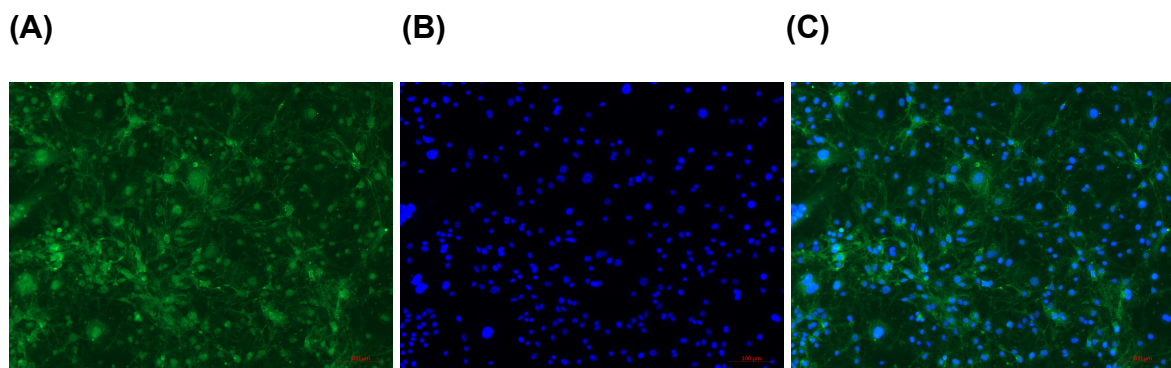
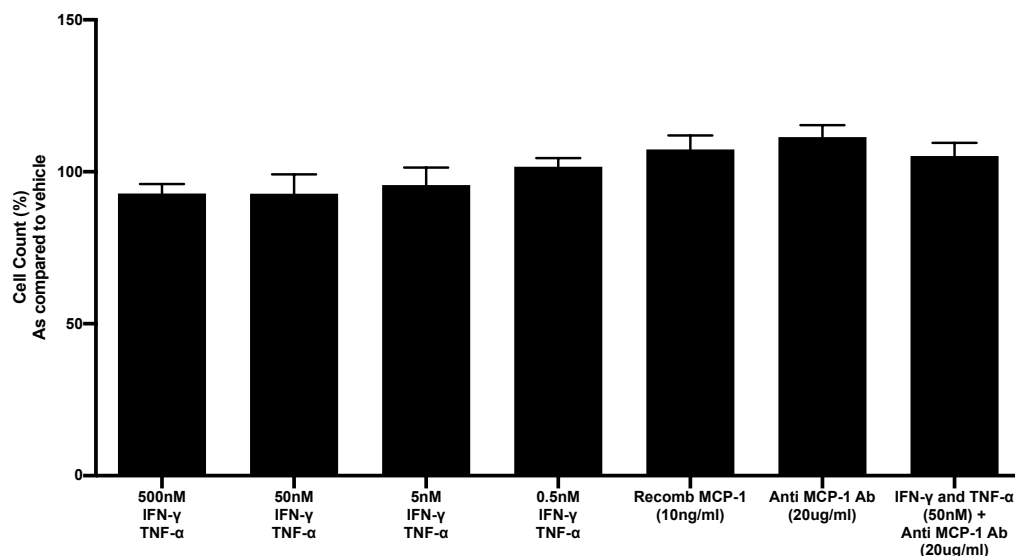


FIGURE 2. Primary murine pulmonary endothelial cell characterization. Primary murine pulmonary endothelial cells were characterized using *Griffonia Simplicifolia* Lectin I Isolectin B4 antibody using immunofluorescence analysis. (A) Isolectin B4 stained primary murine endothelial cells at passage 5. (B) Nuclear staining of DAPI. (C) Merged image of Isolectin B4 and DAPI staining.

3.2 Cell Viability

Primary mMSCs at passage 5 in culture were subjected to a combination of IFN- γ and TNF- α ranging from 500 nM to 0.5 nM in concentrations. The cells were also subjected to recombinant MCP-1 (10ng/ml) and anti-MCP-1 neutralizing antibody (20ug/ml) individually. The viability of the cells was calculated by comparing the percentage change in cells vs. the vehicle. There was no significant change in the overall viability of primary mMSCs at 24 hours after exposure to various conditions tested in this experiment (*Figure 3 A*). The changes in the viability of primary mPECs at passage 5 were also measured after exposure to the different conditions as compared to the vehicle. Cells exposed to recombinant MCP-1 (10ng/ml) had a significantly increased viability percentage – significantly increasing their proliferation rate – compared to the vehicle group, while the rest of the conditions had no significant change in the overall viability of the mPECs (*Figure 3 B*).

(A)



(B)

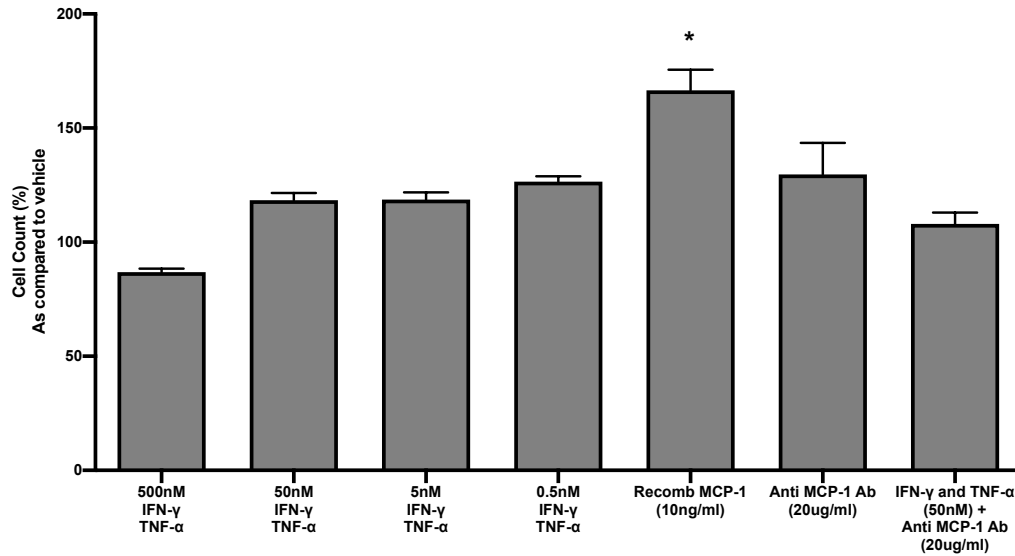


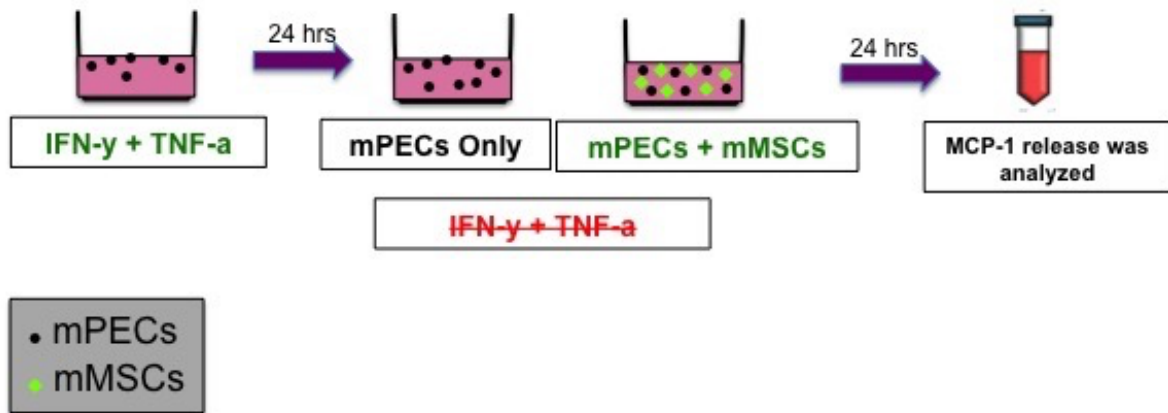
FIGURE 3. Cell Viability of primary mMSCs and mPECs. Passage 5 primary mMSCs and mPECs in culture were subjected to the following conditions: to a combination of IFN- γ and TNF- α ranging from 500 nM to 0.5 nM, to recombinant MCP-1 (10ng/ml) and to anti-MCP-1 neutralizing antibody (20ug/ml). mPECs exposed to recombinant MCP-1 (10ng/ml) had a significant increase in viability compared to the vehicle. The other conditions did not show any significant difference in viability for both (A) mMSCs and (B) mPECs.

3.3 MCP-1 Secretion Levels Increase Under Inflammatory Conditions

We subjected mPECs in culture to a combination of IFN- γ and TNF- α , inflammatory stimuli, ranging from 500 nM to 0.5 nM concentrations for 24 hours. Following this, the inflammatory stimuli were removed and the cells were divided into two groups: the control group (mPECs only) and the group exposed to mMSCs (mPECs + mMSCs). 24 hours after mMSCs exposure, we collected the media and

measured the release of MCP-1 from the two groups (*Figure 4 A*). We found that MCP-1 release in the mPECs only group had significantly increased across the entire range of inflammatory concentrations tested (*Figure 4 B*). Similarly, the group exposed to mMSCs (mPECs + mMSCs) also had a significant increase in MCP-1 release across the entire range of inflammatory concentrations tested (*Figure 4 C*).

(A)



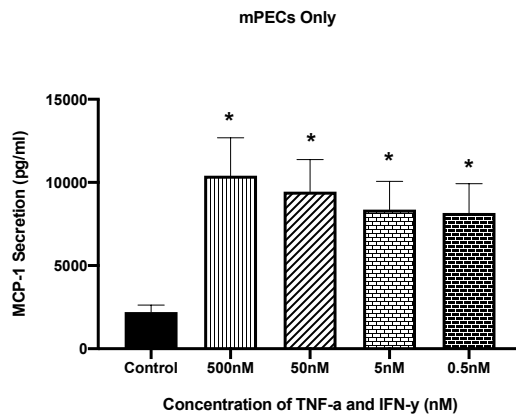
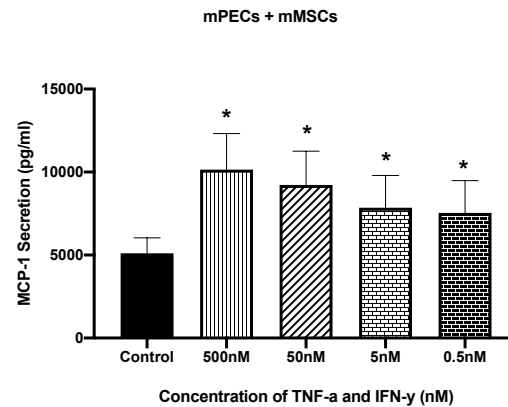
(B)**(C)**

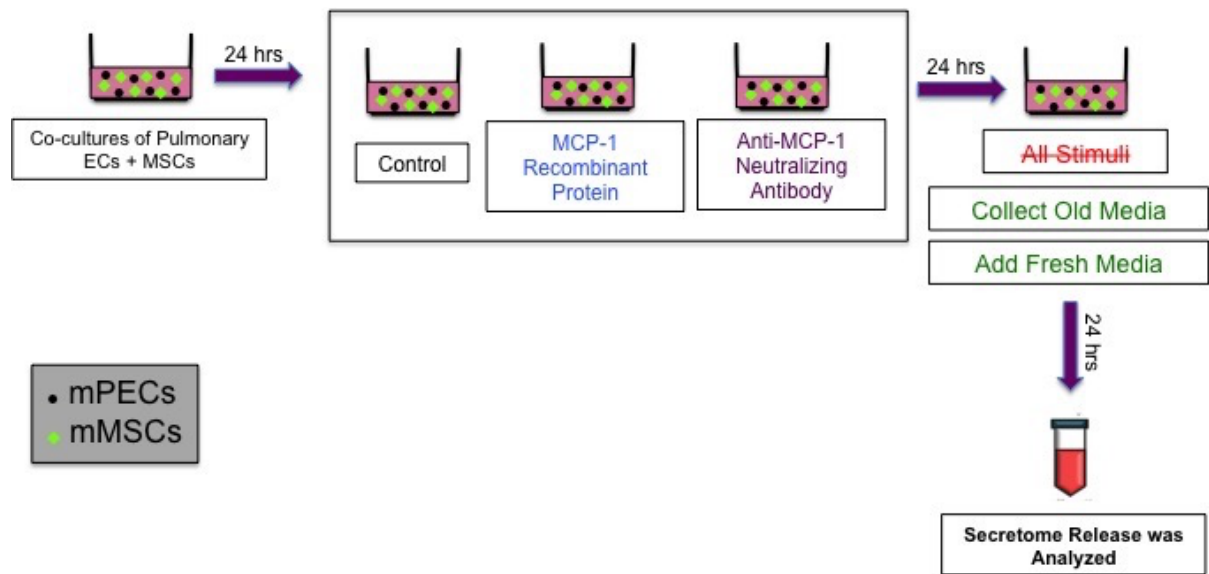
FIGURE 4. Inflammation Increases MCP-1 secretion. (A) Schematic of exposing mPECs to various ranges of IFN- γ and TNF- α inflammatory stimuli and co-culturing with mMSCs and measuring the release of MCP-1. (B) MCP-1 release from mPECs (mPECs only) was significantly increased across all concentrations of IFN- γ and TNF- α . (C) MCP-1 release in mPECs treated with mMSCs (mPECs + mMSCs) had significantly increased across all concentrations of IFN- γ and TNF- α . Significant p -value are denoted with asterisk (*), with p -value <0.05 considered as significant.

3.4 MCP-1 Released by Inflammation Modulates Secretome Release

As we had shown earlier MCP-1 release is modulated under inflammatory conditions. We further wanted to see if over stimulation of MCP-1 or neutralization of MCP-1 changes the secretome release in co-cultures of mPECs and mMSCs. We subjected the co-cultures of mPECs and mMSCs in culture to three conditions: 1) Control, 2) Over Stimulation of MCP-1 with recombinant MCP-1 (Recomb MCP-1) at 10ng/ml concentration and 3) Neutralization of MCP-1 with anti MCP-1 neutralizing

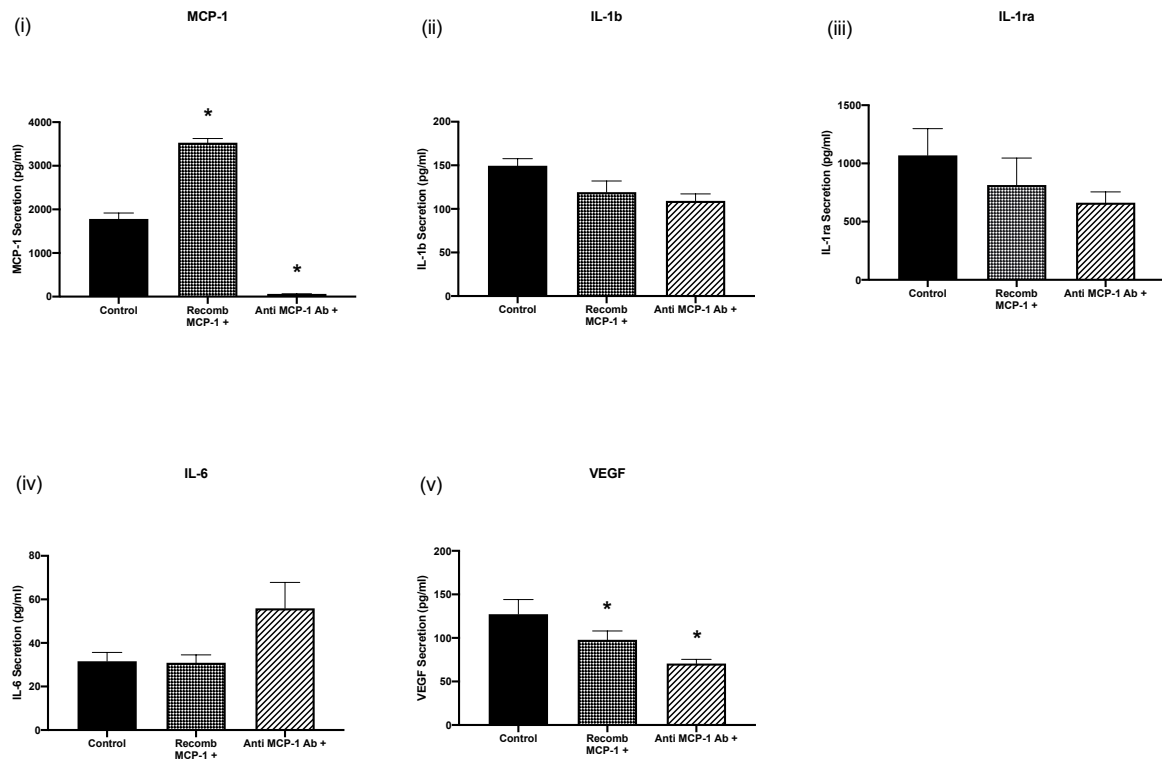
antibody (Anti MCP-1 Ab) at 20ug/ml concentration for 24 hours. Following this we collected the media (Old Media) and added fresh media with no stimuli present. 24 hours later, the media (Fresh Media) was collected (*Figure 5 A*). We showed that MCP-1 was effectively neutralized in the presence of the Anti MCP-1 Ab and was significantly higher in the presence recombinant MCP-1 (*Figure 5 B (i)*). We found that, in the Old Media, VEGF release was significantly reduced when MCP-1 was over stimulated using Recombinant MCP-1 protein and when MCP-1 was effectively neutralized with anti MCP-1 neutralizing antibody (*Figure 5 B (v)*). There were no significant changes in the release of IL-1 β , IL-1ra and IL-6 (*Figure 5 B (ii), (iii) and (iv)*). After the stimuli were removed (Fresh Media), MCP-1 levels had bounced back (*Figure 5 C (i)*). We found that IL-1ra secretion levels, in the Fresh Media, had significantly reduced in the group where MCP-1 was previously neutralized (*Figure 5 C (ii)*). We also found that IL-6 secretion levels had significantly increased in the previously neutralized MCP-1 group (*Figure 5 C (iii)*). We were unable to detect the presence of IL-1 β or VEGF in the fresh media.

(A)



(B)

Old Media (Media after 24 hours with the Stimuli Present)



(C)

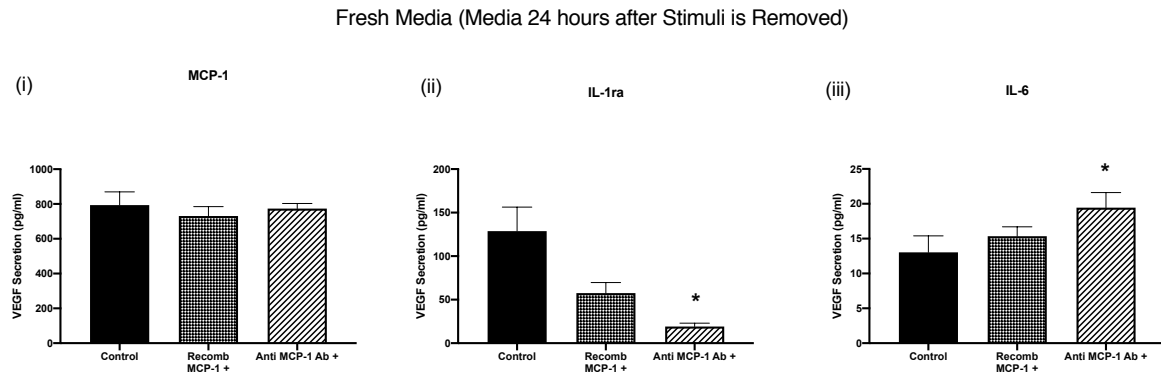


FIGURE 5. MCP-1 modulates secretome release profile. (A) Schematic of exposing co-cultures of mPECs and mMSCs to over stimulation of MCP-1 (Recomb MCP-1) or neutralization of MCP-1 (Anti MCP-1 Ab). (B) Old Media: (i) MCP-1 was effectively neutralized in the presence of Anti MCP-1 Ab. (ii) - (iv) The release of IL-1 β , IL-1ra and IL-6 had no significant difference, respectively. (v) In the presence of Recomb MCP-1 and Anti MCP-1 Ab VEGF secretion had significantly reduced. (C) Fresh Media: (i) After the stimuli were removed, MCP-1 release bounced back. (ii) IL-1ra release, however, was significantly reduced in the groups were MCP-1 was previously neutralized. (iii) The release of IL-6 had significantly increased in the group that had MCP-1 previously neutralized. IL-1 β and VEGF presence were not detectable in the fresh media. Significant p-value are denoted with asterisk (*), with p-value <0.05 considered as significant.

3.5 Secretome Release Profile of mPECs and co-cultures of mPECs and mMSCs changes after neutralization of MCP-1 in inflammation

We subjected mPECs (mPECs Only) and co-cultures of mPECs and mMSCs (mPECs + mMSCs) in culture to three conditions: 1) Control, 2) Inflammatory Stimuli IFN- γ and TNF- α at 50nM concentration (TNF- α + IFN- γ), and 3) Inflammatory stimuli IFN- γ and TNF- α at 50nM concentration + Anti MCP-1 neutralizing antibody (Anti MCP-1 Ab) at 20ug/ml for 24 hours. Following this we removed the media and added fresh media with only anti MCP-1 neutralizing antibody present in the appropriate groups. Downstream, this media was also used as conditioned media (CM) in splenic monocytes experiment. 24 hours later, the media (Fresh Media) was collected (*Figure 6 A*).

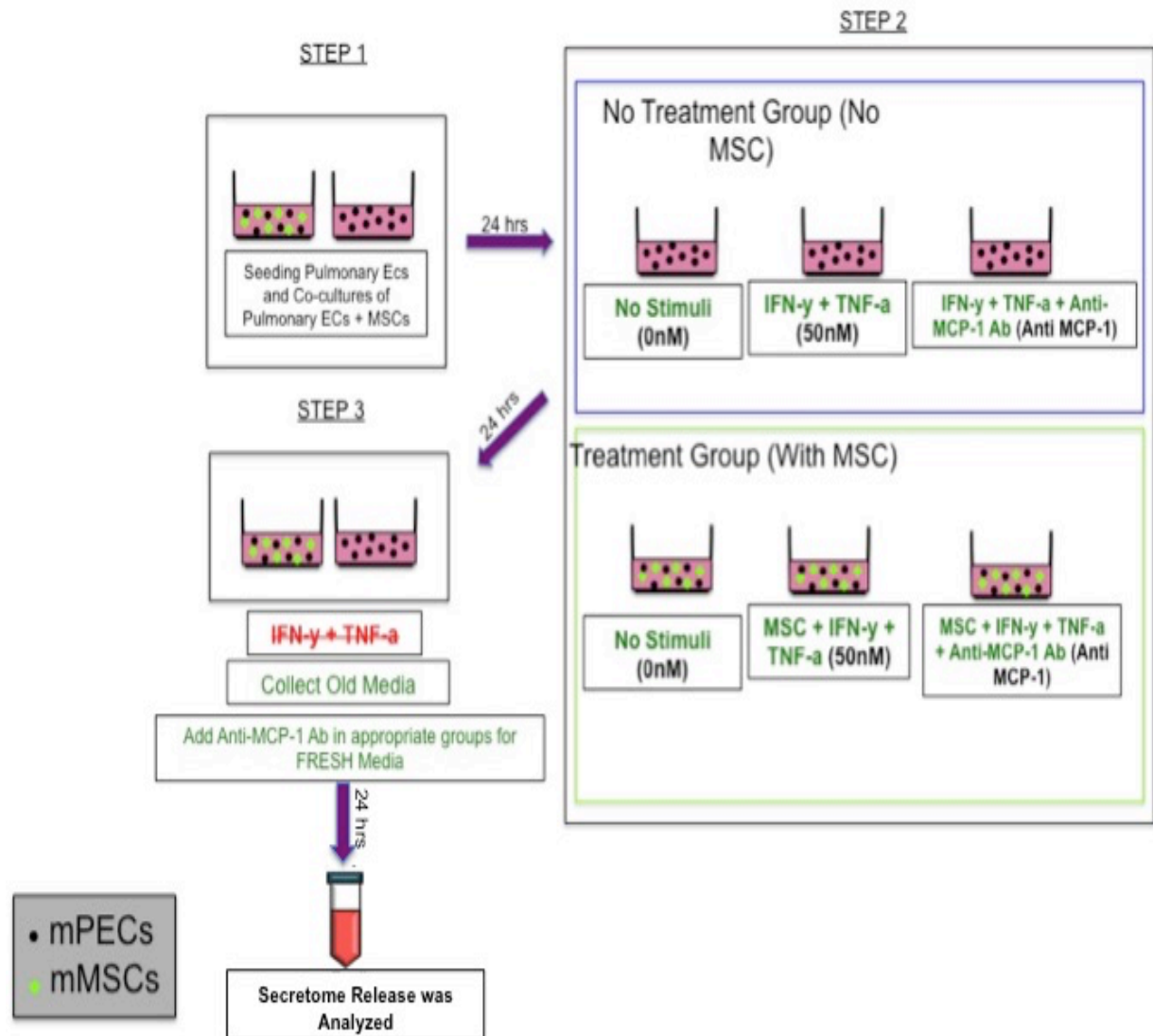
In the Fresh Media from mPECs, MCP-1 is neutralized in the presence of Anti MCP-1 Ab (*Figure 6 B (i)*). The release of IL-6 levels had significantly increased when mPECs group that was previously exposed to inflammatory stimuli and in the group when MCP-1 was neutralized. (*Figure 6 B (iv)*). There was no significant difference in the release of IL-1 β , IL-1ra, and VEGF from mPECs (*Figure 6 B (ii), (iii), and (v)*).

The Fresh Media in the presence of mMSCs (mPECs + mMSCs group), had neutralization of MCP-1 with Anti MCP-1 Ab (*Figure 6 C (i)*). We also found a significant increase in the release of IL-6 and VEGF in the Fresh Media of mPECs + mMSCs group, that was previously treated with the inflammatory stimuli (*Figure 6 C (iv) and (v)*). Although in the Fresh Media, we saw that in the presence of mMSCs (mPECs + mMSCs group) there was an increase in the secretion of IL-6 and VEGF, when MCP-1 was neutralized, it was not statistically significant (*Figure 6 C (iv) and*

(v)). There was no significant difference in the release of IL-1 β and IL-1ra from any of the groups (*Figure 6 C (ii) and (iii)*).

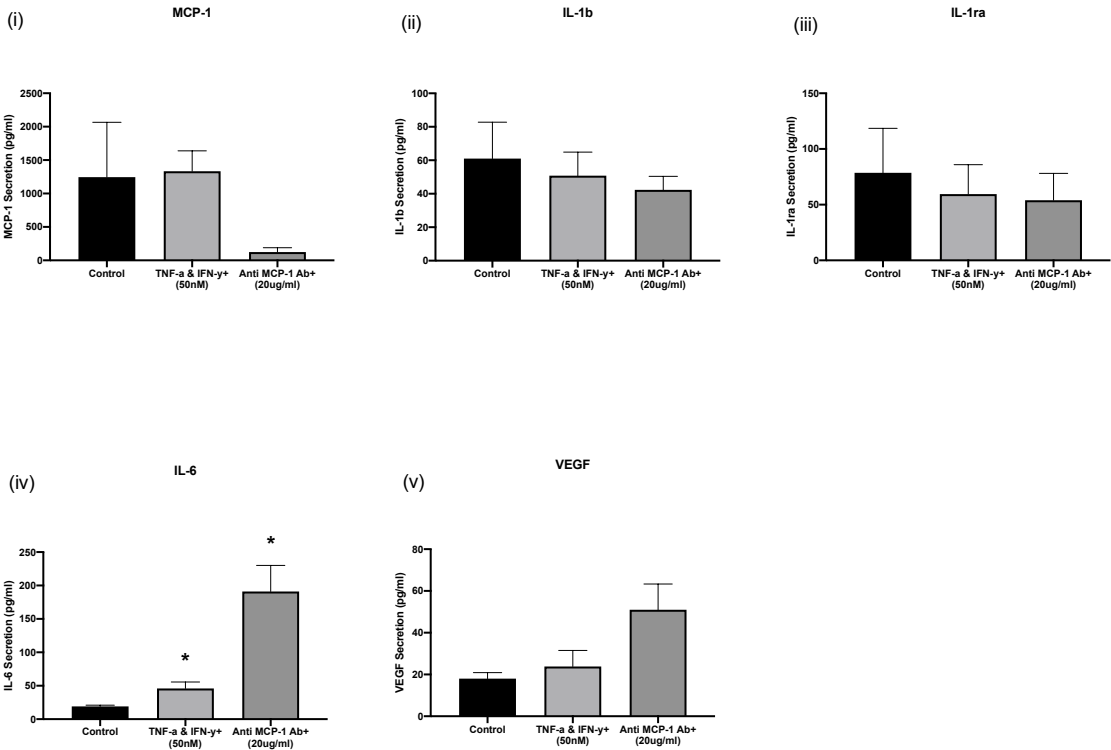
Logarithmic Fold change data was used to appropriately compare the secretome release between mPECs only groups and mPECs + mMSCs groups. In the fresh media, IL-1 β had a reduced logarithmic fold change difference in the mMSCs treated group in the presence of MCP-1 neutralizing antibody (*Figure 6 D (ii)*). We also observed that in the fresh media there was an increased logarithmic fold change difference in the secretion of IL-6 and VEGF release in groups treated with mMSCs that was previously exposed to inflammatory stimuli (*Figure 6 D (iv), and (v)*). There was no significant logarithmic fold change difference observed in the secretion of IL-1ra in any of the groups (*Figure 6 D (iii)*).

(A)



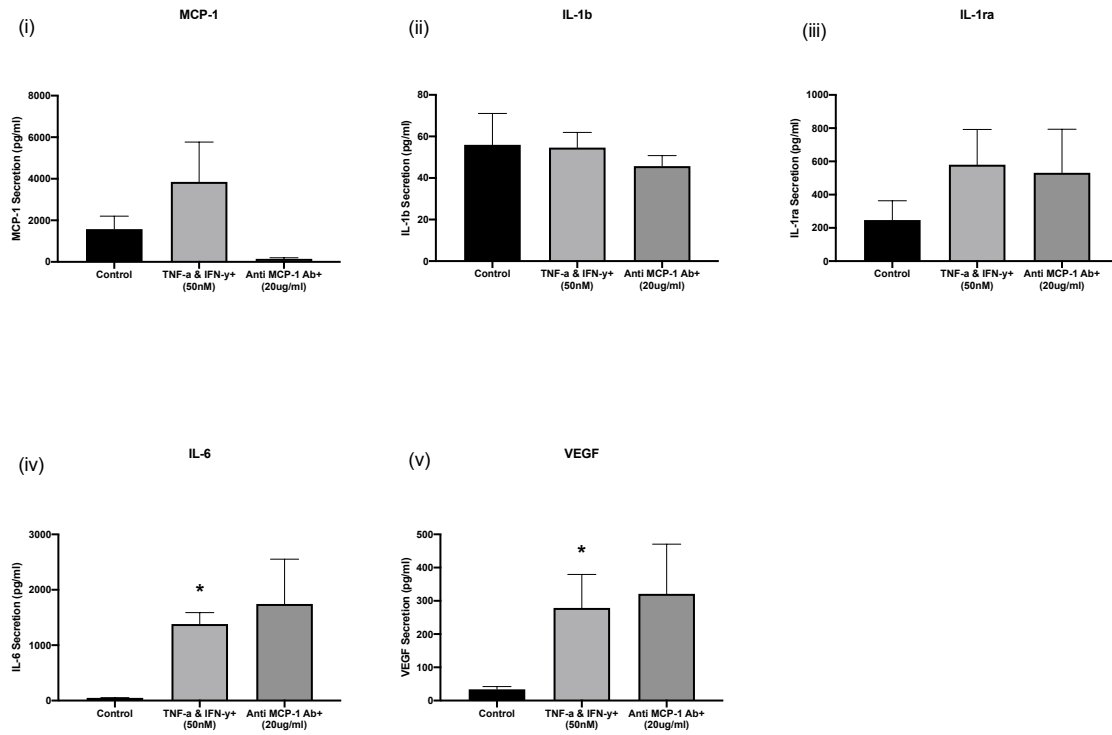
(B)

mPEC Secretome Profile Fresh Media (Media 24 hours after Stimuli is Removed)



(C)

mPEC + mMSC Secretome Profile Fresh Media (Media 24 hours after Stimuli is Removed)



(D)

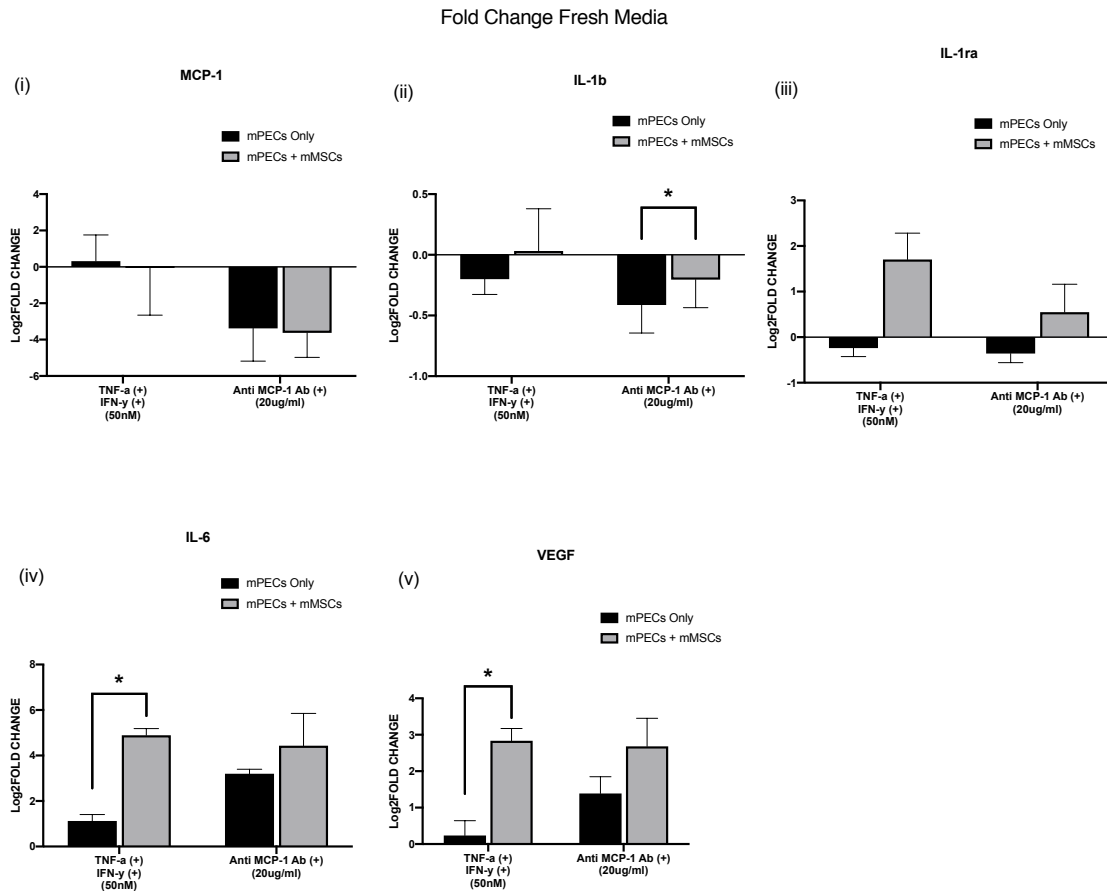


FIGURE 6. Changes in Secretome Release of mPECs and co-cultures of mPECs and mMSCs Previously Exposed to Inflammatory Stimuli. (A) Schematic of exposing mPECs or co-cultures of mPECs and mMSCs to inflammatory stimuli (TNF- α and IFN- γ (50 nM)) or inflammatory stimuli + MCP-1 neutralizing antibody (Anti MCP-1 Ab (20ug/ml)). (B) mPECs Only: (i) In the presence of MCP-1 neutralizing antibody, MCP-1 secretion appears to be neutralized. (ii), (iii) and (v) The release of IL-1 β , IL-1ra and VEGF had no significant difference, respectively. (iv) IL-6 secretion levels were elevated in the groups

previously exposed to inflammatory stimuli and also in the presence of MCP-1 neutralizing antibody. (C) mPECs + mMSCs: (i) In the presence of MCP-1 neutralizing antibody, MCP-1 secretion appears to be neutralized. (ii) and (iii) There was no significant difference in the release of IL-1 β and IL-1ra, respectively (iv) and (v) IL-6 and VEGF secretion were increased in cells that were previously exposed to the inflammatory stimuli. (D) Logarithmic Fold change data was used to appropriately compare the secretome release between mPECs only groups and mPECs + mMSCs groups. (i) Logarithmic Fold Change of MCP-1 secretion. (ii) Logarithmic fold change of IL-1 β secretion levels were significantly reduced in the mMSCs treated group in the presence of MCP-1 neutralizing antibody. (iii) There was no significant change observed in the logarithmic fold change in the secretion of IL-1ra. (iv) and (v) There was increase in the logarithmic fold change difference in the secretion of IL-6 and VEGF in the mMSCs treated groups that were previously exposed to the inflammatory stimuli. Significant p-value are denoted with asterisk (*), with p-value <0.05 considered as significant.

3.6 MCP-1's Modulation of the Secretome Release Response of Splenic Immune Cells and Monocytes

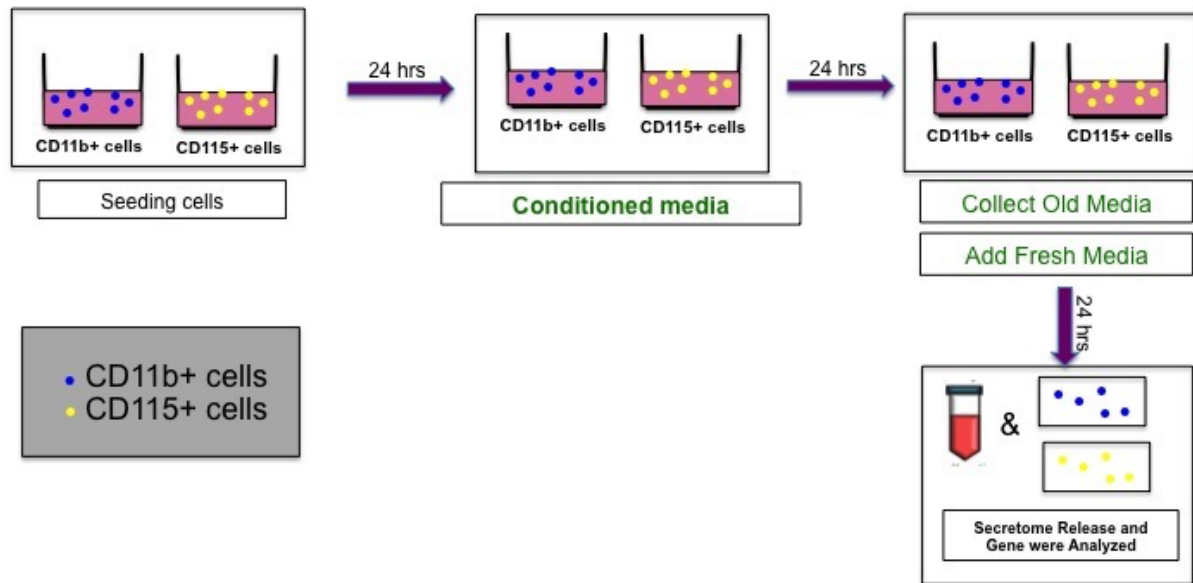
We subjected splenic immune cells CD11b⁺ cells and splenic monocytes CD115⁺ cells in culture to six conditions 1) CM from mPECs only group, 2) CM from mPECs previously exposed to inflammatory stimuli (IFN- γ and TNF- α (50nM)), 3) CM from mPECs exposed to MCP-1 neutralizing antibody (Anti MCP-1 Ab), 4) CM from mPECs + mMSCs group 5) CM from mPECs + mMSCs previously exposed to

inflammatory stimuli (IFN- γ and TNF- α (50nM)), and 6) CM from mPECs exposed to MCP-1 neutralizing antibody (Anti MCP-1 Ab) (*Figure 7. A*).

In the CD11b⁺ Fresh Media, the results were similar to that of the CD11b⁺ Old Media groups. No significant differences in the release of IL-1 β and VEGF were observed in the groups previously treated with conditioned media from mPECs only (*Figure 7. B (i) and (ii) and Figure 7. C (i) and (ii)*). IL-1ra release was observed to reduced in the group that was previously treated with mMSCs and MCP-1 neutralizing antibody. MCP-1 and IL-6 secretion were not detected in the Fresh Media. Logarithmic fold change data was used to appropriately compare the secretome release between CD11b⁺ groups previously treated with CM from mPECs only and CM from mPECs + mMSCs groups. There were no significant logarithmic fold change differences observed in the release of IL-1 β , IL-1ra and VEGF in any of the groups (*Figure 7. D (i) – (iii)*).

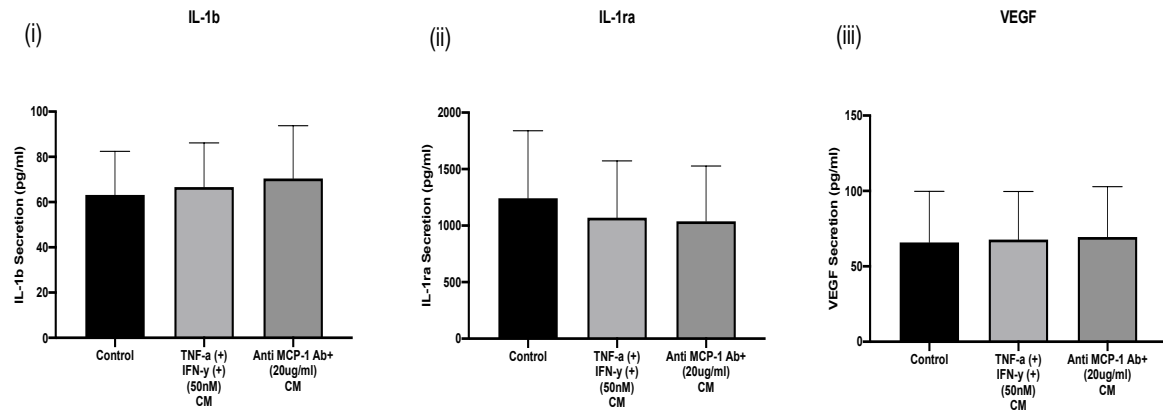
The secretome release of CD115⁺ cells in Fresh Media we observed no significant differences in the release of IL-1 β or VEGF any of the groups (*Figure 8. A (i) – (ii) and Figure 8. B (i) – (ii)*). MCP-1 release was not detected in Fresh Media. Logarithmic fold change data was used to appropriately compare the secretome release between CD115⁺ groups previously treated with CM from mPECs only and CM from mPECs + mMSCs groups. There were no significant logarithmic fold change differences in the release of IL-1 β and VEGF in any of the groups (*Figure 8. C (i) – (ii)*).

(A)



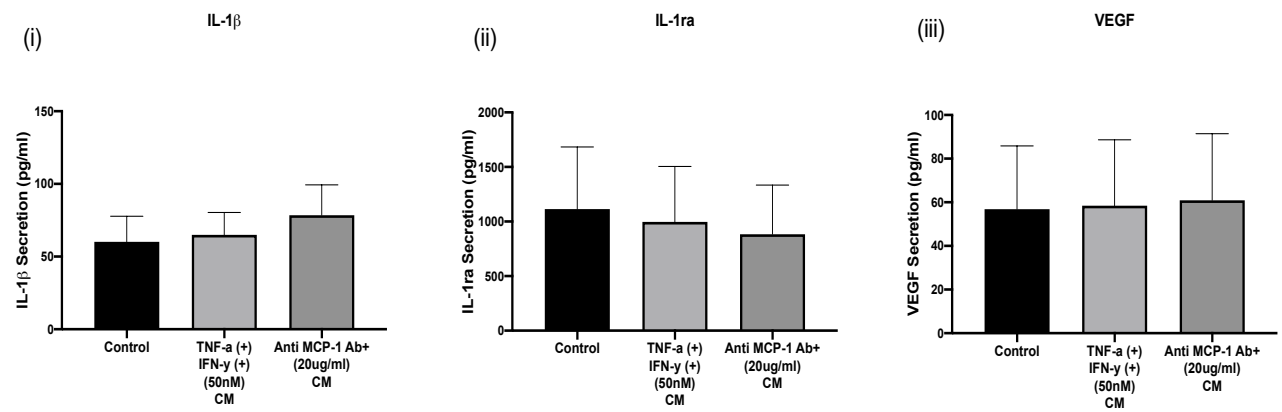
(B)

CD11b Previously Treated with CM from mPEC Only Secretome Release Fresh Media



(C)

CD11b Previously Treated with CM from mPEC + mMSCs Secretome Release Fresh Media



(D)

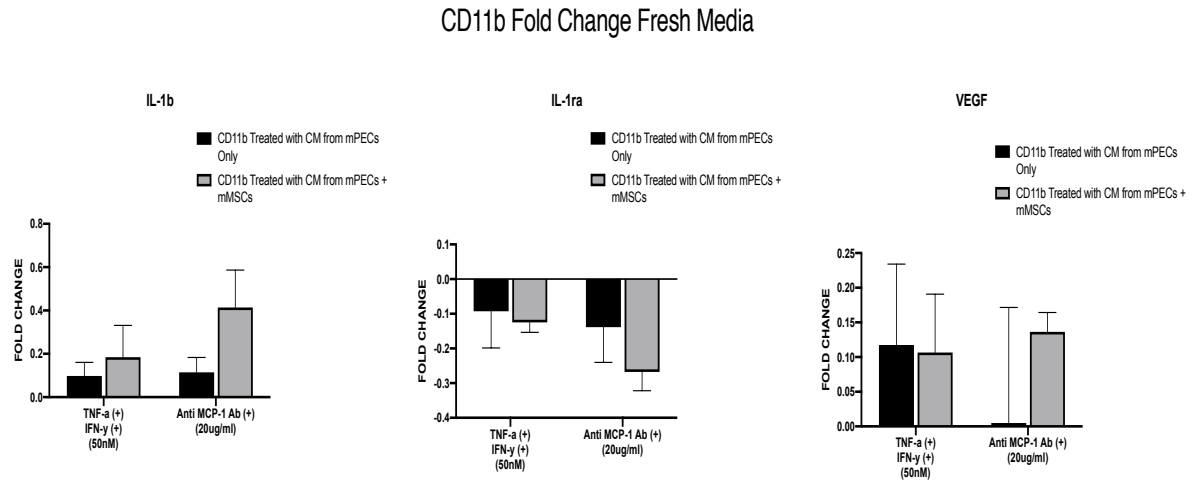


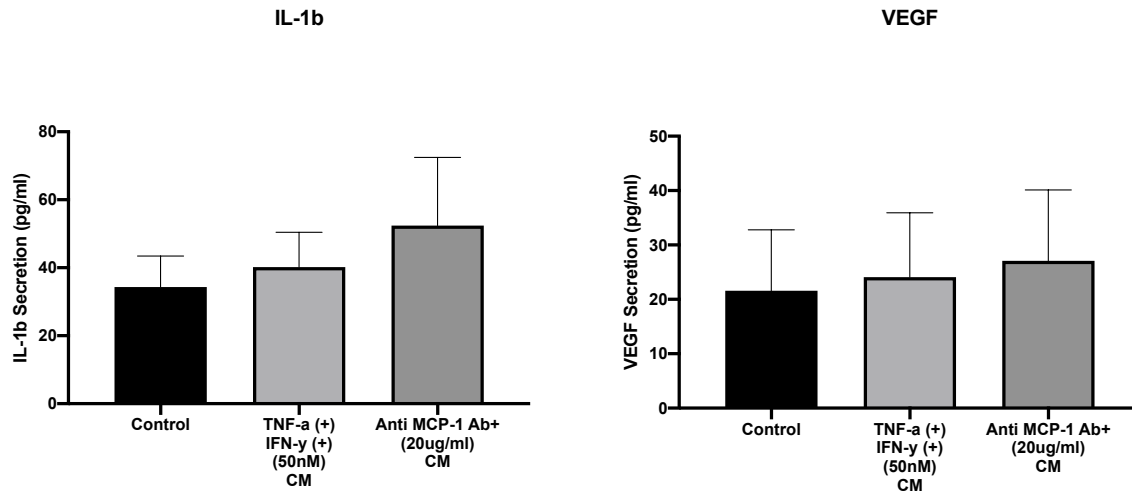
FIGURE 7. Changes in Secretome Release of CD11b⁺ Splenic immune cells.

(A) Schematic of exposing CD 11b⁺ immune cells to CM media collected from the previous experiment where mPECs or co-cultures of mPECs and mMSCs were exposed to inflammatory stimuli (TNF- α and IFN- γ (50 nM) Anti MCP-1 Neutralizing Antibody. (B) CD11b⁺ exposed to CM from mPECs only: (i) – (iii) There was no significant difference in the release of IL-1 β , IL-1ra and VEGF, respectively. (C) CD11b⁺ exposed to CM from mPECs and mMSCs: (i) and (iii) There was no significant difference in the release of IL-1 β and VEGF, respectively. (ii) IL-1ra release was reduced in the group were MCP-1 was previously neutralized. (D) Logarithmic fold change data was used to appropriately compare the secretome release between CD11b⁺ cells previously treated with CM from mPECs only groups and mPECs + mMSCs groups. (i) – (iii) There was no significant logarithmic fold change differences observed in the release of IL-1 β , IL-1ra, and VEGF, respectively.

Significant *p*-value are denoted with asterisk (*), with *p*-value <0.05 considered as significant.

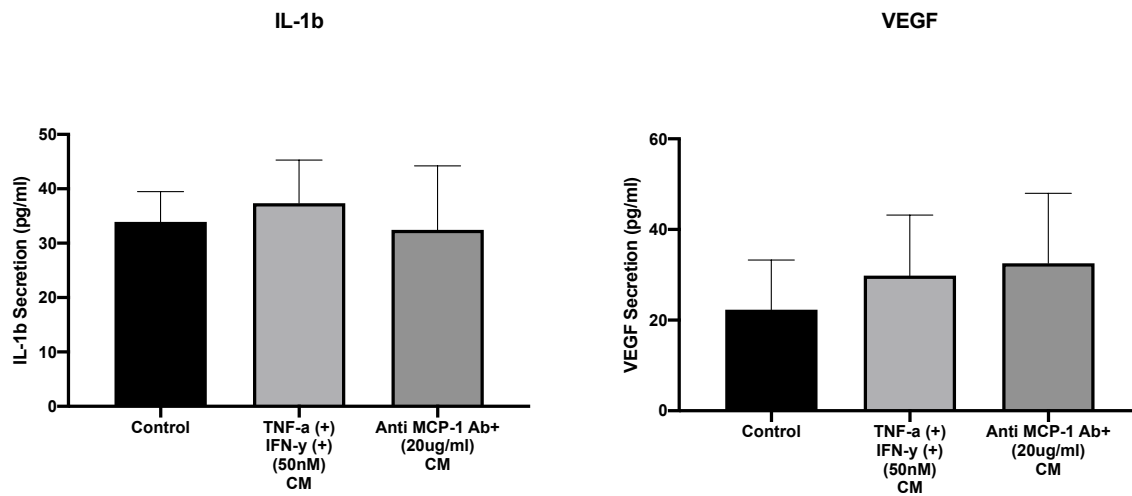
(A)

CD115 Treated with CM from mPEC Only Secretome Release Fresh Media



(B)

CD115 Treated with CM from mPEC + mMSCs Secretome Release Fresh Media



(C)

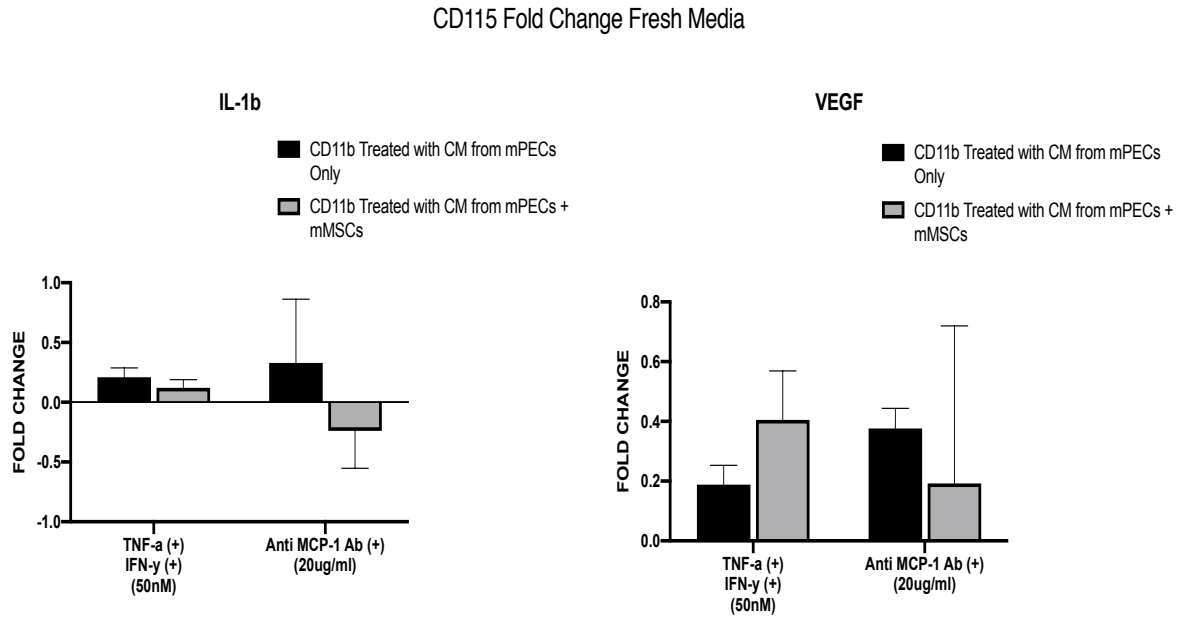


FIGURE 8. Changes in Secretome Release of CD115⁺ splenic monocytes.

(A) CD115⁺ exposed to CM from mPECs only: (i) – (ii) There was no significant difference in the release of IL-1 β and VEGF, respectively (B) CD115⁺ exposed to CM from mPECs and mMSCs: (i) – (ii) There was no significant difference in the release of IL-1 β and VEGF, respectively (C) Logarithmic fold change data was used to appropriately compare the secretome release between CD115⁺ cells previously treated with CM from mPECs only groups and mPECs + mMSCs groups. (i) – (ii) There was no significant logarithmic fold change differences observed in the release of IL-1 β and VEGF, respectively. Significant p-value are denoted with asterisk (*), with p-value <0.05 considered as significant.

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS

Stroke is a leading cause of serious long-term disability in the world. Cell-based therapies such as MSCs therapy have increasingly shown great promise for ischemic stroke recovery, with some therapies already in various stages of clinical trials. Although a majority of the IV administered MSCs lodge in the lungs, studies still observed an endogenous restorative effect in the brain, either the release of trophic factors or through immunomodulation ^{8, 9, 13, 16, 37}. During this 'Pulmonary First Pass', the MSCs mainly interact with the pulmonary microvascular endothelial cells ³⁷. Many studies have previously shown that MCP-1 plays a critical role in immune modulation under inflammatory conditions ⁴³⁻⁴⁵.

In this study, we aimed to look at the interaction between mPECs and mMSCs under inflammatory conditions, similar to the stroke milieu. We specifically focused on the secretome release after this interaction, including MCP-1 release. We found that under inflammatory conditions, MCP-1 release increases in mPECs and co-cultures of mPECs and mMSCs. Previous studies have shown that MCP-1 release is increased during inflammatory events ^{47, 55-58}. However, many studies seldom look at the release modulation of MCP-1 in the presence of stem cells, like MSCs.

In this study, we also looked at how secretome release was affected, when co-cultures of mPECs and mMSCs were either over-stimulated with recombinant MCP-1 protein or neutralization of the secreted MCP-1 using a neutralization antibody. We observed when MCP-1 was effectively neutralized, the secretome release of VEGF had significantly decreased. VEGF is a trophic factor that acts as a potent pro-angiogenic and promotes neovascularization ⁵⁹⁻⁶¹. Many studies have shown that after MSC treatment, the secretion of VEGF increases ⁶¹⁻⁶³. In our study,

we observed that MCP-1 plays a role in modulating the release of VEGF, through the interaction of mPECs and mMSCs. A study by Jay, SM., 2010, ⁶⁴ showed that MCP-1 and VEGF together play an important role in supporting the survival of transplanted endothelial cells, while also increasing the formation of functional vessels from the transplanted endothelial cells ⁶⁴. Some studies have also shown that MCP-1 by itself can modulate angiogenesis under certain conditions ⁶⁵⁻⁶⁷.

In the same experiment, we also observed the release modulation of the pro-inflammatory cytokine IL-1 β , under certain conditions. The presence of MSCs is more than likely causing these observed changes in the secretome release. Studies have shown that after MSC therapy, IL-1 β expression and secretion goes down ⁶⁸⁻⁷⁰.

Studies previously suggested that MCP-1 plays a detrimental role under inflammatory conditions. In MCAo animal stroke models, MCP-1 has been shown to increase infarct size and volume, mainly through the role it plays in the recruitment of inflammatory monocytes and macrophages ⁷¹. Studies have also shown that infarct size was much smaller in animals that had MCP-1 expression knocked down ⁴⁸. While this might be true in inflammatory conditions, after administering treatments like cell therapy, the role that MCP-1 plays could be altered. Studies over the past few years have started revealing the importance of MCP-1 in the migration of various stem cells. Stem cells such as NSCs, MSCs, dental pulp derived MSCs, etc., were shown to migrate better and home to regions of insult or injury in the presence of MCP-1 ^{50-52, 72-74}.

In our study, we wanted to see under therapeutic conditions how MCP-1 produced after the interaction between pulmonary endothelial cells and mesenchymal stem cells change secretome release following inflammation. We

observed that when MCP-1 was neutralized, there was an increased expression of IL-6 levels, which was an interesting observation. IL-6 has been long associated as a pro-inflammatory cytokine and had been associated with detrimental outcomes in a disease condition ^{75, 76}. More recent studies are showing that IL-6 plays a dual role and is also capable of acting as an anti-inflammatory factor. The anti-inflammatory response of IL-6 is carried out through its interaction with membrane-bound IL-6 receptor in *classical signaling*. Whereas, the pro-inflammatory activity is in response to trans-signaling, where IL-6 interacts with soluble IL-6 receptor ⁷⁷. Similar to MCP-1, IL-6 is also involved in the recruitment of leukocytes. It has been shown that animals that were knockdown of IL-6 exhibited poor recruitment of leukocytes. They also found that the interaction between IL-6 and endothelial cells through the soluble IL-6 receptor plays a central role in the leukocyte recruitment ⁷⁸.

Many studies previously have looked at the roles of IL-6 and MCP-1 independently. However, results from our study indicate there might be a co-dependent relationship between the two trophic factors. A recent study by Hosaka, K., et al., 2017⁷⁹, has also shown that the interplay between IL-6 and MCP-1 plays a vital role in intra-aneurysmal tissue healing ⁷⁹. They suggest that IL-6 is a downstream mediator of MCP-1 ⁷⁹. A few other studies have also shown a co-dependent relationship between these two trophic factors ^{80, 81}.

Another important aim of this study was to look at MCP-1's (released after the interaction of PECs and MSCs) immunomodulatory role on the differential recruitment of monocytes. In our study, we were not able to find any significant differences in the secretome release of the monocytes, when they interacted with the conditioned media. One main reason for this could have been the minimal

number of monocytes that we were able to isolate from the splenic tissue. Reports say that there are only about 3.5-5% of splenic cells that are monocytes.

A few studies have reported that following MSC treatment, monocyte and macrophages polarize towards an anti-inflammatory M2 subtype ^{70, 82}. Cancer studies, however, in recent years, have provided more evidence to support the MCP-1 plays an essential role in this M2 type polarization. A recent study by Su, W., et al., 2019 ⁸³, showed that in tumor microenvironments, MCP-1 plays an important role in recruiting tumor-associated macrophages and polarizing them to an M2 phenotype. This is achieved through the interaction it has with the tumor stem cells ⁸³. Studies have shown that MCP-1 promotes angiogenesis and M2 polarization of macrophages in tumor conditions ^{84, 85}. Studies have also shown the role of IL-6 in the recruitment of M2 polarized macrophages in conditions such as cancer and obesity ^{86, 87}. As previously mentioned, IL-6 and MCP-1 seem to have a co-dependent relationship. Cancer studies have also explored this and have shown that MCP-1, along with IL-6, has an increased expression in tumor microenvironments. This increased expression helps in the protection of the tumor-recruited monocytes, while at the same time aiding in their differentiation towards the M2 phenotype ⁸⁸. While these conditions might be detrimental in a cancer setting, they could be beneficial in stroke.

Although this study tried to elucidate how MCP-1 released through the interaction of pulmonary endothelial cells and mesenchymal stem cells modulates its immunomodulatory effects through monocytes, the results were inconclusive. All the experiments conducted for this study were done in an *in-vitro* setting. Although this could give us a preliminary idea of what's going on, to get more precise answers, we

would have to move to an *in-vivo* setting. Secondly, a major limitation we had, as mentioned above, was the limited number of splenic monocytes that we were able to isolate. To overcome this issue, we could move towards the collection of monocytes from other sources such as bone marrow and peripheral blood, while also examining the tissue macrophages and the effects on them. In an *in-vivo* setting, we can better attain stroke-like conditions, and further explore if any of the changes seen through the interaction of PECs and MSCs affect different types of brain cells, especially the resident microglial population. To get a better understanding of the IL-6 and MCP-1 co-dependent relationship, we would include experiments where we block both and see what outcome we get. Further experiments will also be required to understand the exact mechanism behind these observations.

In conclusion, our study was able to elucidate that MCP-1 release under stroke like conditions was modulated through the interaction of PECs and MSCs. However, our study was unable to explain MCP-1's role in immunomodulation through monocytes.

CHAPTER 5: BIBLIOGRAPHY

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VITAE

Kaavya Giridhar was born in India to Umavathy Giridhar and Giridhar K. Kanapadi. She completed her high school graduation from Herndon High School, Herndon, Virginia. She went on to pursue her undergraduate studies in Biology and Psychology at Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia and graduated with a *cum laude*. She proceeded to do research at Virginia Tech, with a cerebrovascular research team. This experience highly influenced her love for research. To pursue her research path, she entered the Master's in Biomedical Sciences program at GSBS, UT Health, Houston, Texas. Since then, under the mentorship of Dr. Sean I. Savitz, she has been working on her thesis project.