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The Impact of SOCS1 and SOCS3 Peptide Mimetics on RHO and CDC42 Proteins Expression, F-actin Cytoskeleton Rearrangements, and Cytokines Production of Uninfected and HSV-1 Infected M1 and M2 RAW 264.7 Murine Macrophages

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THE IMPACT OF SOCS1 AND SOCS3 PEPTIDE MIMETICS ON RHO AND CDC42 PROTEINS EXPRESSION, F-ACTIN CYTOSKELETON REARRANGEMENTS, AND CYTOKINES PRODUCTION OF UNINFECTED AND HSV-1 INFECTED M1 AND M2 RAW 264.7 MURINE MACROPHAGES.

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Maha A. Elwardany ENTITLED The Impact of SOCS1 and SOCS3 Peptide Mimetics on RHO and CDC42 Proteins Expression, F-actin Cytoskeleton Rearrangements, and Cytokines Production of Uninfected and HSV-1 Infected M1 and M2 RAW 264.7 Murine Macrophages BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Elwardany, Maha A. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2017. The Impact of SOCS1 and SOCS3 Peptide Mimetics on Rho and Cdc42 Proteins Expression, F-actin Cytoskeleton Rearrangements, and Cytokines Production of Uninfected and HSV-1 Infected M1 and M2 RAW 264.7 Murine Macrophages.

The immune system plays an essential role in the pathogenesis of HSV-1 during the lytic phase of the disease, orchestrating the inflammatory response, retaining the virus in its latent phase and preventing the recurrence of HSV-1 infection. Macrophages display a vital role in the innate and adaptive immune responses during multiple phases of HSV-1 infection. Polarized macrophages are categorized into two distinct classes with diverse functions. The classically activated M1 can engulf and destroy the microbial agents, produce proinflammatory cytokines, and participate in the pathogenesis of many inflammatory diseases. The alternatively activated M2 induces anti-inflammatory mediators and stimulates tissue remodeling and wound healing. The reorganization of filamentous actin is a prerequisite for polarized macrophages functions. Rho and Cdc42 are highly expressed Rho GTPase proteins in Raw 264.7 murine macrophages. Rho and Cdc42 proteins play a critical role in altering the organization of structural networks of actin microfilaments. These GTPases are cycling between an active (GTP bound) and inactive (GDP bound) form. Activation of Rho proteins leads to dynamic changes in macrophage’s cytoskeleton, cell migration, and phagocytic function. SOCS1 and SOCS3 are structurally related proteins that are induced endogenously in macrophages. They are involved in the down-regulation of the JAK-STAT pathways that lead to production of inflammatory cytokines. The present study indicated that SOCS1 challenged M1 macrophages expressed lower levels of Cdc42 and Rho A proteins and higher level of F-actin in the presence or absence of HSV-1. SOCS1 challenged M1 also exhibited significant reduction in TNF-α production and percentage of viable cells. This study also indicated that SOCS1 challenged M2 polarized macrophages did not show change levels of Cdc42 and Rho proteins and F-actin staining intensities from that of unchallenged M2 cells. These cells expressed a significant increase in IL-10 as compared to unchallenged M2 cells. In contrast, this study demonstrated that SOCS3 has a transient effect on M1 and M2 polarized cells in the early hours of infection. At 2, 4, and 6 hours of infection, SOCS3 potentiated the effect of M1 by promoting the activation of Rho GTPase proteins, and increasing proinflammatory TNF-α cytokine secretion. On the other hand, SOCS3 challenged M2 cells expressed a
significant increase in levels of RhoA and Cdc42 proteins. They also showed a significant decrease in F-actin staining intensity and IL-10 secretion compared to unchallenged cells. SOCS3 effects on polarized macrophages disappeared at late (24 and 48) hours. This data suggests that during an active infection, SOCS1 regulates M1-macrophages indirectly by constraining the activation of Cdc42 and Rho GTPase proteins. This hinders their intracellular signaling pathways and impacts F-actin organization which can interfere with the phagocytic properties of macrophages. During the resolution phase of infection, SOCS1 upregulates M2 polarization and potentiates the M2 secretion of IL-10. SOCS3 has a transient effect on M1 and M2 cells in the early hours of infection. SOCS3 potentiates the effect of M1 cells, promotes the activation of Rho GTPase proteins, and increases proinflammatory cytokine secretion. SOCS3 downregulated M2 cell function that may help to make M1 more efficient, favoring the inflammatory process to eliminate invading pathogens. During the resolution phase of infection, SOCS3 may display an endogenous regulation role in macrophages through the activation of the JAK-STAT3-IL10 pathway.
Hypothesis

SOCS1 peptide mimetic downregulates expression of Rho and Cdc42 proteins in uninfected and HSV-1-infected RAW264.7 macrophages accompanied by decreases in TNF-α production and change in cell morphology. In contrast, SOCS3 peptide mimetic upregulates expression of these proteins.
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List of Abbreviations

APCs           Antigen presenting cells
Arp2/3        Actin related proteins 2/3
Cdc42          Cell division Cycle 42
CR             Complement receptor
DAMPs          Dangerous associated molecular patterns
Dia 1          Diaphanous protein-1
EGF            Epidermal growth factor
FAK            Focal adhesion kinase
FcR            Fc receptor
GAPs           GTPase activating proteins
GDIs           Guanine nucleotide dissociation inhibitors
GEfs           Guanine exchange factors
GM-CSF         Granulocyte macrophage colony stimulating factor
IFN            Interferon
IFNR           Interferon receptor
IRF            Interferon regulatory factors
JAK            Janus Kinase
LIMK  LIM- domain containing kinase
MAPK  Mitogen activating protein kinase
M-CSF  Macrophage colony stimulating factor
MHC  Major histocompatibility complex
MLC  Myosin light chain
MLCK  Myosin light chain Kinase
NFKB  Nuclear factor kappa B
NKs  Natural killers
PAK  p21 activating kinase
PAMPs  Pathogenic associated molecular patterns
pDCs  Plasmacytoid dendritic cells
PI3K  Phosphoinstol 3 kinase
PPR  Membrane bound pattern recognition receptor
ROKs  Rho activating Kinase
STAT  Signal Transducer and Activator of Transcription
TESKs  Testis- specific kinases
TGF-β  Transforming growth factor-beta
VEGF  Vascular endothelial growth factor
DEDICATION

This thesis is dedicated to the soul of my father, who encouraged and supported my education since I was a child, who believed in me more than I believe in myself. I would not stand here if it were not for his support. Special thanks to my husband and my children for their encouragements, love, and support that made it possible for me to keep up my dedication to this project.
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Introduction

Herpes simplex is one of the most widespread infectious diseases of the skin and mucosa in humans. HSV-1 is responsible for 300,000 cases of ocular infection and 3% of corneal transplants annually in the United States; it is estimated to affect one third of the population worldwide (Keadle et al., 2008). Recurrent episodes of ocular HSV-1 affect 60% of individuals over the age of 50. The infection is long-lasting and is usually contracted in early childhood (Arduuino & Porter, 2016). HSV-1 is a double stranded DNA human pathogen that has two phases: lytic and latent (Zaichick et al., 2011). During the lytic phase, the virus enters the epithelia of skin or mucosa via visible or nonvisible injuries (Petermann et al., 2009), replicates in the epithelia of the host and induces its pathogenesis, which varies from symptomless papules and painful sores, such as HSV-1 labialis and stomatitis, to more serious conditions such as keratitis (Huber et al., 2001). Because of improper management of the disease in underdeveloped countries, HSV-1 ocular infection is one of the leading causes of blindness (Johnson et al., 2011). Although less common, fetal encephalitis can also be caused by HSV-1 brain infection (Kigerl et al., 2009; Zaichick et al., 2011). During the latent phase, the virus remains in an inactive state in the neural ganglia of the sensory nerve that innervates the primary lesions (Wakimoto et al., 2003). HSV-1 reorganizes the host cytoskeleton and modulates different cellular signals for directing the cytoskeleton during viral replication and intracellular trafficking (Turowska et al., 2010; Campbell et al., 2005). HSV-1 uses the cell cytoskeleton during entry, assembly, and egress during the lytic phase (Lyman et al., 2008). HSV-1 infection causes radical changes in the reorganization of F-actin (Taylor et al., 2011; Compellone et al., 2010). During the initial stages of entry of HSV-1 into host cells, several capsid glycoproteins (mainly gD) bind to cell surface nectin receptors (Petermann et al., 2009). Viral components such as Us3 kinase stimulate small GTPase proteins, causing them to switch to their active forms (Bauer et al., 2008). This stimulates downstream intracellular signaling pathways leading to development of a wide range of F actin network structures (Honess and Roizan 2006). These structures include stress fibers, lamellipodia, and filopodia, induced by Rho A, Rac1, and CDC42, respectively (Roberts K. and Baines J., 2011). These structures facilitate endocytosis of viral particles into the cytoplasm of host cells (Chimini et al., 2000; Campos et al., 2009). Rho GTPase proteins display a regulatory role in other stages of the HSV-1 lifecycle (Favoreel et al., 2009).

The immune system plays an essential role in the pathogenesis of HSV-1 during the lytic phase of the disease, orchestrating the inflammatory response, maintaining the virus in its latent phase and preventing the recurrence of HSV-1 infection (Keadle et al., 2008). Macrophages display a vital role in the innate and adaptive immune responses during multiple phases of inflammation, containing the infection,
and are also involved in the pathogenesis of many inflammatory diseases (Murray and Wynn, 2011). Tissue infiltrating macrophages are derived from peripheral blood monocytes in response to changes in the extracellular environment. Polarized macrophages are categorized into two distinct classes with diverse functions. The classically activated M1 can engulf and destroy the microbial agents, produce proinflammatory cytokines, and participate in the pathogenesis of many inflammatory diseases. The alternatively activated M2 induces anti-inflammatory mediators and stimulates tissue remodeling, wound healing, and immune regulation (Erwig and Henson, 2007).

Macrophages receive physical and chemical stimuli from the extracellular environment such as growth factors, hormones, bacterial products, chemokines and viral components (Alflaki et al., 2011). These stimuli interact with cell surface receptors and act locally to regulate guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) leading to the upregulation and downregulation of Rho GTPase protein activities, respectively (Sit and Manser, 2011). Guanine nucleotide dissociation inhibitors (GDIs) have an inhibitory role on Rho GTPase proteins by sequestering them and preventing their activation (Heasman et al., 2008). Stimulation of Rho GTPases regulates downstream intracellular signaling cascades of proteins, drastically impacting the assembly and disassembly of F-actin network structures and affecting the dynamics of the cytoskeleton, morphology, and functions of macrophages (Alflaki et al., 2011; Hoppe et al., 2006). Rho GTPase proteins cycle between active (GTP bound) and inactive (GDP bound) forms (Ridley et al., 2001). Mammalian cells demonstrate wide variations in types, levels of expressions, and roles of Rho GTPase proteins between species and between different types of cells within the same species. In mammalian cells, there are about 20 Rho GTPase proteins, 70 GAPs, and over 80 GEFs that elaborate the complexity of this family of proteins in regulating intracellular signaling pathways (Heasman and Ridley, 2008). Rho GTPase proteins in mammals form three subfamilies: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3), and Cdc42 (Cell division cycle 42). Rho A, Rac1, and Cdc42 are the most researched and studied Rho GTPase proteins by clinical researchers to characterize these protein families (Ridley, 2006). These three proteins are best known for their roles in the organization of F-actin networks of structures, including RhoA’s promotion of stress fibers organizations, Rac1’s role in lamellipodia development, and that of Cdc42 in filopodia formation (Etienne-Manneville, 2002). Raw 264.7 murine macrophages are known to express high levels of Rho and Cdc42 proteins, which may be essential to their phagocytic function (Godek et al., 2006).

Macrophages are extremely dynamic cells that are continually undergoing changes between M1 and M2 functional states (Davis M., 2013; Chandak et al., 2010). Regulation of macrophages is a complex
and essential process that involves numerous interconnected intracellular signaling pathways which regulate many target proteins that are crucial during the acute phase and recurrent episodes of infections including herpes. Together, they are responsible for bringing about measured and efficient immune responses and preventing the recurrence of the disease as well as promoting the healing process during the resolution phase of the infection.

Suppressors of cytokine signaling, SOCS1 and SOCS3, are structurally related proteins induced endogenously in macrophages (Babon et al., 2012). They were investigated for their roles in the down regulation of JAK-STAT intracellular pathways, which are crucial for the induction of pro-inflammatory and anti-inflammatory mediators of macrophages (Bigley, 2014; Dalpke et al., 2008). SOCS1 controls M1 polarization through direct inhibition of the IFN-γ JAK2/STAT1 intracellular signaling pathway or through indirect inhibition of the TLR-4-IRF pathway (Liu et al., 2008). SOCS1 also is involved in M2 activation in vitro as well as in vivo (Yu et al., 2004). SOCS3, on the other hand, contributes to M1 polarization through direct inhibition of the cytokine receptors that abrogate the IL-6-STAT1 pathway, which promotes M2 polarization (Hanada et al., 2005; Duluc et al., 2007).

Our current study is designed to examine the regulatory roles of SOCS1 and SOCS3 peptides on M1 and M2 polarized Raw 264.7 murine macrophages. We hypothesized that SOCS1 and SOCS3 proteins regulate RhoA and Cdc42, Rho GTPase proteins that are highly expressed in Raw 264.7 murine macrophages cell lines. These proteins can affect F-actin reorganization in uninfected and HSV-1 infected M1 macrophages, and in IL-4 polarized M2 macrophages. Using immunohistochemistry, we can evaluate in situ changes of cytoskeleton organization and cytokine production in SOCS1 and SOCS3 challenged M1 and M2 at 2, 4, and 6 hours from cell treatments and we compare the outcomes with those observed after 24 hours and 48 hours of treatment.
Herpes simplex type one (HSV-1) is a human pathogen of the alpha-herpes virus and a subfamily of the Herpviridea family. The subfamily also includes other human pathogens such as HSV-2 and varicella-zoster virus (Geraghty et al., 1998). HSV-1 is a large virus consisting of a linear double stranded DNA genome of about 100 kbps which is enclosed in an icosahedral protein capsid that is enveloped in glycoproteins (gB, gC, gD, gH, and gL); this envelope is essential for HSV-1 entry (Shelly et al., 2012). The viral envelope encompasses a plethora of spikes that are essential to viral infectivity as it attaches to host cells (Whitley and Roizman, 2001). The space between the viral capsid and envelope is occupied by tegument proteins which play a crucial role during viral replication (Kim et al., 2012).

Depending on the cell’s receptors, the virus enters host cells either by endocytosis or by fusion with the plasma membrane. Interaction of the viral glycoprotein D (gD) with the host’s nectin receptors is essential for the virus’ entry and fusion with the cell membrane (Frampton et al., 2007). This trans-interaction leads to the activation of Cdc42 and Rac1, which in turn promotes the reorganization of F-actin that is involved in adherence junctions of epithelia (Takai et al., 2008). In vivo, HSV-1 is allowed entry into wounded epithelia as the epithelial barriers of the skin are impaired at injury sites due to the disassembly of protective F-actin microfilaments of keratinocytes (Lyman and Enquist, 2009). Upon entry, HSV-1 replicates in epithelial cells of skin and mucosa and initiates a lytic cycle in the epithelia of the host. From the primary site, HSV-1 can be transported in a retrograde fashion through the sensory nerve axons that innervate the primary lesions (Bigley, 2014) and remains inactive and establishes the latent phase within the trigeminal ganglion of infected individuals (Wakimoto et al., 2003). Immune suppression due to medication or illness, prolonged exposure to ultraviolet (UV) radiation, longstanding uncontrolled diabetes, and emotional stress can reactivate the latent virus and cause recurrence of infection (Woo and Challacombe, 2007). HSV-1 is disseminated from one primary site to another within the infected individual, mainly targeting the trigeminal nerve and its distributed branches to disseminate from a primary sore to infect the eyes and cornea (Woo and Challacombe, 2007). The virus can also be spread through cross contamination of the nails and fingers of medicinal specialists who handle equipment.
contaminated with HSV-1 (Whitley and Roizman, 2001). If untreated, the virus can be transported through the sensory branches of the trigeminal nerve to infect brain tissue and can cause severe fatal encephalitis (Fatahzadeh and Schwartz, 2007).

Host cytoskeleton roles in HSV-1 infection

HSV-1 uses different elements of host cells’ cytoskeleton for virus replication and intracellular trafficking (Turowska et al., 2010; Campbell et al., 2005). From entry to assembly and egress, the virus utilizes the host cytoskeleton for its advantages (Lyman et al., 2008). The three main elements of the cytoskeleton consist of microfilaments, intermediate filaments, and microtubules. They play crucial and dynamic roles in many stages of HSV-1 infection (Parker et al., 2014).

F-actin form micro filamentous structures comprised of interwoven polymeric units of filaments of 5nm in diameter. Each microfilament consists of two strands of monomers of globular protein subunits (G-actin) that are bound to ATP molecules (Taylor at el., 2011). HSV-1 depends on F-actin networks for its entry into the host cell as well as its exit (Turowska et al., 2010). F-actin is a polar structure with a plus barbed end and a minus pointed end (Lyman et al., 2008). During infection, HSV-1 causes a dramatic change in F-actin reorganization leading to severe changes in the morphological appearances of the host cells (Taylor at el., 2011). The binding of HSV-1 gD to surface nectin receptors on host cells upregulates Rho GTPase proteins and causes the disassembly of F actin, enabling the virus to enter the cell cytoplasm. The interaction between HSV-1 and F-actin involves Rho GTPase’s signaling system during different phases of viral entry, replication, assembly, maturation, and egress (Favoreel et al., 2009; Lang et al., 2003). HSV-1 enters the host cell via attachment to host membrane receptors on the epithelia of mucosa and skin (Petermann et al., 2009). HSV-1 can enters human keratinocytes via endocytosis (Nicola et al., 2005), which demonstrate the dynamic effect of F actin on the viral entry regardless of the favored way of internalization. Upon entry, viral particles diffuse into the cytoplasm of host cells. Passing through the nuclear pores, the DNA of HSV-1 is released into the nucleus and incorporated into DNA of the hosts and the virus starts to replicate its genomic DNA. It makes new HSV-1 particles that egress the host nucleus and transports to host cells endosomes passing through Trans- Golgi network (TGN) where the virions’ secondary envelopes are developed. virions are then released from host cells via exocytosis (Owen et al., 2015). Intermediate filaments (IF) are a tetrameric polypeptides 10 nm in diameter composed of monomers that twist together to form a stable rope-like structure (Lyman et al., 2008). All cells have IFs, but differ on the types of protein monomers. IFs are responsible for preserving the cell-shape and strength
Microtubules are polymers of α- and β-tubulin proteins which form a spindle of 24 nm in diameter (Parker et al., 2014). Microtubules play important roles in viral growth and replication. HSV-1 travels along the microtubules across long distances from the host entry site to the nucleus (Lyman et al., 2008). HSV-1 also utilizes microtubules to transport through the sensory nerve from dendritic processes to neural ganglia during latency phase in retrograde manner and transport from ganglia to nerve ending passing by the axon in anterograde manner during the recurrence of infection. Microtubules have two polymeric ends that direct transport path; the plus end that guides the movement of virus toward the host plasma membrane (anterograde transport), and the minus end that guides the viral particles toward the host nucleus (retrograde transport) (Lyman et al., 2009; Saksena et al., 2006). Two families of microtubule associated proteins (MAPs), dynein and kinesin, are associated with the cargo movements depending on the polarity of the microtubules (Dohner et al., 2002).

Macrohage polarization and functions

Macrophages are mononuclear granulocytic white blood cells (WBCs) that are derived from peripheral blood monocytes which account for 5-10% of total WBCs. Monocytes originate from bone marrow-derived precursors (Mantovani et al., 2005; Sica and Mantovani, 2012). Monocytes leave the blood circulation and mature into a morphological and functional diverse group of cells in response to environmental changes (Martinez et al., 2008; Mantovani et al., 2005). Differentiation of monocytes into macrophages occurs in response to two major factors: granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF), which are essential to the differentiation process (Stefater et al., 2011). Based on differences in marker expression, morphology, and function, activated macrophages can be classified into classically activated-macrophages (M1) or alternatively activated macrophages (M2). M1-macrophages are pro-inflammatory immune cells that play crucial roles as phagocytic cells and antigen presenting cells (APCs) (Martinez and Gordon, 2014). During inflammation, the classically activated macrophages M1 are induced by interferon gamma (IFN-γ), and lipopolysaccharides (LPS). M1 macrophages produce a significant amount of proinflammatory cytokines (IL-1, IL-6, TNF-α, IFN-γ) and reactive oxygen and nitrogen species (Kigerl et al., 2009; Sommer et al., 2005). M1 also secrete high levels of interleukin-12 (IL-12) and interleukin-23 (IL-23) cytokines, which recruit the professional Th1 (T-helper cell type1) and Th-17 cells to the sites of inflammation. These Th cells promote cell mediated immunity and cytotoxic activity, inducing huge amounts of ROS and NO (Martinez and
Gordon, 2014) that enhance the tumoricidal and microbiocidal activities of macrophages. Upon infection, M1 macrophages recognize pathogen associated molecular patterns (PAMPs) and danger/damage-associated patterns (DAMPs) via toll like receptors (TLRs). IFN-γ is induced by natural killer cells (NKs) and Th1 at the site of inflammation and promotes tissue macrophages to produce high levels of proinflammatory cytokines ((Reichard et al., 2015).
Macrophage Polarization and Function

- **Classic activated**
  - Pro-inflammatory cytokines: TNF, INOS, ROS, IL-6, IL-12, and IL-23
  - Antigen presenting cells
  - Phagocytosis
  - Cytotoxicity

- **Alternative activated**
  - Anti-inflammatory mediators: IL-10, PDGF, EGF, and TGF-B
  - Tissue remodeling
  - Wound Healing
  - Tumor Promotion

**Figure 1: Macrophage Polarization and Function**
Macrophages and HSV-1

Macrophages are key players in immune responses to HSV-1 infection. During an acute infection, macrophages are involved in the intrinsic resistance of HSV-1, constraining the virus growth inside the macrophages themselves (Ojala et al., 2000). They play a crucial role in the extrinsic resistance to inactivate extracellular viruses, decrease HSV-1 replication in infected cells, and impair its spread to adjacent cells (Ellermann-Eriksen, 2005). Macrophages engulf, phagocytize, and eliminate virally infected cells. Activated macrophages express toll-like receptors (TLRs), which are membrane-bound pattern recognition receptor (PPR) proteins. PPR proteins can identify pathogen associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) via the N-terminals of the receptors while their carboxyl-terminals incite the intracellular responses (Kawai et al., 2005). TLRs bind to their specific PAMPs, leading to stimulation of the NF-κB signaling pathway and activation of the MAPK pathway (Fuchs et al., 2013). Human macrophages express about 10 TLRs, while murine macrophages express about 12 TLRs. TLR3, as well as TLR7 through TLR9, bind to PAMPs that reside in the endosomes of the phagocytic macrophages, while the remaining TLRs reside on the cell surface. TLR2, TLR3, and TLR9 recognize the viral particles in the early stages of the phagocytic process. TLR2 binds to viral structures and mediates inflammatory cytokine production, as well as the induction of IFN-γ and recruitment of Th1 (Doyle et al., 2006). TLR9 can recognize viral DNA by identifying unmethylated CG regions of HSV-1 genome. HSV-1 infection stimulates downstream TLR9-MyD88-dependent and TLR9-MyD88 independent signaling pathways (Kawai et al., 2005). In HSV-1 infection, TLRs interact with specific PAMPs to activate NF-κB-MAP kinase pathway, and subsequently induce the proinflammatory cytokines and co-stimulatory molecules. In HSV-1 infections, macrophages express high levels of nitric oxide in trigeminal ganglia through TLR2 and TLR9 activation and contribute to controlling HSV-1 (Takaoka and Yanai, 2006).

Interferons (IFNs) are produced by immune cells in response to HSV-1 infection, and govern the activation of the NK, pDCs, and macrophages. IFNs induce co-stimulators on the surface of immune cells such as MHCI and MHCII, which activates B7 receptors on naïve T cells (Colonna et al., 2002). This promotes the induction of high levels of pro-inflammatory cytokines and reactive oxygen and nitrogen species, helping in the destruction of viral particles (Minami et al., 2005). In mammals, there are about 10 IFNs, of which 7 are found in humans. They are grouped into 3 types: type I (IFN-I), type II (IFN-II), and type-III (IFN-III) (Kawanokuchi et al., 2006). In humans, IFN-I consists of IFN-α and IFN-β, both of which
bind to IFN-α receptors. IFN-II consists of IFN-γ, which can be recognized by IFN-γ receptor. IFN-I and IFN-II are produced by almost all immune cells, while IFN-γ is induced mainly by NK, T helper, and CD8 cells (Kawanokuchi et al., 2006). Type I IFN plays an essential role in HSV-1 infection, while type II IFNs are vital during HSV-2 infection (Gill et al., 2006).

M2 polarizations

The alternatively activated M2 macrophages are polarized and activated by IL-4 or IL-13 or IL10 cytokines, which are induced by Th2 cells in response to tissue damage (Gabhann et al., 2014). M2 macrophages participate in tissue remodeling, phagocytize apoptotic cells at the site of inflammation, and secrete mediators that promote angiogenesis such as epidermal growth factor (EGF), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) (Gabhann et al., 2014). M2 macrophages also induce high levels of IL10. These mediators participate in apoptotic cells’ clearance in addition to the activation of fibroblasts and epidermal cells at the site of inflammation. Anti-inflammatory mediators from these cells act in association with other extracellular matrix proteins to orchestrate tissue repair, promote wound healing, and bring about hemostasis (Espenosa- Jimenez et al., 2010; Forssell et al., 2007).

M2 macrophages are further subdivided into M2a, M2b, and M2c (Martinez and Gordon, 2014). M2a is polarized by IL-4 or IL-13, while M2b is activated by ligands binding to IL-1 receptors or to TLRs. M2c, on the other hand, is activated by IL-10 and glucocorticoid hormones (Martinez and Gordon, 2014).

Regulation of macrophages is a complex and essential process that involves numerous interconnected intracellular signaling pathways. These pathways regulate many target proteins crucial to the inflammatory process (Poon et al., 2014). They are responsible for bringing about measured and efficient immune responses in acute phases of infections, preventing the recurrence of chronic diseases, and promoting wound healing and tissue remodeling during the resolution phase of infections.

Roles of SOCS1 and SOCS3 in macrophages regulation

The suppressors of cytokine signaling (SOCS) are eight regulatory proteins, SOCS1 to SOCS7 and CIS (cytokine-inducible Src homology 2 protein) (Lu et al., 2006). SOCS proteins consist of a central SH2 domain, a C-terminal domain of variable length, and an N-terminal SOCS box domain (Murray et al., 2011). They also possess a kinase inhibitory region (KIR) at their N-terminal domain. SOCS1 and SOCS3 are structurally related proteins that are induced endogenously in macrophages. These proteins have been the subject of many investigations for their roles in the down-regulation of JAK-STAT pathways (Bigley
SOCS1 controls M1 polarization by direct inhibition of the IFN-Y JAK2/STAT1 intracellular signaling pathway (Baccala et al., 2005) or through indirect inhibition of the TLR-4 -IRF pathway (Baetz et al., 2004). SOCS1 is involved in M2 activation in vitro as well as in vivo (Lang et al., 2003). SOCS3, on the other hand, contributes to M1 polarization through direct inhibition of cytokine receptors (Babon et al., 2012). Cytokine receptors downregulate the IL-6-STAT3 pathway (Yasukawa et al., 2003). SOCS1 and SOCS3 regulate JAK-STAT pathway by proteasome degradation through ubiquitination of signaling pathway proteins (Yoshimura et al., 2007). SOCS1 and SOCS3 also regulate MAPK and NFkB mediators of macrophages (Androulidaki et al., 2009).

During the acute phase of inflammation, activated macrophages induce SOCS1 and SOCS3 (Puhr et al. 2010). SOCS1 impedes macrophage responses to IFNγ/LPS and acts as a direct regulator to enhance PI3K, driving M2 polarization (Frobose et al., 2006). SOCS1 plays a crucial role as an endogenous down regulator of the STAT1 signaling pathway of proinflammatory cytokines (Nakagawa et al., 2002). It also regulates MHC class II and NO; both mediators are essential in the inflammatory process (Ryo et al., 2003). SOCS1 deficiencies in murine macrophages have been associated with sustained disseminated infections and severe systemic autoimmune diseases (Paetz et al., 2004). SOCS3, on the other hand, reduces the IL-6 activation of STAT3 that regulates the anti-inflammatory pathway (Gingras et al., 2004). SOCS3 inhibits IL-4-activated PI3K, which stimulates gene expression of anti-inflammatory cytokines. SOCS3 inhibits the IL-6/STAT3 signaling pathway through binding with gp130 at Tyr759 residue and acts as a noncompetitive inhibitor for tyrosine kinase (Cheng et al., 2014). A recent study of experimental nephritis shows that M1 polarized macrophages within the inflamed glomeruli express high levels of SOCS3 (Lyman, 2009). In other studies, SOCS3-deficient mice models express enhanced STAT3 levels in M1, while Th1 and Th17 immune response dominated in neuroinflammation of model mice (Ruggiero et al., 2009) figure (2).
Figure 2; Role of SOCS1 and SOCS3 in macrophage regulation via JAKs/STATs intracellular signaling pathways and their impact on macrophage polarization and function.
The role of Rho GTPase proteins in F-actin Polymerization of macrophages

In many cells, including macrophages, the dynamic F-actin protein plays an important role in cell functions; this include cell adhesion, division, and motility (Etienne S. and Hall A., 2002). F-actin also plays an essential part in cell structure integrity, intracellular trafficking, and cytoskeleton organization. (Van den Broeke and Favoreel., 2009). The actin filaments are assembled into interconnected structural networks such as short contractile stress fibers in which F-actin arranges into bundles with plus and minus ends. These ends are alternatively held together with α-actinin and myosin II proteins that enable the stress fibers to contract. This allows the cell to change morphology and maintain its attachment to other structures (Soon-Tuck Sit and Manser, 2011). Filopodia are other essential assemblies of F-actin networks which are organized in rigid parallel bundles, and bound firmly to each other. They form spikes-like constructions that project from the plasma membrane (Pollard, 2007). Filopodia contain many surface receptors, integrins, and cell adhesion molecules that act as sensors for extracellular stimuli (Ridley, 2001). Lamellipodia are different forms of branched F-actin networks in which the branching increases toward the plasma membrane in dendritic like structures. Lamellipodia participate in membrane ruffles as moving edges for migratory cells (Urban et al., 2010).

Rho GTPases play a dynamic role in F-actin structure reorganization. They also are crucial in the phagocytic property of macrophages. Macrophages receive various extracellular stimuli such as cytokines, growth factors, and hormones; these ligands bind to and stimulate cell membrane receptors (Ridley, 2006). The interaction between these stimuli and their respective receptors rouse local Rho GTPase proteins regulators. These stimuli act specifically on local (GEFs) and (GAPs) that upregulate and downregulate Rho GTPase proteins respectively. When stimulated, Rho GTPase proteins act on downstream signaling pathways that target many effector proteins. Many of these target proteins are essential for F-actin polymerization and facilitate the phagocytic process of immune cells (Zhao and Manser, 2005), see Figures (3A and 3B).

Biochemical role of Rho GTPase proteins in F-actin polymerization in macrophages

F-actin related protein 2/3 (Arp2/3) is one of the downstream target proteins of Rho GTPase activation. Arp2/3 promotes formation of the network of dendritic-like structures of F-actin filaments at the tips of lamellipodia in polarized macrophages (Pollard, 2007). It is believed that RhoA activation initiates the protrusion of lamellipodia while Cdc42 and Rac1 activation stabilizes the newly formed extension
Myosin II protein is another target protein that interacts with actin at the lamella, causing actin-myosin contraction that moves the cells toward the chemotactic stimuli and facilitates endocytosis of invading particles into the cytoplasm of macrophages (Alexandrova et al., 2008; Choi et al., 2008). HSV-1 uses Rho GTPases during its entry and trafficking in host cells (Akhtar et al., 2009). After endocytosis reaches its end, F-actin filaments mature to become stress fibers that are associated with Rho A protein activation and constant myosin II activity. Formins are another category of target proteins for Rho GTPase proteins which initiate new F-actin polymerization (Nicholson-Dykstra et al., 2005; Rafelski and Theriot, 2004). Diaphanous proteins (Dia1, 2 and 3) are well known mammalian formins (Chesarone et al., 2010). Rho proteins can activate Dia1, while Rac1 and Cdc42 activate Dia 2 and Dia 3 (Lammers et al., 2008). In mammalian cells, Dia2 is enhanced at the tip of lamellipodia and filopodia (Yang et al., 2007). Wiskott-Aldrich Syndrome Protein (WASP) and Wiskott-Aldrich Verproline (WAVE) are two vital proteins in mammalian cells. These two proteins are activated by Cdc42 and Rac1 and participate in extensive F actin polymerization; these proteins also promote endocytosis through binding with Cdc42 Interacting Proteins 4 (CIP4) (Feng et al., 1999). Macrophages and hemopoietic cells in general demonstrate cytoskeletal abnormalities in WAS patients (Thrasher and Bruns, 2010). Cofilin is another target protein that is influenced by activated Rho GTPase proteins. Cofilin binds the barbed ends, capping F actin filaments and preventing further polymerization and extension. Cofilin dissociates Arp2/3 from F-actin dendrites (Murakoshi et al., 2011). Activation of RhoA and Rac1 by different macrophage stimuli target a cascade of downstream signaling protein kinases such as LIM-domain containing Kinase (LIMK) and testis- specific kinases (TESKs). Those kinases phosphorylate specific serine residue, inactivating cofilin, and promoting F-actin elongation (Nishita et al., 2005; Huang et al., 2009). PI3K has similar effects on cofilin. Rho-associated kinases (ROKs) and PAKs activate LIMK via phosphorylation of Thr(508) of LIMK. Rac1 can activate PAK1, Pak2, and target cofilin in lamellipodia (Soon-Tuck Sit and Ed Manser 2006). Rho A and Cdc42 activate Ca²⁺/calmodulin dependent myosin light chain kinase (MLCK) as well as others kinases that target myosin II (MLC II) that enrich the F- actin on lamellar boundaries of most cells (Petermann et al., 2009). The interaction of actin and MLCII provide non-muscular cells with a contractile element that is important for macrophage phagocytic properties as they move the engulfed particles from the cell surface into the cytoplasm and between different components of cells during digestion (Petermann et al., 2009). The actin myosin interaction is used by HSV-1 to transport viral particles between compartments of host cells. The actin-myosin II interaction provides migratory cells with the proper retraction needed for movement (Pertez et al., 2006). Rho
GTPase proteins have different roles in many other activities such as in mitotic division and cell cycle among others.

Phagocytosis is initiated when external stimuli activate cell surface receptors and mediates activation of Rho GTPase proteins to active forms. It later mediates the rearrangement of cortical actin. Two receptors are involved in the phagocytic process, Fc receptor (FcR) and complement-mediated phagocytosis receptor (CR) (Chimini et al., 2000; Mitra et al., 2005). Mast cells deficient in Cdc42 display FcR-mediated phagocytosis but do not express pseudopodia. Knockout Rac1 results in particles wrapped with pseudopodia that do not fuse with the mast cell membrane (Lyman, 2009). Complement-mediated phagocytosis receptor (CR) activates RhoA which in turn recruits Arp 2/3 to specific foci of F-actin at the particle attachment site. Arp 2/3 participates in phagocytosis, but not in filopodia formation. Since F-actin is crucial in many cell activities, including endocytosis and short distance transport between intracellular compartments, it is no surprise that HSV-1 uses all the previous activities of F-actin structures of the host cells during the infection (Van den Broeke and Favoreel, 2009) figure (3).

**Impact of Rho GTPase proteins on the biochemical activity of HSV-1 infected host cells**

HSV-1 uses the active form of Rho GTPases to manipulate the host cells in all the three major phases of the viral infection including HSV-1 entry, replication, and spread of the virus to neighboring cells (Pettermann et al., 2009). HSV-1 entry starts with the binding of viral glycoproteins specifically (gD) and the nectin-1 surface receptors of the host cells. The interaction stimulates local Rho GTPase proteins (Rho A, Rac 1, and Cdc42) leading to a drastic change in cortical F-actin rearrangement (Akhtar et al. 2008). HSV-1 infection of keratinocytes increases Rac1 and CDC42 expression in the infected keratinocytes (Pettermann et al., 2011). Sensory neuronal cells of trigeminal ganglia express high levels of Rho GTPase proteins in HSV-1 infections, indicating their roles in lytic and latent viral phases (Celine et al 2011; Hall A., 1998). During viral entry, HSV-1 gD binds to nectin-1 associated Cdc42 protein and participates in filopodia formation (Clement et al., 2006). During assembly of the stress fibers, alternating polar end (+/-) F-actin fibers are held with α-actinin and myosin II proteins and form the contractile bundles of F-actin that enable the host cell to contract in a retrograde fashion toward the cell body of the host. This facilitates endocytosis and trafficking of viral particles between cell compartments, as well as translocation to the nucleus. There, Rho A is a crucial mediator that targets a cascade of intracellular kinases downstream (Cheshenko et al., 2005; Naranett et al., 2003). Activation of a cascade of kinases can also lead to acetylation of host microtubules boosting viral trafficking. This process has been associated with Rho A activation (Frampton et al., 2007). The highly branched F-actin fibers also form the lamellipodia that help
cell migration and plasma membrane ruffling. The active form of Rac1 is associated with the lamellipodia action (Kari et al 2011). The lytic phase of the herpes infection is associated with Rho GTPase protein activation which leads to rearrangement of the F-actin structural networks. Rho GTPase protein activates viral US3 kinase, which encourages F-actin rearrangements that forms long filopodia and stress fibers [Finnen et al., 2010]. The F-actin structural networks are induced through the activation of Cdc42, Rac1, and Rho that target downstream (PAKs). These network structures have been associated with an increase in HSV dispersion (Van den Broecke et al, 2009) Figure 3.
Figure 3 A: Role of Rho GTPases in F-actin structural networks in macrophages
Cdc42   Rho MRCK/ROCK/MLCK/LIMK/PAKs
GTP/GTP  Lamellipodia
GDP/GDP  Filopodia
GAPs
Arp2/3  Stress fibers
MLC-II  Cortical actin
WASP-complexes  Endosome
Dias 1, 2, and 3  Membrane
Cofilin  ruffle
Focal adhesions

HSV-1 and RhoGTPases

Entry
FAK->PI3K->Rho GTPases ----
>stress fibers, filopodia, viral
surfing, endocytosis.

Lytic phase
US3→ PAK1----> cell projection
- viral spreading
US3→  PAK2----->stress fibers
disassembly-viral spread

Figure 3 B: The biochemical roles of Rho and Cdc42 proteins in organizing the actin cytoskeleton in macrophages and host cells
Methods and Materials

Raw 264.7

RAW 264.7 cell line is an Abelson murine leukemia virus-induced tumor derived from an adult male BALB/C mouse. It was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. The cell line was incubated at the optimum conditions of 37 °C in 5% CO2 in a humidified incubator.

Virus

HSV-1 (syn17+) (originally supplied by Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH) is replicated in Vero cells (CCL-81, ATCC). HSV type 1 Virus stocks were grown in Vero cells with multiplicity of an infection of three for 2-3 days. Infected cells are left to grow to achieve the maximal cytopathic effect where infected cells reaches a confluency of 90-100% and the cells are rounded up. Following centrifugation, the supernatant is harvested, aliquoted and stored at -80°C. The virus is titered and the virus titer is then determined by the standard plaque assay (Milne., et al 2005). RAW 264.7 cells were infected with 0.1 multiplicity of infection (MOI).

Polarization of macrophages

Three sets of test group were examined in which RAW 264.7 murine macrophage cells were grown for 2-3 days to reach an observable confluency of 70 %. In the first and second groups set, cells were treated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) to polarize the cells to M1. Tested cells of the second group were also challenged with 0.1 MOI of HSV-1. The third group of cells was polarized to the M2 phenotype using 20 ng/ml of IL-4. Murine cytokines IFN-γ and IL-4 were purchased from Peprotech, Rocky Hill, NJ and LPS was purchased from Chondrex, Redmond, WA. All three groups were challenged with SOCS1 mimetic (35μM/ml) or SOCS3 mimetic (35μM/ml), and untreated cells (M0) served as the control. Cell cultures in all tested groups were incubated in 37°C and 5% CO2 in a humified incubator. Cultures were polarized for 2, 4, 6, 24, or 48 hours. Cells were then collected from the culture dishes to analyze cell viability. The immunofluorescence intensities of Rho GTPases (Cdc42 and Rho proteins), cytokines (TNF-α and IL-10), and F-actin were evaluated using an immunofluorescence microscope.
**Cell Viability Trypan blue exclusion test (Hemocytometer method)**

Trypan Blue staining was performed during cell culture to monitor continued viability of cells between passages. Raw264.7 cells were grown to 70% confluency (by observation under the microscope). Untreated cells were used as experimental controls. At 2, 4, 6, 24, and 48 hours, cells were detached and collected using a cell scraper from the 6-wells cluster plates and centrifuged at 15,000 revolutions per minute (rpm) at 4°C for 5 minutes. Cells were then suspended in 1 mL of 10% FBS DMEM culture medium. A hemocytometer was cleaned thoroughly with 70% ethanol and a 20mm glass coverslip was positioned over the counting area on the hemocytometer. The cells were stained with trypan blue (Fisher Scientific, Pittsburgh, PA) at a dilution ration of 1:2. 10μl of the cell suspension was diluted into trypan blue twice and 10μL of the cell-stain mixture was pipetted into the loading channel on the hemocytometer. Live cells and dead cells were counted separately counted from the four corner squares of the sixteen-grids on the hemocytometer. The live cells were identified by their transparent appearance due to their lack of staining, while dead cells took up the stain and appeared blue. The following equation was used to calculate the percentage of cell viability:

**Percentage of Cell Viability** = [Live cells count / Total cells count (Live + Dead)] X 100.

In accordance to the following equation: % Cell Viability = [Total Viable Cells (Unstained) / Total Cells (Viable + Dead)] X 100

**Immunofluorescent Staining**

Cells were grown on sterilized 12-wells chambers on removable microscope glass slides (Ibidi, Madison, WI) to approximately 50%-70% confluency, at which point, cells were treated with IFN-γ and LPS at a concentration of 20ng/ml and 100ng/ml respectively (M1) with or without HSV-1. At the same time, another set cells was treated with IL-4 with a concentration of 20ng/ml (M2) and all polarized cells (M1 and M2) were challenged with either SOCS1(35μM/ml) or SOCS3(35μM/ml). All testing groups were incubated for 2, 4, 6, 24, and 48 hours at which time, the cells were rinsed using a phosphate buffered saline (PBS) (pH: 7.4) for three times to remove non-adherent cells. Cells were then fixed using 4% paraformaldehyde solution in PBS for 15-20 minutes at room temperature. The fixative was aspirated and the cells were rinsed with PBS three times; cells were permeabilized using 0.25% Triton X-100 in PBS for 5-10 minutes at room temperature and were rinsed using PBS for three times. After permeabilization, cells were blocked with non-specific blocker (5% Goat serum, 3% BSA, and 0.05 % Tween solution in PBS)
and kept at room temperature for 1-2 hours. Following blocking, the primary antibodies against Cdc42 and Rho proteins (BioVision, Milpitas, CA) (Thermofisher Scientific, Rockford, IL), TNF-α and IL10 (Life Technologies, Fredrick, MD) were diluted in PBS and added as specified by the manufacturer (Table 1). The 12-wells slide chambers were then incubated overnight at 4°C (6-12 hours). The following day, the primary antibodies were aspirated and cells were rinsed with 1% BSA in PBS three times for 5 minutes per rinse. The secondary antibody (rabbit IgG Fab’2 Goat anti-Rabbit Polyclonal) antibody for Cdc42 and Rho proteins purchased from (Lifespan BioSciences, Seattle, WA) was added. [To detect F-actin, Texas Red-Phalloidin X (Lifespan BioSciences, Seattle, WA) was added to all fixed, permeabilized cells at a dilution as recommended by the manufacturer (Table-1) for 2 hours in the dark at room temperature]. The secondary antibody was aspirated and slides were washed with PBS. A drop of hard-set mounting medium, Vectashield (H-1400) from Vector Laboratories was used to mount the stained cells onto the microscope slide. An Olympus Epi-fluorescence microscope with a ‘spot imaging’ digital camera was used to make observations.

Table 1

<table>
<thead>
<tr>
<th>Antibody/Stain</th>
<th>Dilution</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdc42 synthetic peptide polyclonal rabbit IgG</td>
<td>1:50</td>
<td>BioVision, Milpitas, CA</td>
</tr>
<tr>
<td>Rho Lyophilized synthetic peptide polyclonal rabbit IgG</td>
<td>1:50</td>
<td>BioVision, Milpitas, CA</td>
</tr>
<tr>
<td>Secondary antibodies (rabbit IgG Fab’2 Goat anti-Rabbit Polyclonal Antibody) (Atto 425) (cyan)</td>
<td>1:500</td>
<td>Lifespan BioSciences, Seattle, WA</td>
</tr>
<tr>
<td>Texas Red-Phalloidin X (stain) To detect F-actin</td>
<td>1:40</td>
<td>Lifespan BioSciences, Seattle, WA</td>
</tr>
<tr>
<td>IL10 Rat Anti-mouse Monoclonal fluorescence conjugate (green).</td>
<td>1:50</td>
<td>Life Technologies, Fredrick, MD</td>
</tr>
<tr>
<td>TNF-α Anti-mouse monoclonal TNF-α with pacific (blue) fluorescence conjugate</td>
<td>1:50</td>
<td>Life Technologies, Fredrick, MD</td>
</tr>
</tbody>
</table>
Image analysis and quantitative procedures

Visualization of the stained chamber slides was performed on an Olympus inverted fluorescence microscope in the imaging facility core located at Wright State University. The optimal wavelengths found for these assays were TRITC (tetramethylRhodamine, red) 500 nms, FITC (Fluorescein isothiocyanate, green) 1500 nms, (cyan) 2000 nms, and the (Pacific blue conjugate) 2500 nms. The cyan fluorescent intensity represents secondary antibody that binds to and stains Cdc42and Rho proteins and demonstrates their localization, while the red (TRITC) fluorescent intensity represents the level of F-actin staining. The green fluorescent intensity (FITC) represents IL10 and the Pacific blue fluorescent conjugate to detect TNF-α cytokine. Optical and software settings were identical when capturing images. The captured immunofluorescent images were analyzed using ImageJ software (National Institutes of Health, http://imagej.nih.gov/ij/) to detect the relative cytoskeletal changes at 2, 4, 6, 24, and 48 hours. This software aided us to isolate the qualified region of interest (qROI) of the fluorescent, exclude uneven background and artifacts, and quantify number of cells. The aim of the image processing steps was to isolate the fluorescence that best represents the presence of the protein of interest. Processing is based on the concept of lookup tables (LUT). The target metrics are total ROI area (square. pixels) and cell count.

The image processing and analysis steps:

First, the uneven background after converting the single-color image had to be converted to eight-bit image. Background subtraction is essential when quantifying image intensities; subsequent operations on artifacts pixels can be easily done without background interference. Next, before measuring the fluorescence intensity of an image, the pixel values (bins 0-255) of the exclusion regions must be identified. The two exclusion regions are the very black areas like background and the very bright (saturated) artifacts spots. Repeated measurements identified that pixel values from 0-19 represent black and values from 112-255 are present only in the (very bright) artifacts. In each image, the final range of pixel values representing the ROI were bins 20-111. The total area in the image that contained qualifying pixel intensity was limited after isolating the threshold (ROI). Within the ImageJ “threshold feature”, the pixel values are fixed to the 20-111 range. At this point, the image is composed of area that will or will not be counted in analysis. After that, “image analysis” step is used to measure the total area restricted within these ROI “islands” and other properties related to the size and distribution. The “measure particles” function within the ImageJ software calculates the area of individual ROI and provides an image total. Finally, the averages of the areas (Total area / Number of counted cells) of each image that represent immunofluorescence intensities within the ROI was compared using One-way ANOVA.
Statistical Analysis

Data were collected from at least three independent trials on separate days/cell passages to measure cell viability, and the immunofluorescence intensity of Rho proteins, Cdc42 protein, IL10, and TNF-α with contrast to the immunofluorescence of Texas Red-Phalloidin X (to detect F-actin). Cells were fixed and stained at 2, 4, 6, 24, and 48 hour time intervals of cells treatments. The statistical significance of experimental control groups and challenge groups were analyzed. One-way ANOVA was used to analyze the differences between the experimental testing groups using Sigma Plot 13.5 Software. Statistical significance was measured in accordance to P-values < 0.001 (less than or equal to 0.001) and the data were depicted as mean +/-standard deviation (SD) of the mean.
Results

Three sets of test groups were examined in which RAW 264.7 cells were grown to reach an approximate confluency of 70-80%. In the first and second group sets, cells were treated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) to polarize the cells to M1. The tested cells of the second group were also challenged with 0.1 MOI of HSV-1. The third set of cells was polarized to the M2 phenotype using 20 ng/ml of IL-4. All three groups were challenged with SOCS1 (35μM/ml) or SOCS3 (35μM/ml) peptide mimetics, and were compared to untreated cells (M0), which served as the control. Cell cultures in all tested groups were incubated at 37°C and 5% CO₂ in a humified incubator. Cultures were polarized for 2, 4, 6, 24, or 48 hours and all cells were fixed and stained with immunofluorescent antibodies for Cdc42 (Cyan), Rho (Cyan), and the Texas red phalloidin staining to detect F-actin. Another set of M1 cells was fixed and stained with TNF-alpha (blue) and M2 cells with IL10 (green) immunofluorescent antibodies. Cells were observed using an Olympus inverted fluorescent microscope and three images were taken per field. During each experiment, different filters were used to take images: between 500nms to 2500nms. Cell viabilities were also determined for each time frame. All comparisons were evaluated for significance by one-way ANOVA using Sigma Plot 13.5 Software. Statistical significance was measured in accordance to P-values ≤ 0.001 (less than or equal to 0.001) and the data were depicted as mean ± standard deviation (±SD) of the mean.

SOCS1 down regulates M1 and SOCS3 upregulates M1 in the early (2 and 4) hours following an infection.

At 2 and 4 hours of cell treatment with SOCS1 expression of Cdc42 and Rho proteins decreased significantly in polarized M1 cells in the presence or absence of HSV-1 infection. Conversely, SOCS3 significantly increased the expression of Cdc42 and Rho proteins. Figures 4, 5, 6, and 7 show that at 2 and 4 hours of cell treatment in the presence or absence of HSV-1 infection, polarized M1 and SOCS3 challenged M1 macrophages expressed significant increases in the fluorescence intensities of Rho proteins (Figure 4A, Figure 5A, 6A, 6C and Figure 7A and C) and Cdc42 protein (Figure 4B, Figure 5B, 6B, 6D and Figure 7B and D) when compared to un-polarized M0 (P ≤ 0.001). The immunofluorescence images showed that Rho and Cdc42 were distributed mainly in the central zone (nucleus area) and para central zone (around the nucleus) of polarized macrophages. These figures also demonstrate that SOCS1
challenged M1 macrophages showed significant reductions (P ≤ 0.001) in the levels of expression of Cdc42 and Rho proteins (P ≤ 0.001).
Levels of expression of Rho and Cdc42 in macrophages at 2 hours

Figure (4): After 2 hours of cell treatment with SOCS1 the expression of Cdc42 and Rho proteins decreases in polarized M1 cells in the presence or absence of HSV-1 infection, while SOCS3 increases the expression of Cdc42 and Rho proteins. Cyan fluorescence images show un-polarized and polarized Raw 264.7 murine macrophages challenged with SOCS1 or SOCS3 fixed after 2 hours and stained with an antibody for Rho proteins (A) or Cdc42 proteins (B). Cells are polarized with LPS and IFN-γ, cells in the right column of (A) and (B) are also challenged with 0.1 MOI of HSV-1. The insets demonstrate digital enlargement to detect changes in the protein expression. (Images captured at 60X oil magnification, scale bar =20μm and 50μm for the insets).
Levels of expression of Rho and Cdc42 in macrophages at 4 hours

Figure (5): After 4 Hours of cell treatments with SOCS1, the expression of Cdc42 and Rho proteins decreases in polarized M1 cells in the presence or absence of HSV-1 infection, while SOCS3 increased the expression of Cdc42 and Rho proteins. Cyan fluorescence images show unpolarized and polarized murine macrophages challenged with SOCS1 or SOCS3 fixed after 4 hours and stained with an antibody for Rho proteins (A) or Cdc42 proteins (B). Cells are polarized with (LPS) and (IFN-γ), cells in the right column of (A) and (B) are also challenged with 0.1 MOI of HSV-1. The insets demonstrate digital enlargement to detect change in the protein expression. (Images captured at 60X oil magnification, scale bar =20μm and for the insets= 50μm).
Figure (6): levels of expression of Rho and Cdc42 proteins in M1 at 2 hours of cell treatments. After 2 Hours of cell treatment, SOCS1 significantly decreases the expression of Cdc42 and Rho proteins in polarized M1 cells in the presence or absence of HSV-1 infection. SOCS3 significantly increases the expression of Cdc42 and Rho proteins. Rho (A) and Cdc42 (B) levels of expression of polarized M1 cells after 2 hours of cell treatments are shown. Figures C and D show HSV-1 challenged M1 levels of expression in Cdc42 and Rho proteins respectively. A One-way ANOVA with a Tukey’s post hoc t-test was applied. Black stars (*) indicate the significance level: p< 0.01 (**) and p <0.001 (**). Values are means +/- SD; n = 3.
Figure (7): Rho and Cdc42 levels of expression in unpolarized and polarized M1 at 4 hours. After 4 hours from cells’ treatments, SOCS1 significantly decreased the expression of Cdc42 and Rho proteins in polarized M1 cells in the presence or absence of HSV-1 infection. SOCS3 significantly increases the expression of Cdc42 and Rho proteins. Rho (A) and Cdc42 (B) levels of expression of unpolarized and polarized M1 cells after 4 hours of cell treatments are shown. Figures C and D show levels of expression in Cdc42 and Rho proteins respectively in HSV-1 challenged unpolarized and polarized M1 Raw macrophages. A One-way ANOVA with a Tukey’s post hoc t-test was applied. Black stars (*) indicate the significance level: p< 0.01 ** and p <0.001*** . Values are means +/- SD; n = 3.
Percentage of viable cells, morphological changes, and TNF-α in M1 at 2 and 4 hours of cell treatments

At 2 and 4 hour of cell treatments (figures 9-14), M1 and SOCS3 challenged M1 show morphological changes in the form of irregular protrusions from the cell surface and the appearance of pseudopodia-like structures in the presence or absence of HSV-1. These morphological changes are associated with significant increases (P ≤ 0.001) in Cdc42 and Rho proteins and significant reduction (P ≤ 0.001) of phalloidin staining F-actin (figure 14). The cells show drastic changes in morphology; the cell stretches with the formation of filopodia-like structures that protrude from the cell surface of many cells in the images (figure 10-13). In contrast, SOCS1-treated M1 cells show significant increases (P ≤ 0.001) in intensities of phalloidin stain for F-actin and the cells are rounded in their morphological appearance. SOCS1-treated M1 macrophages demonstrated a sharp decrease in percentage of viable cells when compared to M1 (figure 9).

After 2 or 4 hour of cell treatments, M1 and SOCS3-challenged M1 macrophages showed a significant increase (P ≤ 0.001) in TNF-α production in the presence or absence of HSV-1. Alternatively, SOCS1-challenged M1 cells expressed a significant reduction (P ≤ 0.001) in TNF-α production (figures 15-18).
Figure (9); **Percentage of viable cell in unpolarized or polarized M1 at 2 and 4 hours of cell treatments** Percentage of viable cells in polarized and unpolarized M1 at 2 hours (A) and 4 hours (B) of cell treatment. SOCS1 challenge of uninfected and infected M1 cells show a significant decrease in the percentage of viable cells in comparison of SOCS3 challenge and unchallenge M1 cells. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; ** p < 0.01 and *** p < 0.001. Values are means +/- SD; n=3.
Cdc42 protein expression and F-actin staining of macrophages at (2 hours)

(A) F-actin  (B) Cdc42  (C) Merge

Figure (10): After 2 Hours of cell treatments, SOCS1 decreases the expression of Cdc42 proteins in polarized M1 cells, while SOCS3 increases the expression of Cdc42 proteins. Red fluorescence (TRITC) images stained phalloidin (for filamentous actin arrangement) of polarized (M1) and un-polarized 264.7 murine macrophages. (A) Cells demonstrate the morphological changes and cytoskeleton rearrangements of F-actin at 2 hours of cell treatment. (B) Cyan fluorescence to detect (Cdc42) protein. Images show un-polarized and polarized murine macrophages challenged with SOCS1 or SOCS3, cells were fixed at two hours of cell treatment and stained with an antibody for Cdc42 proteins. Merged images (C) of (A) and (B) are shown. The insets on the upper left corner of (C) are digital enlargement to detect the morphological changes. (Images captured at 60X oil magnification, scale bar = 20μm for full images and 50μm for insets).
Cdc42 protein expression and F-actin staining in HSV-1 infected macrophages at 2 hours

(A) F-actin  (B) Cdc42  (C) F-actin/Cdc42

Figure (11): After 2 Hours of cell treatments, SOCS1 decreases the expression of Cdc42 proteins in polarized M1 cells, while SOCS3 increases the expression of Cdc42 proteins. Red fluorescence (TRITC) (A) images stained phalloidin (for filamentous actin arrangement images) of HSV-1 infected unpolarized and polarized 264.7 murine macrophages (M1). Cells demonstrate the morphological changes and cytoskeleton rearrangements at 2 hours of cell treatment. Cyan fluorescence (B) images show unpolarized and polarized murine macrophages challenged with SOCS1 or SOCS3, cells were fixed after two hours of treatments and stained with an antibody for Cdc42 proteins. Merged images (C) of (A) and (B) are shown. The insets on the upper left corner of (C) are digital enlargements to detect the morphological changes. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for the insets).
Cdc42 Protein and F-actin staining in macrophages at 4 hours

(A) F-actin  (B) Cdc42  (C) F-actin/Cdc42

Figure (12): After 4 Hours of cell treatments, SOCS1 decreases the expression of Cdc42 proteins in polarized M1 cells, while SOCS3 increases the expression of Cdc42 proteins. Red fluorescence (TRITC) (A) images stained phalloidin (for filamentous actin arrangement images of polarized and un-polarized 264.7 murine macrophages (M1). Cells demonstrate the morphological changes and cytoskeleton rearrangements at 2 hours of cell treatment. Cyan fluorescence images (B) show unpolarized and polarized murine macrophages challenged with SOCS1 or SOCS3, cells were fixed after 4 hours of treatments and stained with an antibody for Cdc42 proteins. Merged images (C) of (A) and (B) are shown. The insets images on the upper left corner of (C) are digital enlargements to detect the morphological changes. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for the insets).
Cdc42 Protein and F-actin staining in HSV-1 infected macrophages at 4 hours

Figure (13): After 4 hours of cell treatments, SOCS1 decreases the expression of Cdc42 proteins in HSV-1 infected M1 cells, while SOCS3 increases the expression of Cdc42 proteins. Red fluorescence (TRITC) (A) images stained phalloidin (to detect filamentous actin arrangement) in HSV-1 infected unpolarized (M0) and polarized 264.7 murine macrophages (M1). (A) immunofluorescence phalloidin staining, cells demonstrate the morphological changes and cytoskeleton rearrangements at 4 hours. Cyan fluorescence (Cdc42; B) images show un-polarized and polarized murine macrophages challenged with SOCS1 or SOCS3, cells were fixed after 4 hours of treatments and stained with an antibody for Cdc42 proteins. Merged images (C) of (A) and (B) are shown. The zoomed pictures on the upper left corner of (C) are digital enlargements to detect the morphological changes. (Images captured at 60X oil magnification, scale bar = 20μm for full images and 50μm for insets).
Figure (14): Phalloidin intensities detect (F-actin) in uninfected and in HSV-1 infected unpolarized and polarized M1 at 2 and 4 hours of cell treatment. Phalloidin immunofluorescent intensity for control and polarized macrophages (M1) following 2 and 4 hours of cell treatments in uninfected (A) and HSV-1 infected (B) M1 murine macrophages. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; ** p < 0.01, *** and p < 0.001. Values are means +/- SD; n=3.
Figure (15): TNF-α production in unpolarized and polarized M1 cells at 2 hours of cell treatment. (A) Immunofluorescent images of polarized and un-polarized Raw 264.7 macrophage cells show morphological changes and TNF-alpha cytokine immunostaining at 2 hours of cell treatment. Images show unpolarized and polarized macrophages that were fixed after 2 hours of cell treatment and stained with red fluorescence (TRITC) for phalloidin (to detect filamentous actin arrangement) in the first column, and a pacific blue fluorescence conjugate for (TNF- alpha) in the second column, and the last column show the overlay between TNF-alpha and phalloidin staining. (Images captured at 40X oil magnification, scale bar =20μm and 50μm for insets). (B) TNF-alpha immunofluorescent intensity for control and polarized macrophages following 2 hours of cell treatments. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; ** p <0.01 and *** p <0.001. Values are means +/- SD; n=3.
Figure (16): TNF-α production in HSV-1 infected unpolarized and polarized M1 cells at 2 hours of cell treatment. (A) Immunofluorescent images of polarized and un-polarized Raw 264.7 macrophage cells show morphological changes and TNF-alpha cytokine immunostaining at 2 hours of cell treatments with HSV1 infection. Images show unpolarized and polarized macrophages that were fixed after 2 hours of cell treatments and stained with an antibody for red fluorescence (TRITC, F-actin) images show phalloidin (for filamentous actin arrangement) in the first column, and pacific blue fluorescence conjugate for (TNF-α) in the second column, and the last Column show the overlay between TNF-α and phalloidin staining (Images captured at 40X oil magnification, scale bar =20μm and 50μm for insets). (B) TNF-alpha immunofluorescent intensity for control and polarized macrophages following 2 hours of cell treatment. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; ** p <0.01 and *** p <0.001. Values are means +/- SD; n=3.
TNF-a in unpolarized and polarized M1 cells at 4 hours of cell treatment

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(B) TNF-alpha levels in M1 at 4 hours

Figure (17): TNF-α production in unpolarized and polarized M1 cells at 4 hours of cell treatments. (A) Immunofluorescent images of polarized and un-polarized Raw 264.7 macrophage cells show morphological changes and TNF-alpha cytokine immunostaining at 4 hours of cell treatments. Images show unpolarized and polarized macrophages that were fixed and stained with an antibody for red fluorescence (TRITC) images show phalloidin (for filamentous actin arrangement) in the first column, and Pacific blue fluorescence conjugate for (TNF-α) in the second column, and the last Column show the overlay between TNF-alpha and phalloidin staining (images captured at 40X oil magnification, scale bar =20μm and 50μm for insets). (B) TNF-alpha immunofluorescent intensity for control and polarized macrophages following 4 hours of cells treatments. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; ** p <0.01 and *** p <0.001. Values are means +/- SD; n=3.
TNF-alpha in HSV-1 infected unpolarized and polarized M1 cells at 4 hours

(A) F-actin  TNF-alpha  F-actin/TNF-alpha

Figure (18): TNF-α production in HSV-1 infected unpolarized and polarized M1 cells at 4 hours of cell treatments

(A) Immunofluorescent images of polarized and un-polarized Raw 264.7 macrophage cells show morphological changes and TNF-alpha cytokine immunostaining at 4 hours of cell treatments with HSV1 infection. Images show unpolarized and polarized macrophages that were fixed and stained with red fluorescence (TRITC) phalloidin (to detect filamentous actin arrangement) in the first column, and pacific blue fluorescence conjugate for (TNF- alpha) in the second column, and the last Column show the overlay between TNF-alpha and phalloidin staining (Images captured at 40X oil magnification, scale bar =20μm for full images and 50μm for the insets). (B) TNF-alpha immunofluorescent intensity for control and polarized macrophages following 4 hours of cells treatments. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; *** p <0.001. Values are means +/- SD; n=3.
**SOCS1 and SOCS3 effects on M1 cells at 6 hours** (Figures 19-22)

At the 6-hour time interval of cell treatments, SOCS1 led to a significant decrease of the Cdc42 and Rho proteins in polarized M1 cells in the presence and or absence of HSV-1 infection. On the other hand, we noticed a significant decrease in Cdc42 and Rho proteins intensities in SOCS3 challenged M1 in presence or absence of HSV-1 in comparison to unchallenged cells (figures 19 and 20). The cells started to round and express high F-actin intensities with less TNF-α production as compared to M1 polarized macrophages (figures 21 and 22). M1 polarized macrophages were impacted by the endogenous induced SOCS1 and SOCS3 that regulate their TNF-α production and morphological appearances, as well as their Cdc42 and Rho proteins expression. [Figures 23 and 24 show the comparison between 2, 4, and 6 hour time intervals of the cell treatments in F-actin and Rho GTPases intensities].

![Figure 19: levels of expression of Rho and Cdc42 in unpolarized and polarized M1 macrophages at 6 hours of cell treatment.](image)

Figure (19): levels of expression of Rho and Cdc42 in unpolarized and polarized M1 macrophages at 6 hours of cell treatment. After 6 Hours of cell treatments, SOCS1 significantly decreases the expression of Rho and Cdc42 proteins in polarized M1 cells in the presence or absence of HSV-1 infection. SOCS3 significantly increases the immunofluorescence intensity of Rho proteins and Cdc42. However, M1+SOCS3 had a significantly lower expression than M1 alone. Figures (A) show the immunofluorescence intensities of Rho (B) show the immunofluorescence intensities of Cdc42 proteins of unpolarized and polarized M1 cells after 6 hours of cell treatments. HSV-1 challenged unpolarized and polarized M1 expression of Rho proteins and Cdc42 (right column) of (A) and (B). The insets are digital enlargement to detect the morphological changes. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for insets).
After 6 hours of cell treatments, SOCS1 significantly decreases the expression of Rho proteins and Cdc42 in polarized M1 cells in the presence or absence of HSV-1 infection. SOCS3 significantly increases the expression of Rho proteins and Cdc42. However, M1+SOCS3 had a significantly lower expression than M1 alone. Figures A and B show levels of expression of Rho and Cdc42 in polarized M1 cells and unpolarized macrophages after 6 hours of cell treatments. Figures C and D show the levels of expression of Rho and Cdc42 proteins in HSV-1 challenged macrophages. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level: p <0.001***. Values are means +/- SD; n = 3.
Cdc42 and f-actin staining in HSV-1 infected unpolarized and polarized M1 cells at (6 hours)

Figure (21): After 6 Hours of cell treatments, SOCS1 decreases the expression of Cdc42 proteins in polarized M1 cells, while SOCS3 increases the immunofluorescence intensity of Cdc42 proteins. Red fluorescence (TRITC) (A) images stained phalloidin (to detect filamentous actin arrangement) of unpolarized and polarized 264.7 murine macrophages (M1). Cells demonstrate the morphological changes and cytoskeleton rearrangements at 6 hours. Cyan fluorescence (B) images show unpolarized and polarized murine macrophages challenged with SOCS1 or SOCS3, cells were fixed at 6 hours of cell treatment and stained with an antibody for Cdc42 proteins. Merged images (C) of (A) and (B) are shown. The insets on the upper left corner of (C) are digital enlargements to illustrate the morphological changes. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for insets).
Figure (22): F-actin reorganization in M1 cells at 6 hours of cell treatment. (A) phalloidin immunofluorescent intensity (to detect F-actin) for control and polarized macrophages (M1) following 6 hours of cell treatment. (B) phalloidin immunofluorescent intensity for control and polarized macrophages following 6 hours of cell treatment after HSV-1 infection. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level: *p< 0.010 ** and *p <0.001 ***. Values are means +/- SD; n = 3.

Figure (23): Comparison in F-actin reorganization in M1 at 2, 4, and 6 hours. Phalloidin immunofluorescent intensity (to detect F-actin) for control and polarized macrophages at 2, 4, and 6 hours of cell treatments prior to and after HSV-1 infection. HSV-1 Infected cells were compared to the uninfected cells at mentioned time frame.
Rho proteins in M1 cells at 2, 4, and 6 Hours

Cdc42 protein in M1 cells at 2, 4, and 6 Hours

Figure (24): Comparison between 2, 4, and 6 hour treatments in the levels of expression of Rho and Cdc42 proteins. In the presence or absence of HSV-1 infection, SOCS1 has a sustained down regulation effect on polarized M1 expression of Rho and Cdc42 proteins while SOCS3 has a transient up-regulation of both proteins which peak at 2 hours and start to decline by 6 hours from cell treatments.
At 24 hours:

Following cell treatments, HSV-1 challenged and unchallenged M1 macrophages showed a dramatic decrease in the Rho proteins and Cdc42 proteins activities. Figure (25 A) and Figure (27) show HSV-1-challenged and unchallenged M1 macrophages after 24 hours of cell treatment express significant reductions in immunofluorescence intensities of Rho and Cdc42 proteins which was accompanied with decreased levels of TNF-α production by all challenged cells (Figure 26 B). By comparing SOCS1 challenged cells with SOCS3-challenged M1 cells, no significant differences in protein activity or TNF-α production were observed. In comparison to unchallenged M1 cells, significant decreases in TNF-α levels of production were seen in both SOCS1 and SOCS3 challenged cells (Figure 26 B). The F-actin staining intensities and the morphological appearance of all polarized surviving cells showed no significant difference (Figure 26 C). The cell viability percentage was higher in SOCS3 challenged M1 cells compared to SOCS1 challenged or unchallenged M1 cells (Figure 26 A).

At 48 hours:

Rho proteins were sequestered at the periphery of all surviving macrophages as shown in (Figures 25B and 28) and Cdc42 protein (Figures 25B and 29) was sequestered in the perinuclear area of polarized and unpolarized macrophages. All cells rounded up with no significant differences in F-actin staining intensities or in TNF-α production (Figures 26B and 26C).
Level of expression of Rho and Cdc42 proteins at 24 and 48 hours

(A) HSV-1 infected macrophages at 24 hours

(B) HSV-1 infected macrophages at 48 hours

Figure 25: At 24 hours and 48 hours of cell treatment, the expression of Cdc42 and Rho proteins in polarized M1 cells in the presence of HSV-1 infection decrease. Figure A shows the immunofluorescence intensities of Rho and Cdc42 in HSV1 infected M1 at 24 hours of cell treatments. Figures (B) shows HSV-1 challenged M1 expression of Rho and Cdc42 proteins at 48 hour of cell treatments. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level: p <0.1* and p< 0.010 **. Values are means +/- SD; n = 3.
Figure 26: The percentage of viable cells, TNF-α, and F-actin staining intensities of HSV-1 infected M1 cells at 24 and 48 hours (A) shows the percentage of viable cells in HSV-1 challenged unpolarized or polarized M1 macrophages at 24 hours and 48 hours of cells treatments. (B) shows TNF-α production of polarized and unpolarized HSV-1 infected M1 at 24 and 48 hours of cell treatments. (C) shows immunofluorescence intensities of phalloidin (for F-actin) of challenged cells at 24 and 48 hours of cells treatments. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level: \( p < 0.1 \), \( p < 0.010 \) ** and \( p < 0.001 \) ***. Values are means \( +/- SD; n = 3 \).
Rho proteins 24 hours after HSV-1 infection of macrophages

Figure (27): After 24 Hours of cell treatments, the immunofluorescence intensities of Rho proteins in polarized M1 cells and in unpolarized M0 show significant reduction in the presence of HSV-1 infection. (A) images stained Texas red phalloidin (for filamentous actin) and demonstrate the morphological changes and cytoskeleton rearrangements after 24 hours after cell treatments. (B) images stained Cyan fluorescence illustrate (Rho proteins) in unpolarized and polarized murine macrophages, cells at the 3rd and 4th row were challenged with SOCS1 or SOCS3 respectively, cells were fixed after 24 hours of treatments and stained with an antibody for Rho proteins. Merged images (C) of (A) and (B) are shown. The insets on the upper left corner of (C) are digital enlargements to detect the morphological changes. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for insets).
Level of expression of Rho proteins in macrophages at 48 hours

Figure (28): After 48 Hours of cell treatments, the expression of Rho proteins in polarized M1 cells and in unpolarized M0 can not be detected in the presence or absence of HSV-1 infection. (A) Red fluorescence (TRITC) stained phalloidin used to detect filamentous actin arrangement of polarized and unpolarized macrophages to demonstrate the morphological changes and cytoskeleton rearrangements at 48 hours. (B) Cyan fluorescence (Rho proteins). (C) Merged images of (A) and (B). Cells were fixed after 48 hours of treatments and stained with antibody for Rho proteins and TRITC for F-actin. The insets in (C) are digital enlargements to detect the morphological changes. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for insets).
Cdc42 protein in M1 macrophages after 48 hours of cell treatment

Figure (29): After 48 Hours of cell treatments, the expression of Cdc42 proteins in polarized M1 cells and in unpolarized M0 cannot be detected in the presence or absence of HSV-1 infection. (A) Red fluorescence (TRITC) stained phalloidin for filamentous actin demonstrates the morphological changes and cytoskeleton rearrangements. (B) Cyan fluorescence images show Cdc42 of unpolarized and polarized murine macrophages. Cells were fixed after 48 hours of cell treatment and stained with an antibody for Cdc42 protein. (C) Merged images of (A) and (B) are shown. The insets on the upper left corner of (C) are digital enlargements to detect the morphological changes. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for insets).
SOCS3 down regulates IL-4 polarized M2 and SOCS1 promote M2 polarization.

As noted in figures 30-36 at 2 and, to a lesser extent, at 4 hours of cell treatments and following M2 polarization, SOCS3 challenged M2 macrophages expressed significant increases (P ≤ 0.001) in expression of Rho and Cdc42 proteins (figures 30-33). A significant reduction (P ≤ 0.001) of F-actin expression (figure 34) and IL-10 cytokine secretion was noted in SOCS3 challenged M2 macrophages when compared to that of unchallenged M2 cell (figures 35 and 36). Morphologically, SOCS3 treated M2 cells showed projections (filopodia) similar to those of M1 cells at the 2hour observation time as shown (figure 30). Conversely, SOCS1 challenged M2 cells demonstrated no change in Cdc42 expression, Rho proteins expression, or F-actin staining intensity. They did express significant increases (P ≤ 0.001) in IL-10 when compared to unchallenged M2 macrophages (figures 34 and 35).

At 4 hours following treatment, SOCS3 challenged M2 macrophages showed a significant reduction in Cdc42 and Rho protein staining intensities (figure 36). This reduction in staining intensities of these proteins continued for 24 to 48 hours, although significant differences in levels of IL-10 expression were not evident between challenged and unchallenged M2 macrophages (figure37).
Level of expression of Cdc42 and F-actin staining in unpolarized and IL-4 polarized M2 at (2 hours)

![Immunofluorescent images of polarized(M2) and un-polarized Raw 264.7 macrophages cells show Cdc42 expression, morphological changes and cytoskeleton immunostaining at 2 hours after cell treatments. Images show unpolarized and polarized M2 macrophages that were fixed and stained with (A) red fluorescence (TRITC, F-actin) images show phalloidin (for filamentous actin arrangement) first column, cyan immunofluorescence (B) Cdc42 antibody in the second column, and (C) the last column show the overlay between Cdc42 and phalloidin staining. The insets are digital enlargements to detect morphological changes. SOCS3 treated M2 cells showed projections (filopodia-like structures) similar to those of M1 cells. (Images captured at 60X oil magnification, scale bar =20μm) for full images and 50μm for insets).
**Cdc42 and F-actin staining in unpolarized and IL4 polarized M2 macrophages at 4 hours**

![Immunofluorescent images of M2 polarized and un-polarized Raw 264.7 macrophages cells show Cdc42 expression, morphological changes and cytoskeleton immunostaining at 4 hours after cell treatments. Immunofluorescence images show unpolarized and polarized M2 macrophages that were fixed and stained with (A) red fluorescence (TRITC) phalloidin staining (to detect filamentous actin arrangement) first Column, and (B) cyan fluorescence (Cdc42) in the in the second Column, and (C) the last Column show the overlay between Cdc42 and phalloidin staining. The insets are digital enlargements to detect morphological changes. (Images captured at 60X oil magnification, scale bar =20µm for full images and 50µm for the insets). M0 control unpolarized, and M2 macrophages polarized by IL-4. Cells in the 3rd and 4th raw challenged with SOCS1 and SOCS3 respectively.**

Figure (31): Immunofluorescent images of M2 polarized and un-polarized Raw 264.7 macrophages cells show Cdc42 expression, morphological changes and cytoskeleton immunostaining at 4 hours after cell treatments. Immunofluorescence images show unpolarized and polarized M2 macrophages that were fixed and stained with (A) red fluorescence (TRITC) phalloidin staining (to detect filamentous actin arrangement) first Column, and (B) cyan fluorescence (Cdc42) in the in the second Column, and (C) the last Column show the overlay between Cdc42 and phalloidin staining. The insets are digital enlargements to detect morphological changes. (Images captured at 60X oil magnification, scale bar =20µm for full images and 50µm for the insets). M0 control unpolarized, and M2 macrophages polarized by IL-4. Cells in the 3rd and 4th raw challenged with SOCS1 and SOCS3 respectively.
Levels of expression of Rho proteins in unpolarized and IL-4 polarized M2 cells at 2, 4, and 6 hours

**Figure (32):** After 2, 4 and 6 Hours of cell treatment, SOCS1 decreases the expression of Rho proteins in IL-4 polarized M2 macrophages, while SOCS3 increases the expression of Rho proteins. Cyan fluorescence images show IL4 polarized Raw 264.7 murine macrophages challenged with SOCS1 or SOCS3 fixed after 2 hours (A), 4 hours (B), and 6 hours (C) from cell treatments and stained with an antibody for Rho proteins. The insets are digital enlargements to detect changes in Rho proteins expression. Cells in the 2nd and 3rd rows were challenged with SOCS1 and SOCS3 respectively. (Images captured at 60X oil magnification, scale bar =20μm for full images and 50μm for the insets).
Levels of expression of Rho and Cdc42 proteins in unpolarized and IL-4 polarized M2 cells at 2 and 4 hours

Figure 33: The immunofluorescence intensities of Rho and Cdc42 proteins in IL4 polarized M2 at 2 and 4 hours. (A) After 2 Hours of cell treatments, SOCS3 significantly increased the expression of Cdc42 and Rho proteins in IL4 polarized M2. (B) Rho and Cdc42 levels of expression of polarized M2 cells after 4 hours of cell treatments. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level: \( P<0.01 \) ** and \( P<0.001 \) ***. Values are means +/- SD and \( n = 3 \).
Figure (34): Phalloidin immunofluorescent staining intensity for (F-actin rearrangements) in control and IL4 polarized macrophages (M2) following 2, 4 and 6 hours of cell treatments. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; ** p < 0.01, *** and p < 0.001. Values are means +/- SD (n = 3).
Secretion of IL-10 in unpolarized and IL-4 polarized M2 at 2 Hours

Figure (35): Immunofluorescent images of polarized and un-polarized Raw 264.7 macrophages cells show morphological changes and IL10 cytokine immunostaining at 2 hours after cells treatments. (A) Images show unpolarized and IL4 polarized M2 macrophages that were fixed and stained with antibody for red fluorescence (TRITC, F-actin) images show phalloidin (for filamentous actin arrangement) in the first Column, and green fluorescence (FITC for IL10) in the second column, and the last Column show the overlay between IL10 and phalloidin staining (Images captured at 40X oil magnification, scale bar =20μm for full images and 50μm for insets). (B) upper graph shows the levels of Il10 in unpolarized and IL4 polarized M2, at 2 hours of cell treatments. Lower graph shows the percentage of viable cells in unpolarized and polarized M2 at 2 hours after cell treatment. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; *** p <0.001 and ** p <0.01. Values are means +/- SD (n = 3).
Secretion of IL-10 in unpolarized and IL-4 polarized M2 at 4 Hours

Figure (36): Immunofluorescent images of IL4 polarized M2 and un-polarized Raw 264.7 macrophages cells show morphological changes and IL10 cytokine immunostaining at 4 hours after cell treatment. (A) Images show unpolarized and IL4 polarized M2 macrophages that were fixed and stained with antibodies for IL-10 cytokines. Images show red fluorescence phalloidin staining (TRITC for filamentous actin arrangement) in the first Column, and green fluorescence (FITC for IL10) in the second column, and the last Column show the overlay between IL10 and phalloidin staining (Images captured at 40X oil magnification, scale bar =20μm for full image and 50μm for insets).

(B) Upper graph shows the levels of IL10 in unpolarized and IL 4 polarized M2 cells at 4 hours of cell treatment. The lower graph shows the percentage of viable cells in unpolarized and polarized M2 cells at 4 hours of cell treatment. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; ** p <0.01 and *** p <0.001. Values are means +/- SD (n=3).
Figure 37: IL-10 levels in IL-4 polarized M2 and unpolarized M0 at 24 and 48 hours (A) shows at 24 hour following cell treatments; there was a significant decrease in the production of IL10 from M2 of all macrophages but the cytokines released from M2 was higher than SOCS1 challenged M2 and from SOCS3 challenged M2. (B) 48 hours after cell treatments, the levels of IL10 production of all treated maintained at lower levels with no significant difference between polarized M2. A One-way ANOVA with Tukey’s post hoc test was applied. Black stars (*) indicate the significance level; * p <0.1 and *** p <0.001. Values are mean+/- SD and (n=3).
At 2 and 4 hours of cell treatment
unchallenged and SOCS3 challenged M1 macrophages express high levels of Rho and Cdc42 accompanied with F-actin cytoskeleton reorganization and morphological changes.

At 6-24 hours time interval
unchallenged and SOCS3 challenged M1 macrophages express low levels of Rho and Cdc42 accompanied with F-actin cytoskeleton reorganization and morphological changes.

At 48 hours
Rho and Cdc42 proteins sequestered in all cells in nuclear and para-nuclear zones with no apparent changes in cell morphology.

At 0 hour
Rho and Cdc42 are sequestered in M0

At 2 hours
SOCS3 challenged M2 macrophages express high levels of Rho and Cdc42 proteins. SOCS3 challenged M2 cells show morphological changes similar to that observed in M1 cells. SOCS1 challenged and unchallenged M2 cells express low levels of Rho and Cdc42 proteins.

At 4 hours
SOCS3 challenged M2 macrophages express high levels of Rho and Cdc42 proteins but lower than the levels of expression at 2 hours.

At 6 hours
SOCS3 challenged M2 macrophages express Rho and Cdc42 proteins at a level similar to that observed in unchallenged and SOCS1 challenged M2.
Discussion

HSV-1 infection elicits an innate immune response, in which neutrophils, pDCs, and NKs produce INFs. Interferons activate MHC I, MHC II, macrophages and T cells. Macrophages are dominant feature in pathological conditions in which classically activated M1 induce a high levels of pro-inflammatory cytokines, such as TNF-α, IL6, IL12, and IL23. M1 macrophages also produce NO and ROS, which act as cytotoxins destroying and eliminating the virally engulfed particles during phagocytosis and promoting apoptosis (Wang et al., 2010). Alternatively, activated M2 produces anti-inflammatory cytokines such as IL-10 and other mediators that regulate the immune response and promote the healing process (Wang et al., 2014).

The reorganization of filamentous actin is a prerequisite for polarized M1 macrophage functions. Cdc42 and Rho A proteins play a critical role in altering the reorganization of structural networks of actin microfilaments which leads to dynamic changes in macrophage’s cytoskeleton, cell migration, and phagocytic function. SOCS1 and SOCS3 are structurally related proteins that are induced endogenously in macrophages. They are involved in the down-regulation of the JAK-STAT pathways. SOCS1 inhibits LPS-induced IL-12, TNF-α, and NO production through direct inhibition of the STAT-JAK1 pathway (Cheng et al., 2014). SOCS1 inhibits the INF-Y JAK2/STAT1 pathway (Wong et al., 2006). The present study demonstrated that SOCS1 and SOCS peptide mimetics alter the endogenously induced SOCS molecules and alter Cdc42 and Rho A protein expression and F-actin polymerization in uninfected or HSV-1 infected M1 polarized macrophages. SOCS1 challenged M1 macrophages expressed significantly lower levels of Cdc42 and Rho proteins and higher level of F-actin in the presence or absence of HSV-1 comparing to that observed in unchallenged M1 cells. This study confirms the observation that unpolarized or polarized HSV-1-infected M1 inflammatory macrophages exhibited significant reduction in cell viability compared to unpolarized M0 control cell and M2 polarized macrophages (Reichard and Bigley, 2015) and (Al Sharif, Almutairi, and Subahi, 2016). SOCS1 challenged M1 macrophages, especially in the presence of HSV-1 infection, showed a decrease in the percentage of viable cells and TNF-α production. Our finding suggests cells challenged with the M1 polarizing substances at the same time as SOCS1 peptide mimetic exhibited impaired M1 phagocytic functions and led to apoptosis of many cell. This study also indicated that SOCS1 challenged M2 cells did not change expression levels of Cdc42 and Rho proteins and F-actin staining intensities from that of unchallenged M2 macrophages. These cells expressed a significant increase in IL-10 as compared to unchallenged M2 at 2 and 4 hours. During HSV-
1 infection, macrophages induce SOCS1 to down regulate the inflammatory process and decrease the level of destruction associated with high numbers of M1 macrophages at the site of inflammation. Lu., et al (2006) demonstrated that SOCS1 knockdown macrophages exhibited increased levels of IL-6, IL-12, NO, and MHCII. Collectively, these results suggest that during the resolution phase of an infection, SOCS1 peptide contributes to M2 polarization and increased production of anti-inflammatory mediators such as IL-10 that promote wound healing and hemostasis.

SOCS3 on the other hand upregulates M1 polarization through direct inhibition of cytokine receptors for IL-6-STAT pathway, which then upregulates M2 polarization (Gingras et al., 2004). SOCS3 impends IL-4-activated PI3K, which stimulates gene expression of anti-inflammatory cytokines (Cheng et al., 2014; Lui et al., 2013). A recent study of experimental nephritis shows that M1 polarized macrophages within the inflamed glomeruli express high levels of SOCS3. SOCS regulates JAK-STAT pathway by proteasome degradation through ubiquitination of signaling proteins (Liu et al., 2008). SOCS also regulates MAPK and NFkB mediators of macrophages (Androulidaki et al., 2009). These observations clearly show the dynamic interactions of signaling pathways in which SOCS1 and SOCS3 molecules are intimately involved in the regulation of the functions of one another.

Our study demonstrated that SOCS3 exerts a transient effect on M1 and M2 in the early hours of infection. SOCS3 potentiated the effect of M1 polarization, promoted the activation of Rho GTPase proteins, and increased proinflammatory TNF-α cytokine secretion. On the other hand, SOCS3 challenged M2 cells expressed a significant increase in levels of RhoA and Cdc42 proteins. They also showed a significant decrease in F-actin staining intensities and IL-10 secretion compared to unchallenged M2 macrophages. This finding is of particular interest because it counters what was shown in previous studies of IL-4 polarized M2 macrophages which demonstrated that M2 polarized cells were unresponsive to subsequent M1 stimuli (Erwig et al., 2000). The present study clearly shows that M2 polarized cells challenged with SOCS3 peptide mimetic can mimic M1 polarized macrophages as they produce decreasing amounts of IL-10 and expressed morphology similar to that of M1 cells. This observation could help make M1 cells more efficient, favoring the inflammatory process to eliminate invading pathogens. The delicate balance between the effects of SOCS1 and SOCS3 has also been observed by Lyman (2009) who found that M1 polarized macrophages within inflamed glomeruli expressed high levels of SOCS3. The observations of the present study also match previous studies (Liu et al., 2008) in which it was shown that SOCS3 deficient macrophages demonstrated a decrease in pro-inflammatory properties.
The present data suggest that during an active infection, SOCS1 regulates M1-macrophages indirectly by constraining the activation of Cdc42 and Rho GTPase proteins, hinders their intracellular signaling pathways and impacts F-actin organization which can interfere with the phagocytic properties of macrophages. During the resolution phase of infection, SOCS1 upregulates M2 polarization and potentiates the M2 secretion of IL-10.

At 24 hours, macrophages are only influenced expression of GTPases by endogenously induced SOCS1 and SOCS3. This suggest that infectious stimulants may be destroyed by this time, resulting in a shift in the expression of Cdc42 and Rho proteins as well as TNF-α in all challenged or unchallenged M1, and in the F-actin reorganization and the morphological appearance of surviving cells. It is imperative to note that TNF-α was measured by immunostaining in situ at 24 and 48 hours. Others note the increase in TNF-α that has accumulated and not been degraded by 24 hours in cell culture. At 48 hours following treatment, Cdc42 appeared inactive and sequestered in the center of the cells. Rho proteins showed no activity and were sequestered in the periphery of polarized or unpolarized macrophages.

The present data suggests that M1 macrophages in-vivo induces SOCS1 as a negative regulator to reduce the destructive power (over shooting) of M1 and impede the secretion of toxic molecules. SOCS1 may also constrain the activation of Rho GTPase proteins in M1 polarized macrophages. While SOCS1 potentiates the anti-inflammatory role of IL-4 polarized M2 cells in the resolution phase of infection, SOCS3 on the other hand has a transient effect on M1 and M2 in the early hours of infection. SOCS3 potentiates the effect of M1, promotes the activation of Rho GTPase proteins, and increases proinflammatory cytokine secretion. SOCS3 downregulates M2, helping to make M1 more efficient and favoring the inflammatory process to eliminate invading pathogens. During the resolution phase of infection, SOCS3 appears to display an endogenous regulatory role in macrophages through the activation of the JAK-STAT3-IL-10 pathway.

Limitation of study

In our experiment, we use direct immune fluoresce IF staining techniques where we use a single conjugate antibody directed against F- actin, TNF-alpha, or IL-10. We also used indirect IF techniques in which we used an unconjugated primary antibody directed against Rho or Cdc42 proteins and a secondary fluorophore conjugated antibody directed against the primary antibody to evaluate the levels of expression of Rho and Cdc42 proteins in macrophages. Using direct and indirect IF techniques allows for visualization of target proteins distribution inside macrophages using a fluorescent microscope. IF cell staining is relatively standard and is the optimal staining for specific target proteins that depends on
protein biology, location inside the cell, and its stability. Direct and indirect methods are not limited to IF techniques. We can use them in flow cytometry, western blot, and immunohistochemistry techniques. One limitation in our experiment for investigating GTPase proteins (Rho and Cdc42) as well as cytokines (TNF-α and IL-10) was the limited number of cells that we were able to investigate using IF staining techniques. One can see the advantage of using other techniques such as flow cytometry technique to confirm our results as well as to increase the sample size of tested cells (macrophages). This could eliminate or decrease the percentage of bias that develops from using small sample sizes associated with IF technique even though it will not beneficial to locate the target proteins inside the cells. The second limitation of study that we faced came from cytokines analysis which are commonly secreted proteins. We were unable to measure the secreted portion of the cytokines that was removed during IF staining technique, while we were able only to measure the intracellular cytokines. Although our investigation excluded any cytokines secretion from dead cells before they died, there was a percentage of the secreted cytokines from live cells that our experiments was unable to include while measuring the IF intensities of intracellular cytokines of tested macrophages. Future study can trap those cytokines inside the macrophages using protein transport inhibitors such as BD GolgiStop™ (containing monensin) or BD GolgiPlug™ (containing brefeldin A). This could allow us to measure most of secretory proteins (Cytokines) as they became trapped inside the macrophages and stained with IF staining technique to be included in our results.

**FUTURE STUDIES**

- In the present study, we have taken some steps towards showing some functional differences between SOCS1 and SOCS3 peptide mimetics on M1 and M2 macrophages. We focused on the effects of SOCS1 and SOC3 in Rho and Cdc42 proteins as they are highly expressed in Raw 264.7 murine cell lines. We highlighted the effects of SOCS1 and SOCS3 in changing the cytoskeleton rearrangements and on the morphology of polarized macrophages via altering the activity levels of Rho GTPase proteins. There are several vital questions that remain unanswered. This section is dedicated to unfolding needed future experiments to resolve these questions.

- Rho GTPase proteins (RhoA, Rac1, and Cdc42) cycle between active forms (GTP bound) and inactive forms (GDP bound) via GEFs and GAPs that upregulate and downregulate small GTPases respectively. Since Rho GTPase proteins play crucial roles in macrophages’ phagocytic properties
and are used by the virus during its replication in host cells, it will be interesting to examine the impact of GAPs, such as Rab 5 and Rab7 proteins, which act as negative regulators of Rho GTPases. These GAPs can be closely examined to see their influence on the infection of HSV-1 in host cell lines. Also, these GAPs can be examined to evaluate their roles in regulating Rho GTPase proteins on macrophages’ cytoskeleton rearrangements and functions.

- Future studies of guanine nucleotide exchange factors (GEFs) and their roles in activating Rho GTPase proteins in macrophages and host cells could investigate SOC1 and SOC3 effects on macrophages or in host challenged cells, further disclosing the roles of these regulators. There are newly discovered members of this family of proteins which can be used as activators for Rho GTPase proteins.
- Because of their high expression in the Raw264.7 cell line, we focused on Cdc42 and Rho proteins roles in SOCS1 and SOCS3 challenged macrophages. Future studies can investigate the SOCS1 and SOCS3 effects on the role of Rac1 in polarized macrophage using other cell lines such as J177.4A1 and in primary macrophage isolated from mice and humans.
- Future studies can investigate knockdown, or blocking, of one of the crucial proteins that are targets for Rho GTPase proteins, like cofilin or Arp2/3 or one of the formins (Dia1, Dia2, Dia3) and how that may block viral entry and their spread to host.
- Knowing that the binding of HSV-1 gD to nectin1 surface receptors of host cells of HSV-1 activates Rho GTPases and downstream signaling proteins such as Us3 kinase and PAKs, which promotes viral entry and dispersion (Spear M. and Wu Y., 2014). Future studies where these proteins are knocked down may portray a way to contain HSV infections.
- M1 macrophages are major participants of many pathogenic diseases such as atherosclerosis and arthritis, among others (Wilson, 2010; Gui et al., 2012). In future studies, we can investigate the effect of SOCS1 induction as a negative regulator to M1 and a positive regulator to M2 in animal models, such as in those susceptible to atherosclerotic plaques. We can examine the induction of SOCS1 peptide in controlling such formation of plaques in tested animals.
- Recently in clinical studies, researchers have been able to show a strong correlation between the high expression of Rho GTPases in macrophages and a good prognosis in the early stages of breast cancer (Stout et al., 2009). Future studies of GEFs induction to animal models can further elaborate the role of these regulators on potentiating the effects of M1 GTPases in controlling the spread of tumors. In addition, a study of SOCS3 induction can potentiate the roles of M1 to control tumor cells and prevent their spread in the early stages.
References


