The Impact of HSV-1 Infection, SOCS1 Peptide, and SOCS3 Peptide Mimetic on Cell Viability, Morphology, and Cytoskeleton Proteins of Unpolarized and Cytokine-Polarized M1 RAW 264.7 Murine Macrophages

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The Impact of HSV-1 Infection, SOCS1 peptide, and SOCS3 peptide mimetic on Cell Viability, Morphology, and Cytoskeleton Proteins of Unpolarized and Cytokine-Polarized M1 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 Mubarak Huraysan Almutairi ENTITLED The Impact of HSV-1 Infection, SOCS1 peptide, and SOCS3 peptide mimetic on Cell Viability, Morphology, and Cytoskeleton Proteins of Unpolarized and Cytokine-Polarized M1 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

Mubarak Huraysan Almutairi. M.S. Department of Microbiology and Immunology, Wright State University, 2016. The Impact of HSV-1 Infection, SOCS1 peptide, and SOCS3 peptide mimetic on Cell Viability, Morphology, and Cytoskeleton Proteins of Unpolarized and Cytokine-Polarized M1 RAW 264.7 Murine Macrophages

The immune response against HSV-1 involves macrophages in both innate and adaptive immunity by limiting HSV-1 replication. In this study, the effects of HSV-1 challenge on cell viability, morphology, and cytoskeletal filament in unpolarized and cytokine-polarized murine RAW 264.7 macrophages at 24 and 48 hours were monitored. Because the distribution of cytoskeleton throughout the cell is critical in cell viability and function, effects of HSV-1 challenge on the organization of F-actin and microtubule (tubulin) in unpolarized and cytokine-polarized murine RAW 264.7 macrophages were monitored at 24 and 48 hours post infection. F-actin and tubulin organization were assessed by quantifying the fluorescent intensity of immunofluorescent images using ImageJ analysis. M1 polarized cells displayed a significant decrease (p, 0.001) in cell viability when compared to control cells. At 24 and 48 hours post polarization, M1 cells showed flattened, irregular shapes with intracellular vacuoles, whereas unpolarized control cells (M0) appeared rounded. Following HSV-1 infection, both M0 and M1 macrophages exhibited a rounded shape. HSV-1 infection enhanced the organization of F-actin expression by unpolarized M0 and polarized M1 cells at 24 hours post infection; decreases in F-actin expression for all cells were observed at 48 hours post infection. The effects of treatments with peptide mimetics of suppressor of cytokine signaling (SOCS) proteins, namely SOCS1 and SOCS3, on cell viability and the organization of F-actin and tubulin of uninfected and HSV1-infected unpolarized and cytokine-polarized murine RAW 264.7 macrophages were evaluated at 24 and 48 hours. Treatment with a SOCS3 peptide mimetic increased cell viability of polarized M1 and HSV-1-infected M1 RAW 264.7 cells, whereas treatments with a SOCS1 peptide mimetic decreased
viability of these cells. These observations suggest that SOCS3 peptide mimetic functions as an anti-inflammatory (anti-apoptotic) molecule by preventing cell death. Treatment of HSV-1-infected polarized M1 cells with either SOCS1 or SOCS3 peptide mimetic increased tubulin expression (p<0.001), suggesting that mechanism increased microtubule expression, such as increasing microtubule stability as a consequence of RhoA GTPases activation by SOCS1 and SOCS3 proteins.
HYPOTHESIS

SOCS3 peptide mimetic can cause uninfected and HSV-1 infected, polarized M1 macrophages (RAW 264.7) to convert to the M2 anti-inflammatory state. This conversion should be accompanied by increased levels of IL-10 and decreased levels of TNF-α in supernatant fluids of the SOCS3 treated cells at 24 after M1 polarization. SOCS1 and SOCS3 peptides will cause notable differences in the cytoskeletal F-actin and tubulin proteins of HSV-1 infected polarized and unpolarized cells at 24 and 48 hours.
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LIST OF ABBREVIATIONS

ATCC = American Type Culture Collection
BSA = Bovine Serum Albumin
DMEM = Dulbecco’s Modified Eagle’s Medium
F-actin = Filamentous actin
gD = Glycoprotein D
HSV = Herpes Simplex
HVEM = Herpes Virus Entry Mediator
ICP0 = Infected Cell Protein Null
IFN-γ = Interferon gamma
IL = Interleukin
iNOS = Inducible Nitric Oxide Synthase
JAK = Janus Kinase
KIR = Kinase Inhibitory Region
LPS = Lipopolysaccharide
MHC = Major Histocompatibility complex
MOI = Multiplicity of Infection
PBS = Phosphate buffered saline
qROI = Qualified region of interest
SOCS = Suppressor of Cytokine Signaling
TNF-α = Tumor necrosis factor-alpha
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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is one of the most common contagious infections, and a major cause of illnesses and death in the world (Arduino & Porter, 2016). HSV-1 is an extremely widespread human virus, and is a member of herpesviruses family. HSV-1 mainly infects epithelial cells becoming latent in ganglia of sensory neurons (Bello-Morales et al., 2016). If HSV-1 is reactivated and infection is uncontrolled, HSV-1 may cause oral infections, blindness, and encephalitis. Furthermore, HSV-1 has become one of the major cause of genital infections (Ramakrishna et al., 2016). Recent studies suggest that the immune system regulate reactivation of HSV-1 at the latency site in sensory neurons, and/or control transmission of virus from nerve cells to the other body tissues. Therefore, improving the mechanisms that preserve the virus in a latent state in a nerve cell might be advantageous in controlling HSV-1 pathogenesis (Liu et al., 2016).

Macrophages are phagocytic cells that differentiate from circulating peripheral blood monocytes, and are present in most tissues. They have a significant function in the immune system, especially during bacterial and viral infectious diseases. Macrophages have different mechanisms to protect host immune system against pathogens, for instance, phagocytosis and releasing certain cytokines and chemokines. Depending upon the immediate environment, macrophages can activate different phenotypes responsible for a variety of functions. The main two activated phenotypes are the pro-inflammatory M1 macrophage, and the anti-inflammatory M2 macrophage. The classical M1-macrophages can be activated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ), and have antibacterial functions by producing high levels of pro-inflammatory effector molecules, like interleukins (IL), including IL-1β, IL-6, IL-12, and IL-23, and tumor necrosis factor. On the other hand, the alternatively activated, anti-inflammatory M2-macrophages are
divided into three main subgroups, and play an important function in tissue repair and wound healing by producing a variety of anti-inflammatory cytokines like IL-10 (Wang et al., 2015). Many recent studies show that during HSV-1 corneal infection, the virus invades sensory neurons and transmits to the trigeminal ganglion (TG) starting a latent infection in these cells. Many recent studies show that macrophages and other leukocytes infiltrate the TG during the period of active HSV-1 replication and release certain cytokines, particularly TNF-α, IFN-γ, the inducible nitric oxide synthase (iNOS) enzyme, and IL-12 (Kodukula et al, 1999).

The cytoskeleton of eukaryotic cells is made of filamentous proteins, and its functions include establishing cell shape and providing mechanical strength. Three major filaments form the cytoskeleton, each part has different structure and protein components. Microtubules represent the largest filaments of the cytoskeleton, and mainly made of a highly dynamic structure proteins called tubulin. Microtubules initially form and organized from microtubule organizing centers (MTOCs), and they play an essential role in the cellular growth and movement, intracellular transport of organelles, and the chromosomal separation in mitosis. Actin filaments are intertwined strands filaments composed of g-actin proteins. The actin filaments have characteristics of remodeling and turnover, which are important in many cellular processes, such as motility, differentiation, division and membrane organization (Fife et al., 2014). Microtubules with certain motor proteins, like Kinesin and dynein play an important role in Axoplasmic transport mechanism that used by HSV-1 to move from cytoplasm into the host cell’s nucleus. Therefore, study the morphological and structural changes in the cytoskeleton of the HSV-1 infected cell is a significance method to identify the viral mechanisms of movement along the host cytoskeleton.

Suppressor of cytokine signaling (SOCS) proteins are a family of eight proteins (SOCS1–7 and cytokine-induced protein (CIS)), and they help to control the release of cytokines in polarized
macrophages. They negatively regulate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway (Frey et al., 2009). SOCS1 functions as a negative feedback regulator to reduce the signaling of certain types of cytokines, particularly IFN-γ (Hashimoto et al., 2011). Conversely, SOCS3 can be activated by various cytokines, particularly IL-6/IL10, and takes an important part in a negative feedback loop to prevent excessive activation of the signaling pathway (Li et al., 2016).
Macrophages polarization

Macrophages are "professional" phagocytic cells that are produced by differentiation of monocytes in the tissues and they show high functional diversity with respect to their environments. Macrophages have a range of surface receptors recognizing signals that can change their function and create multiple effector populations making them an important components of the host immune system (Murray & Wynn, 2011). This capability to change function is referred to as macrophage polarization.

Researchers have characterized macrophage subtypes as proinflammatory (M1) or anti-inflammatory (M2). The development of one phenotype depends upon environmental stimuli. M1 macrophages are activated by IFNγ and LPS, and they secrete proinflammatory cytokines, like iNOS, and TNF-α (Kigerl et al., 2009) (Figure 1). The classically activated macrophages (M1) are also defined by a large capacity to present antigen, interleukin-12 (IL-12) and IL-23 production, produce reactive oxygen intermediates, and a polarized type 1 response. The M1 macrophages promote host defense against some bacteria, viruses, and protozoa, and they also have a mediating role in antitumor activity. On the other hand, M2 macrophages are induced by interleukin-4 (IL-4) or interleukin-13 (IL-13) (Reichard et al., 2015). M2 macrophages are anti-inflammatory cells that release molecules, like interleukin-10 (IL-10), and they promote tissue remodeling and angiogenesis (Kigerl et al., 2009).
Figure 1: Macrophage activation and polarization. M1 classical macrophages activated by IFNγ and LPS, and produce TNFα, iNOS, IL-6, and ROS. M1 promote polarized Th1 response and host defense. M2 are activated by various stimuli, like IL-4 or IL-13, and produce IL-10 for the anti-inflammatory response, and wound healing regulation (Reproduced from Bohlson et al., 2015).

Cytoskeleton (Microtubules and Microfilaments)

Microtubules are cellular components responsible for determining the cell's structure, and they are made of hollow rigid, polar cylinders consist of α-tubulin and β-tubulin heterodimers as fundamental units (outer diameter of 25 nm). These tubulin heterodimers link together to form thirteen protofilaments that form the structure of a single microtubule (Howard & Hyman, 2007).
Alpha and beta tubulins create microtubules through polymerization. Polymerization cannot occur without the formation of the γ-tubulin ring complexes (γ-TuRCs), or the framework provided by the gamma form of the tubulin proteins, found in the centrosome or microtubule organization center (MTOC) (Chaffey et al., 2003).

Microtubules filament play an important role in long-distance transport of cargo to and from the cell center. They also serve as a transport mechanism for vesicles including the essential materials to the rest of the cell. Microtubules' rigid core allows microtubule-associated motor proteins (MAPs) such as Kinesin and Dynein to create force and movement within cilia and flagella (Vale et al., 1992). Besides being responsible for determining a cell's structure, microtubules also form the spindle apparatus which is used during mitosis, dividing chromosomes directly (Geoffrey, 2000). In addition, many microtubules organize as a network among the cell in different distribution depending on the cell type. The organization of microtubules by microtubule organizing centers (MTOCs) and their association with certain motor and severing proteins (kinesin, dynein) are important for the dynamic instability mechanism of microtubules as well as microtubule polarity (Döhner et al., 2005).

There are two major motor proteins that associate with microtubules and mediate the direct transport of cargo within the cells: The highly processive and relatively powerful motor proteins include kinesins (Plus-end-directed molecules) move cargo to the cell periphery (Miki et al., 2005), and dyneins (Minus-end transport molecules), which transport cargo from the periphery to the cell center (Pfister et al., 2006).

Microfilaments are composed of monomer globular actin subunits (G-actin) are linked together to form two flexible intertwined strands of filamentous actin (F-actin) with outer diameter of 5 to 9 nm. They organize into few dimensional networks inside the cell to connect the interior
of the cell with its surroundings, and also assisting cell motility and cell division (Falahzadeh et al., 2015). In muscle cells, actin filaments are associated with myosin proteins to generate force leading to muscle contraction (Van et al., 2000).

Actin filaments have a fundamental function in short range movements of cargo within the cell, particularly in parts that display low density of microtubules (Lyman & Enquist, 2009). Myosin V (MyoV) and myosin VI (MyoVI) are the major myosin motor proteins which are responsible for controlling the directed transport of cargo and protein complexes along actin filaments (To and away from the cell surface in case of MyoV, and to the ends of actin filaments in case of MyoVI) (Krendel & Mooseker, 2005).

Suppressor of Cytokine Signaling Proteins (SOCS)

SOCS are a group of inducible proteins that control the JAK STAT signaling pathway which is important for mediating the activity of cytokines during macrophage and dendritic cell regulation. They also have an important role in T cell development and differentiation (Frey et al., 2009). SOCS consists of eight types, SOCS-1 to SOCS-7 and the cytokine-induced SH2 protein. SH2 domain and a C-terminal SOCS box represent the main parts of SOCS structure. SOCS-1 and SOCS-3 have a KIR of 12 amino acids work with SH2 to regulate the activity of JAK STAT pathway. Therefore, SOCS-1 and SOCS-3 molecules are able to control cytokine function by inhibiting JAK activity through proteasomal degradation (Croker et al., 2008) (Figure 2).

SOCS-3 mainley induced LPS stimulation in macrophages, SOCS-3 has a crucial role in regulation of two important cytokines: the pro-inflammatory cytokine IL-6, which has a central role in regulating inflammatory diseases, and the anti-inflammatory cytokine IL-10 following TLR stimulation. (Yoshimura, et al., 2007). Recent research has suggested that SOCS-3 inhibits the
activation of TNF-receptor-associated factor 6 and the growth factor-β (TGFβ)-activated kinase 1 (TAK1), that are important for TLR- and IL-1-induced responses (Frobøse et al., 2006).

**Figure 2:**

A) Structure of SOCS proteins consist of three main parts: SH2 domain, amino terminal region, and a C-terminal SOCS box. B) JAK–STAT signaling pathway diagram. It activated by specific cytokines and transfer the phosphorylated group via stat molecules to nucleus for the gene transcription. SOCS proteins can negative regulate the JAK–STAT signaling pathway through different mechanisms (Reproduced from Shuai, & Liu 2003).
**SOCS proteins and cytoskeleton**

Research up to the present time has emphasized the direct and indirect roles of SOCS proteins in immune system and infectious diseases. Some studies confirm the interaction between certain types of SOCS proteins and the cytoskeleton proteins. Zou et al. (2008) found that the microtubule-associated protein 1S (MAP1S) which is a member of the microtubule-associated protein 1 (MAP1) family and has a crucial role in microtubule stabilization, is highly expressed in nervous tissues and has a significant relation to SOCS3 function in macrophage. Overexpressed MAP1S can increase the translocation of SOCS3 from the nucleus to cytoplasm. After examination of a MAP1S deficient macrophage, they observed that the negative regulation of STAT3 pathway by SOCS3 was delayed during the IL-6 signaling suggesting that MAP1S functions as an adaptor protein for the interaction between SOCS3 protein and microtubule cytoskeleton.

**Herpes simplex virus (HSV-1)**

HSV-1 is a double-stranded DNA virus belonging to Herpesviridae family (Ryan and Ray, 2004). HSV-1 mainly infects the host oral or genital mucous membrane and penetrating the nerve cells starting the latent stage (Cunningham et al., 2006). Latent infection lasts for the lifespan of the host. If the immune system is compromised, the HSV-1 may be reactivated, which leads to a lytic infection. These infections typically show with a cutaneous disease (Reichard et al., 2015). Less frequently, more severe HSV-1 cases can result in an infection of the corneal epithelium, leading to blindness (Jones, 2003).

HSV-1 is a large double stranded, linear DNA genome enclosed inside an icosahedral protein capsid, which is enveloped in a bilayer of lipid connected to the capsid by means of a tegument forming the complete structure of HSV-1 virion. HSV-1 generally contains a minimum
of 74 genes (or open reading frames, ORFs) within its genome, encoding a variety of proteins responsible for regulating the virus replication during infection and developing the virus particle components mainly, the capsid, the tegument and the envelope of the virus (Sigamani et al., 2013).

Forming an opening pore enables HSV-1 to enter the host cell and move to the nucleus. This process depends on a variety of glycoproteins located on the surface of the virus envelope. These glycoproteins bind to specific receptors on the cell membrane, fusing the virus envelope with host cell membrane and forming the opining pore. The virus glycoproteins gD, gB, gC, gH, and gL found on the surface of the virus envelope play the main role of the viral entry into the host cell by interacting with the receptors on host cell (Rajcani et al., 2000). In the case of a herpes virus, the primary binding occur when the viral envelope glycoproteins, glycoprotein C (gC) and glycoprotein B (gB) bind to a host cell surface particle called heparan sulfate. Glycoprotein D (gD) provides a stable attachment to one of three specific entry receptors including nectin-1, herpesvirus entry mediator (HVEM), and 3-O sulfated heparan sulfate. When gD binds to its specific receptor, they form the viral hemi-fusion state with the membrane. Fusion events are further mediated by glycoprotein B (gB) and the gH–gL complex, which eventually result in creation of the entry pore and releasing viral capsid into the cytoplasm (Akhtar & Shukla, 2009) (Figure 3). Next, by using the retrograde and anterograde mechanism of microtubules, the viral capsid is transported to the cell nucleus and releases its DNA through the nuclear entry pores, starting the viral genes transcription process (Wisner et al., 2011).
Figure 3: A) Human herpes virus structure. B) Entry of HSV-1 within the host cell occur in several events involving various glycoproteins (Reproduced from Suenaga & Arase, 2015).

Furthermore, HSV-1 secretes infected cell protein 47 (ICP-47) to prevent the activation of the major antiviral immune cells, cytotoxic T-lymphocytes (CTLs). The inhibition involves blocking the transporter associated with antigen processing (TAP), which is an essential component to create the viral epitope presentation with MHC class I. HSVs may continue in an inactive but persistent form known as latent infection, particularly in neural ganglia (Goldsmith et al., 1998). However, the virus can be reactivated by some illnesses and stresses and be transmitted to the original site of infection, causing cutaneous disease (Alsharif, 2015).

The immune response to the HSV-1 infection concerns innate and adaptive immune system cells. Macrophages have an essential role in controlling HSV-1 replication by targeting and killing the infected cells or slowing the virus replication (Reichard et al., 2015). Macrophages perform a range of anti-herpetic actions during the first stage of the HSV-1 infection. The primary response to HSV-1 is achieved by production of variety of cytokines, especially tumor necrosis factor and type
I interferons (IFN), generating a direct antiviral effect and activated the macrophages. The next response line involves IL12 and other cytokines, which lead to the production IFN-γ mainly by NK cells (Ellermann-Eriksen, 2005). The activated macrophages and other cells function as a network to control the HSV-1 replication (Mantovani et al., 2004). The antiviral activity of macrophages is typically phrased as intrinsic or extrinsic. The intrinsic activity of the resting macrophages against HSV-1 is high as they are not permissive to viral replication. Macrophages can protect other cells from infection, and they are a blind end for the infection. Extrinsic activity relates to the ability of macrophages to inactivate the virus outside of the macrophage and to stop replication in other cells. Intrinsic antiviral activity is dependent upon differentiation factors and IFN activity (Ellermann-Eriksen, 2005). The production and regulation of simple toxic substances, such as NO and ROS is complex in activated macrophages and remains an area of significant research (Ellermann-Eriksen, 2005; Andrews & Sullivan, 2003).

The impact of HSV on the host cytoskeleton

The distribution of host cytoskeleton plays a critical role in the local transport of different cargo (organelles, vesicles, viruses, etc.). Microtubules and microfilaments control the transferring of cargo and cellular organelles. These cytoskeleton complex network with their associated proteins are important in the viral DNA (ex, HSV-1) transmission to and away from the host cell nucleus in multiple viral infection stages, particularly during the viral entry, replication, and egress. (Lyman & Enquist, 2009). So, developing and designing new immunological tests for identifying the composition and the function of the transported complexes at each stage in the virus life cycle is a critical progress to control the virus infection.

The impact of HSV on the host cytoskeleton network begins with the binding of virus
glycoproteins with cell receptors. In the case of HSV infection, both gD and nectin viral glycoproteins play an important part during the viral fusion within cell’s membrane. Once the viral nectin proteins bind to the gD viral glycoprotein, the actin-remodeling proteins, particularly Rho GTPases and Ras are activate and regulate the reorganization of the actin cytoskeleton, and increase the efficiency of the viral trafficking along microtubules to the nucleus (Lyman & Enquist, 2009) (Figure 4). Therefore, inactivation of the actin-remodeling proteins Rho GTPases and Ras is a significant way to decrease the transport of virus to the nucleus by decreasing the stability of microtubules indirectly (Naranatt et al., 2005). Palazzo et al. (2001) found that microtubule stability are induced by a class of Rho effectors known as the diaphanous- related formins (DRFs): mDia and mDia (serum starved 3T3 mouse fibroblasts).
FIGURE 4: Cytoskeleton remodeling during herpesvirus entry at cell surface. Binding of virus gB with host cell α3β1 integrin stimulates RhoA and Rac1 GTPases production, and resulting in microtubule stabilization, facilitating the viral capsids to transport to the host cell nucleus (Reproduced from Lyman & Enquist, 2009).
MATERIALS AND METHODS

Cell Line
The RAW 264.7 murine macrophage cell line (from American Type Culture Collection ATCC, Manassas, VA) is the Abelson murine leukemia virus- induced tumor derived from an adult male BALB/C mice. According to specific instructions, the RAW 264.7 murine macrophages were cultured on 100mm x 20mm culture petri plates (from BD Biosciences) with Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone), 10% fetal bovine serum (heat-inactivated) (from Fisher Scientific) and 1% penicillin-streptomycin antibiotic (from MP Biomedical, LLC). Cells were grown in a humidified 5% incubator at 37ºC. Cells were split two to three times every week.

Vero 76 cells (CCL-81, ATCC) which are African green monkey kidney epithelial cells, were used to propagate the HSV-1 (Syn 17+) (originally supplied by Dr. Nancy Sawtell, Children’s Hospital Medical Center, Cincinnati, OH) and calculating their titers. In our study, we infected the cells with 0.1 multiplicity of infection (MOI).

Polarization Induction
RAW 264.7 murine macrophage cells were treated with lipopolysaccharide (LPS) (100 ng/mL) (from Chondrex) and IFN-λ (20 ng/mL) (from Propech) directly once the cells reached a level of observable confluency ranging from 70-80% in order to induce a M1 phenotype. Cells were polarized for a total of 24 hours, 48 hours, or 72 hours. The cells were collected from the culture dishes, for cell viability. Immunofluorescence staining was used to analyze and evaluate the morphological changes and the immunofluorescence intensities for F-actin and tubulin between the testing groups.
Cell Viability

RAW 264.7 murine macrophages were be grown to an observable confluency of approximately 70%. Once 70% confluency are reached via observation, cells were then stimulated with IFN-λ/LPS, SOCS1 peptide (35 μM/ml), and SOCS3 peptide (35 μM/ml) with a 0.1 multiplicity of infection of HSV-1 or without a multiplicity of infection of HSV-1. Cells were incubated for 24 hours or 48 hours. Cells that were untreated served as the control M0. The cells which were grown on 24-well plates for a period of 24 hours, 48 hours, or 72 hours were collected using cell scraper. The original supernatants were collected and stored at −80°C for cytokine measurements. Centrifugation of the new supernatant at 1500 rpm was done for a total of 5 minutes at a temperature of 4°C. After centrifugation, the supernatant was aspirated. One ml of 10% DMEM medium was used to suspend the cell pellet and a trypan blue stain (Fisher Scientific) was applied to the cells at a ratio of 1:2 to determine the cell viability in accordance to the following equation:

\[% \text{ Cell Viability} = \frac{\text{Total Viable Cells (Unstained)}}{\text{Total Cells (Viable + Dead)}} \times 100\]

**Figure 5:** Trypan blue exclusion test (Hemocytometer method). Live cells appear colorless under microscope, whereas the dead cells stain blue.
Table 1: Cell viability calculations.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Equation</th>
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<tbody>
<tr>
<td>% Cell Viability</td>
<td>[Viable cells / Total cells] X 100</td>
</tr>
<tr>
<td>Viable Cells/ml</td>
<td>Average viable cell x Dilution Factor x 10^4</td>
</tr>
<tr>
<td>Dilution Factor</td>
<td>Total Volume (Volume of sample + Volume of diluting liquid) / Volume of sample</td>
</tr>
</tbody>
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Cytokine Measurements

The frozen supernatants were measured for multiple cytokine concentrations using Luminex Multiplex Immunoassays (R&D Systems) in accordance with manufacturer’s instructions.

Immunofluorescent Staining

The 12 wells removable silicone cultivation chambers (purchased from Ibidi) were used to grow RAW 264.7 murine macrophages. Once the level of confluency reached approximately 50%, the cells were then stimulated with IFN-λ/LPS, SOCS1 peptide (35 μM/ml), and SOCS3 peptide (35 μM/ml) with a 0.1 multiplicity of infection of HSV-1 or without a multiplicity of infection of HSV-1. Cells that were untreated served as the control. Cells were incubated for 24 hours, 48 hours, or 72 hours. Aspiration of the culture media immediately followed the incubation period. One percent (1%) bovine serum albumin suspended in phosphate buffered saline was used to wash the cells (2-3 times, and 3-5 minutes each time) to remove non adherent cells. After that, cells were fixed with
four percent (4%) paraformaldehyde suspended in PBS at room temperature for a total of 15 minutes. Cells were then rinsed three consecutive times (for five minutes each time) with 1% BSA in PBS at room temperature. Cells were then permeabilized using 0.25% Triton X-100 diluted in PBS for 5-10 minutes. Cells were then rinsed three consecutive times (for five minutes each time) with 1% BSA in PBS. Next, to prevent cells from partaking in non-specific binding, cells were blocked with a mixture of 5% Goat serum, 3% BSA, and 0.05% tween for one to two hours at room temperature. Cells were then rinsed three consecutive times (for a five minute each time) with 1% BSA in PBS. Primary antibody concentrations and dilutions were assessed using 3% BSA in PBS (the blocking buffer for the non-specific binding of cells) as demonstrated in Table 2. Following the application of 3% BSA in PBS containing diluted primary antibodies on the cells, cells were incubated at a temperature of 4°C throughout the night. After that, one percent (1%) BSA in PBS was used to rinse the cells three times for approximately three minutes each time (for a total of nine minutes). Next, cells were incubated in the secondary antibody and Texas Red-Phalloidin X (Table 2) for 1–2 hours at room temperature in the dark. The cells were rinsed with 1% BSA in PBS six times (for three to five minutes each time). 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’ digital camera was used to make the observations and visualizations become apparent.
Table 2: A brief summary of the antibodies and the staining used in immunofluorescence experiment.

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<thead>
<tr>
<th>ANTIBODY / STAIN</th>
<th>Concentration / Dilution</th>
<th>COMPANY</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texas Red-Phalloidin X</td>
<td>1:40</td>
<td>Life Technologies</td>
<td>F-actin stain</td>
</tr>
<tr>
<td>α/β-Tubulin Antibody</td>
<td>1:50</td>
<td>Cell Signaling Technology</td>
<td>Primary antibody against Tubulin</td>
</tr>
<tr>
<td>Rabbit iGG FAB2 goat anti-rabbit polyclonal antibody</td>
<td>1:500</td>
<td>LifeSpan BioSciences</td>
<td>Secondary antibody</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Experiments were repeated a minimum of three successive times. Data were gathered and collected from the experiments to measure cell viability, and the immunofluorescence intensity for Texas Red-Phalloidin X and microtubules. The statistical significance of experimental control groups and testing further examined and analyzed the differences between testing and the control groups. One-way ANOVA was used to analyze the differences between the experimental testing groups using Sigma Plot 13.0 Software. Statistical significance was measured in accordance to P-values ≤ 0.05 (less than or equal to 0.05) and the data was depicted as mean ± standard error of the mean.

**Image Processing and Analysis**

The Immunofluorescent images were processed and analyzed in Image J (National Institutes of
Health, http://Image J.nih.gov/ij/) by accepted quantitative fluorescence procedures within the microscopy community. The image processing and analysis steps with some described screenshots.

**STEP1: SELECT AND CONVERT THE IMAGE.** To analyze an image, open the original image by ImageJ software and convert the color image into an 8-bit image as shown in the Figure 6.

![Image J software interface](image)

**Figure 6:** Opening and converting the image type by Image J.

**STEP2: SUBTRACT BACKGROUND.** (Image → Process → subtract background), which is a very important step for measuring image intensity. This process is important to remove the pixel information from a continuous background. Missing this steps result in false data result when the intensity of image analyzed.

**STEP 3: DETECT EXCLUSION REGIONS, AND ISOLATE THE THRESHOLD (ROI).** Before analyzing the immunofluorescence intensity of an image, the pixel values (bins 0-255, by histogram) of the exclusion regions (black background and the bright saturated artifacts) must be
identified to prevent false fluorescence intensity. Many repeated measurements showed that the
black background represent pixel values from 0-20, and pixel values from 111-255 represent the
bright saturated artifacts. Identifying pixels above or below a particular threshold value can be
done as shown in Figure 7.

![Image J screenshot with Isolating Threshold (ROI)](image)

**Figure 7:** Isolating Threshold (ROI) by Image J.

**STEP 4: ANALYZING THE IMAGE.** The Cell Counter tool was operated to count the number
of cells in each image in order to Regulate the image’s ROI area, and then providing a more
accurate evaluation of the cytoskeleton changes within the cells. In ImageJ, the Image → Analyze
→ Analyze Particles... allows you to define total area for the selected image. The average size of
the area value (Total area / Number of counted cells) for each image that represent
immunofluorescence intensities within the ROI were compared using One-way ANOVA and t test
(Sigma Plot 13.0 Software).
Figure 8: Analyzing the image by Image J.
RESULTS

Polarized uninfected and virus-infected macrophages displayed morphological changes when compared to control macrophages

Macrophages were stimulated for 24 or 48 hours with IFN-γ and LPS to induce the M1 phenotype. The M1 macrophages displayed different shapes and structures with and without HSV-1. For the uninfected group, the polarized M1 macrophages appeared enlarged and flattened with intracellular vacuoles (increasing with the incubation period), while the control M0 cells appeared rounded at 24 and 48 hours. On other hand, both HSV-1 infected M0, and HSV-1 infected M1 macrophages displayed a rounded shape at 24 and 48 hours.

Following IFN-γ and LPS, uninfected and virus-infected M1 macrophages showed a significant decrease in cell viability for 24 and 48 hours

Cell viabilities for M1 macrophages were calculated for both groups (with and without HSV-1 Infection) for 24 and 48 hours, and compared to the cell viabilities of the control M0 macrophages. The cell viabilities for M1 macrophages at 24 and 48 hours were extremely similar (47%, 39%, respectively, with p value <0.001) and exhibited a decrease in cell viability compared to M0 control cells. The HSV-1 infected group also showed a significant decrease in the cell viability of M1 macrophages compared to M0 macrophages. At 24 hours, the cell viability of M1 macrophages decreased 40% with a p value of p =<0.001. After 48 incubation period, the cell viability of M1 macrophages decreased compared to M0 control (35%) (Figure 17 and Figure 18).
Following treatment of SOCS3 peptide mimetics, uninfected and virus-infected M1 macrophages showed a significant increase in cell viability when compared to SOCS1 treated M1 macrophages

Cell viabilities were also measured for polarized M1 and HSV-1-infected M1 RAW 264.7 macrophages after treatment with SOCS1 peptide and SOCS3 peptide mimetics, with the same cell suspension used in the above experiments for 24 and 48 hours. All experiments results showed that the SOCS3 peptide mimetic resulted in increase in the cell viability of polarized M1 macrophages compared with SOCS1 treated cells (p<0.001). There was no difference between infected and uninfected groups (Figure 17 and Figure 18).

Pro-inflammatory cytokine TNF-α was increased in M1 cells compared to M1 cells treated with SOCS3 peptide mimetic and M0 cells. Furthermore, M1 macrophages treated with SOCS1 peptide show high levels of TNF-α compared to other cells. However, a decrease in IL-10 production was observed in M1 cells treated with SOCS1 peptide, while M1 cells treated with SOCS3 peptide mimetic increased the production of IL-10 compared to M1 cells (Figure 21).

**HSV-1 promote the overall formation of F-actin structures in macrophages during the 24 post infection**

We quantified the cellular F-actin staining in either HSV-1 infected cells or uninfected cells including control M0 cells, polarized M1 cells, M1 treated with SOCS1 peptide, and M1 cells treated with SOCS3 peptide mimetics for 24 and 48 hours. As shown in Figure 19, F-actin staining of infected cells increased at the 24 hours post infection comparing to the uninfected cells. Both infected and uninfected groups showed a decrease in the F-actin staining at 48 hours that may relatively result from decreased cell viability at that time. These two results indicate that HSV-1
can promote the F-actin structure assembly in macrophage cells during the first hours of infection, which suggesting that the F-actin play an important role in the HSV-1 movement within the macrophage cells.

**Infected and uninfected polarized M1 macrophages, and SOCS1 treated uninfected cells displayed a significant decrease in immunofluorescence intensity of microtubules for 24 hours**

We quantified the cellular tubulin staining in either HSV-1 infected cells or uninfected cells including control M0 cells, polarized M1 cells, M1 treated with SOCS1 peptide, and M1 treated with SOCS3 peptide mimetics for 24 and 48 hours. As shown in Figure 20, tubulin staining of infected and uninfected polarized M1 macrophage cells and SOCS1 treated uninfected cells exhibited a significant decrease at 24 hours after polarization and infection.

**SOCS1 Peptide and SOCS3 Peptide Mimetics increase the immunofluorescence intensity of microtubules during the 24 hours of HSV-1 infection**

The infected polarized M1 macrophage cells showed a significant increase in tubulin staining after treating cells with SOCS1 peptide and SOCS3 peptide mimetics for 24 hours post infection. Both infected and uninfected groups showed a decrease in tubulin staining at 48 hours that may relatively result from the decrease in the cell viability at that time (Figure 20). These peptides exhibited a significant increase in tubulin staining of virus infected polarized M1 cells in contrast to the polarized M1 cells.
Figure 9: Un-polarized RAW 264.7 macrophage and polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 24 hours. Actin stained with Texas-Red Phalloidin X (Scale bar =20 μm).
**Figure 10:** Un-polarized RAW 264.7 macrophage and polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 48 hours. Actin stained with Texas-Red Phalloidin X (Scale bar =20 μm).
**Figure 11**: immunofluorescence images for un-polarized RAW 264.7 macrophage and polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 24 hours using α / β tubulin Antibody (Scale bar =20 μm).
Figure 12: immunofluorescence images for un-polarized RAW 264.7 macrophage and polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 48 hours using α / β tubulin Antibody (Scale bar =20 μm).
Figure 13: HSV-1 infected un-polarized RAW 264.7 macrophage and HSV-1 infected polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 24 hours. Actin stained with Texas-Red Phalloidin X (Scale bar =20 μm).
Figure 14: HSV-1 infected un-polarized RAW 264.7 macrophage and HSV-1 infected polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 48 hours. Actin stained with Texas-Red Phalloidin X (Scale bar =20 μm).
Figure 15: immunofluorescence images for HSV-1 infected un-polarized RAW 264.7 macrophage and HSV-1 infected polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 24 hours using α / β tubulin Antibody (Scale bar =20 μm).
**Figure 16:** immunofluorescence images for HSV-1 infected un-polarized RAW 264.7 macrophage and HSV-1 infected polarized M1 (treated with SOCS1 peptide or SOCS3 peptide mimetics) for 48 hours using α / β tubulin Antibody (Scale bar =20 μm).
Figure 17: Cell viability of un-polarized (M0), polarized macrophages M1, polarized macrophages M1 treated with SOCS 1, and polarized macrophages M1 treated with SOCS 3 peptide Mimetics after 24 hours post polarization and HSV-1 infection. (A) Cells without HSV-1 infection. (B) Cells infected with HSV-1. Each value characterizes mean ± standard error (SE) of three separate experiments. ***, p ≤ 0.001.
**Figure 18**: Cell viability of un-polarized (M0), polarized macrophages M1, polarized macrophages M1 treated with SOCS 1, and polarized macrophages M1 treated with SOCS 3 peptide Mimetics after 48 hours post polarization and HSV-1 infection. (A) Cells without HSV-1 infection. (B) Cells infected with HSV-1. Each value characterizes mean ± standard error (SE) of three separate experiments. ***, p ≤ 0.001.
Figure 19: Quantification of F-actin reorganization in RAW 264.7 macrophage cells with and without HSV-1 infection. M1 cells induced by IFN-λ and LPS treatments, and treated with Socs1 peptide or Socs3 peptide mimetics. Cells were infected with HSV-1 (MOI = 0.01) for 24 (A) or 48 (B) hours. Cells were fixed, permeabilized, and stained with Texas-Red Phalloidin X. The fluorescence intensity was analyzed by immunofluorescence microscope and ImageJ software. Each value characterizes mean ± standard error (SE) of three experiments.
Figure 20: Quantification of Tubulin reorganization in RAW 264.7 macrophage cells with and without HSV-1 infection. Cells were polarized with IFN-λ and LPS, and treated with Socs1 peptide or Socs3 peptide mimetics. Cells infected with HSV-1 (MOI = 0.01) for 24 (A) or 48 (B). Cells were fixed, permeabilized, blocked and incubated α/β-Tubulin Antibody. The fluorescence intensity was analyzed by immunofluorescence microscope and ImageJ software. Each value characterizes mean ± standard error (SE) of three experiments.
**Figure 21:** Production of anti-inflammatory IL-10 (A) and pro-inflammatory TNF-α (B) by RAW 264.7 cells 24 hours after polarization. M0 cells, M1 cells, M1 cells treated with SOCS1 peptide, M1 cells treated with SOCS3 peptide mimetic.
In this study, M1-polarized RAW 264.7 macrophages appeared flattened, elongated, adherent, and more vacuolated than uninfected control macrophages. Reichard (2012) suggested that similar morphological changes in infected and polarized M1 J774.1 macrophages may result from the disruption of the actin cytoskeleton during the HSV-1 replication stage. Staufenbiel and colleagues (1986) noted adenovirus, another DNA virus, induced a distinct sequence of cytoskeletal rearrangement in host cells (TC7 subclone CACO human intestinal cells). These alterations started at the microtubule and eventually the cytoskeleton became nonpolar and rounder. Alterations occurred not only in the microtubules but also in actin and intermediate filaments (Staufenbiel et al., 1986).

Following polarization with IFN-γ and LPS, the M1 macrophages displayed a significant decrease in the cell viability compared to the control M0 cells (Figure 17, 18). M1 macrophages function as pro-inflammatory cells producing certain cytotoxic molecules such as TNF-α, nitric oxide species (iNOS) and reactive oxygen species (ROS). These cytotoxic molecules play an important role in the decline in cell viability of the M1 phenotype (Schachtele et al., 2010).

SOCS molecules are a family of inducible proteins that control the JAK/STAT signaling pathway mediating the cytokine production (Frey et al., 2009). SOCS proteins play a critical role in controlling the innate intracellular immune response during viral infection. Cell viabilities of polarized M1 and HSV-1-infected M1 RAW 264.7 macrophages were determined following treatment with SOCS1 and SOCS3 peptide mimetics for 24 hours. The M1 polarized cells exhibit a significant increase in cell viability after treatment with SOCS3 peptide mimetic compared to SOCS1 treated M1 cells (for both infected and uninfected groups) (Figure 17, 18). Al sharif (2015) found that SOCS3-treated M1 macrophage cell lines (J774.1 and RAW 264.7) exhibited a decrease
in the production of inflammatory cytokines (TNF-α and IL-6), and an increase in anti-inflammatory IL-10 compared with M2 phenotypes of these cell lines. In this study, M1 macrophage cells treated with SOCS3 peptide mimetic exhibited an increase of anti-inflammatory IL-10 production and a decrease in pro-inflammatory cytokines TNF-α production compared to M1 cells. These observations suggested that SOCS3 exerts a critical role in controlling the lytic effect of M1 cells by stimulating the cells to produce a high level of the anti-inflammatory cytokine IL-10.

Using flow cytometry, Reichard (2012) found that the ratios of SOCS1/SOCS3 for polarized J774.1 macrophages suggested differing biological roles for SOCS1 and SOCS3. Marked elevations in SOCS1 over SOCS3 were seen in the M1 polarized cells (7:1) whereas elevations in SOCS3 over SOCS1 (2:1) were seen in the M2 polarized cells, suggesting that SOCS1 expression predominated in the pro-inflammatory M1 cells and the anti-inflammatory SOCS3 expression predominated in the M2 polarized cells.

Microtubules and actin filaments are two components of the cytoskeleton in eukaryotic cells. They provide the structural support and the shape to cells, and are responsible for the majority of the intracellular transport of organelles. Microtubules grow and shrink to produce enough power for the intracellular transport of organelles with the help of certain motor proteins. Alpha-tubulin binds to beta-tubulin forming dimers which bind to GTP to form tubulin; these complexes dynamically assemble and disassemble but permit microtubules to function as the transport system for intracellular transport of cargo, such as HSV-1. The present study involving HSV-1 infection of the macrophages provides an understanding of the effect of this virus on the microtubule cytoskeleton during infection. Microtubule reorganization during HSV-1 infection plays an important role in the intracellular transport of the virus to and from the host cell nucleus (Newcomb
et al., 2007).

In this study, SOCS1 peptide and SOCS3 peptide mimetics were evaluated for their effects on microtubules of uninfected and HSV-1-infected M1 polarized RAW 264.7 macrophages. Treatment with either of these peptide mimetics preserved microtubule integrity in HSV-1-infected M1 RAW 264.7 cells by 24 hours (p<0.01), but not 48 hours (NS), after polarization and infection (Figure 20). These significant changes in tubulin assuming may result from an increase of microtubule stability as a consequence of RhoA activation by SOCS1 and SOCS3 proteins. Naranatt et al. (2015) found that GTPase RhoA has an important function in modulating the microtubule dynamics by increasing microtubule stability, increasing viral transport along microtubules to the nucleus. SOCS1 and SOCS3 proteins activate GTPase RhoA (Stevenson et al., 2010). Palazzo et al. (2001) found that microtubule stability is induced directly by a class of Rho effectors known as the diaphanous-related formins (DRFs): mDia and mDia (serum starved 3T3 mouse fibroblasts).

In the present study, uninfected polarized M1 cells exhibited a significant decrease in tubulin staining in contrast to M0 control cells (Figure 20). This decline in the florescent intensity of tubulin may result from the effect of LPS and cytotoxic molecules such as TNF-α on microtubule dynamics. TNF-α inhibits microtubules assembly (Shivanna et al., 2009).

Also, in the present study, uninfected M1 polarized cells treated with SOCS3 peptide mimetic exhibited a significant increase in tubulin staining in contrast to untreated polarized M1 cells (Figure 16). These observations are consistent with the study of Zou et al. (2008) that the microtubule associated protein 1 (MAP1S) has a crucial role in SOCS3 localization to microtubules and its negative regulating mechanism during cytokine signaling. They found that the overexpressed MAP1S can increase the translocation of SOCS3 from the nucleus to cytoplasm.
After examination of a MAP1S deficient macrophage, they observed that the negative regulation of STAT3 pathway by SOCS3 was delayed during the IL-6 signaling, suggesting that MAP1S function as an adaptor protein for the interaction between SOCS3 protein and microtubule cytoskeleton. Another explanation for this phenomenon might be that increase the immunofluorescence intensity of tubulin may result from the increase in the cell viability of polarized M1 cells when they treated with SOCS3 peptide mimetics as we found in the result of this study.

In this study, the effect of these peptide mimetics on F-actin were similarly examined. After 24 hours of infection, F-actin expression was increased in virus-infected unpolarized or polarized cells where the cells were treated with either of the peptide mimetics (Figure 19). The peptide mimetics had no effect on F-actin expression but HSV-1 infection did. This increase in F-actin expression by the infected cells during the first 24 hours suggests that the F-actin structures may facilitate HSV-1 infection and replication in the macrophage cells. These observations are supported by those of Xiang and colleagues (2012). They found HSV-1 infection enhanced the formation of F-actin–based structures in the early infection of a neuroblastoma cell line.

In future studies it would be beneficial to study the morphological changes and the quantitative immunofluorescence intensities for the cellular cytoskeleton during the early stage of HSV-1 infection. An in vitro approach would include inducing M1 macrophages treated with SOCS1 peptide SOCS3 peptide mimetics (or untreated control cells) with or without HSV-1 (MOI of 0.1) for 4, 12, and 18 hours and then evaluating the changes on F-actin and microtubules via immunofluorescent microscope. Since quantitative analysis of cytoskeletal proteins is critical for understanding HSV-1 infection, immunoblotting approaches should also be used to include unpolarized actin (G-actin) and tubulin filaments in assessing total pools of cytoskeletal
proteins in these cells.

Cell viability studies should include measurements of apoptosis such as immunostaining for anexin V. Since HSV-1 increases activation of RhoA GTPase proteins that are involved in regulating actin filaments, tubulin stability, and motor proteins (dynein and kinesin), immunofluorescent experiments should be included to determine the levels of these molecules in the cytoplasm of SOS1 and SOC3 treated, uninfected and HSV-1-infected polarized and unpolarized RAW 264.7 macrophages.
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