The Effects of SOCS1, SOCS3 and HSV-1 Infection on Morphology, Cell Viability and Rab7 Expression in Polarized M1 and M2 Raw 264.7 Murine Macrophages

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THE EFFECTS OF SOCS1, SOCS3 AND HSV-1 INFECTION ON MORPHOLOGY, CELL VIABILITY AND RAB7 EXPRESSION IN POLARIZED 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

JESSICA RENEE HEY
B.S., Wright State University, 2015

2018
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jessica Renee Hey ENTITLED The Effects of SOCS1, SOCS3, and HSV-1 Infection on Morphology, Cell Viability, and Rab7 Expression in Polarized 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

Hey, Jessica Renee. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2018. The Effects of SOCS1, SOCS3 and HSV-1 Infection on Morphology, Cell Viability and Rab7 Expression in Polarized M1 and M2 Raw 264.7 Murine Macrophages.

HSV-1 causes a life-long infection in its host and has evolved multiple strategies to facilitate infection and evade the immune response. This virus has been found to enter cells by both endocytosis and fusion. The way the virus exploits endocytosis is not fully understood. Recent studies have uncovered roles of Rab GTPases, key regulators in intracellular membrane trafficking pathways, in distinct steps of the HSV-1 life cycle (Raza et al., 2018). This study will focus on analyzing the levels of the late endosomal regulator Rab7 expression in macrophages infected with HSV-1. Revealing the effect of virus on the levels of the Rab7 expression-directed vesicular trafficking pathway in endocytosis will allow insight into possible future therapies. Peptide mimetics of suppressor of cytokine signaling molecules (SOCS1 and SOCS3) were examined for the potential ability to modulate expression of Rab7. Immunofluorescence was used to determine Rab7 expression and F-actin polymerization levels in uninfected and HSV-1 infected unpolarized M0 Raw264.7 murine macrophages and polarized subsets. M1 macrophages were polarized by treatment with lipopolysaccharide (LPS) and interferon gamma (IFN-γ). M2 cells were polarized by treatment with either IL-4 [M2(IL-4)] or IL-10 [M2(IL-10)].
Rab7 expression levels increased over a 24 hour observation period in M0 and M2 cell types independent of virus infection. During this same observation period Rab7 expression levels were significantly decreased in M1 polarized macrophages with HSV-1-infection. Treatment of the various unpolarized and polarized, infected and uninfected groups of RAW264.7 cells with either SOCS1 or SOC3 peptide mimetic caused a significant decrease in Rab7 expression over the 24 hour observation time. During this same time, HSV-1 infection effected a significant decrease in F-actin polymerization levels in virus infected M0 and in both M2 macrophage phenotypes. SOCS1 and SOCS3 peptide mimetics caused an additional decrease in F-actin polymerization in both unpolarized HSV-1-infected M0 cells and in uninfected IL-4 polarized M2 cells.

These results indicate that macrophage polarization and SOCS1 and SOCS3 peptide mimetics impact Rab7 expression and F-actin polymerization levels more significantly than HSV-1 does in unpolarized M0 and polarized M1 and M2 RAW264.7 murine macrophages.
HYPOTHESIS

HSV-1 is expected to obtain entrance into M0, polarized M1, and polarized M2 macrophages by endocytosis upon infection and increase endocytic activity, raising the level of the late endosomal regulator protein Rab7 expression over a 24 hour time period. HSV-1 is known to manipulate rearrangements of F-actin polymerization in the cytoskeleton of host cells to propagate its viral cycle. By 24 hours, at later stages of virus infection, the levels of F-actin polymerization will be decreased. Addition of SOCS1 and SOCS3 peptide mimetics will decrease both Rab7 expression and F-actin polymerization levels over time. The LPS and INF-γ being used to induce polarization of the M1 phenotype will alter cell morphology and decrease viability after 24 hours because of its induction of various anti-inflammatory molecules and cytotoxic properties.
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LIST OF ABBREVIATIONS:

HSV-1: Herpes Simplex 1 Virus
SOCS1: Suppressor of Cytokine Signaling 1
SOCS3: Suppressor of Cytokine Signaling 3
LPS: Lipopolysaccharide
IFN-γ: Interferon-gamma
M0: Uninfected Unpolarized RAW 264.7 Macrophages
M0V: HSV-1 Infected Unpolarized RAW 264.7 Macrophages
M0S1: Uninfected Unpolarized RAW 264.7 Macrophages Treated with SOCS1 Peptide Mimetics
M0S1V: HSV-1 Infected Unpolarized RAW 264.7 Macrophages Treated with SOCS1 Peptide Mimetics
M0S3: Uninfected Unpolarized RAW 264.7 Macrophages Treated with SOCS3 Peptide Mimetics
M0S3V: HSV-1 Infected Unpolarized RAW 264.7 Macrophages Treated with SOCS3 Peptide Mimetics
M1: Uninfected LPS & IFN-γ Polarized RAW 264.7 Macrophages
M1V: HSV-1 Infected LPS & IFN-γ Polarized RAW 264.7 Macrophages
M1S1: Uninfected LPS & IFN-γ Polarized RAW 264.7 Macrophages Treated with SOCS1 Peptide Mimetics
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M2(IL-10): Uninfected IL-10 Polarized RAW 264.7 Macrophages

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INTRODUCTION

Herpes simplex 1 virus (HSV-1) is a common and highly contagious disease that affects people of all ages. It is also known as oral herpes and is spread by both oral to oral and skin to skin contact. HSV-1 establishes lifelong latent infections and resides in the host’s nervous system. It may be transmitted even when the host is asymptomatic. People are affected in different ways by HSV-1. While some are unaware they have contracted the virus others experience recurrent flare ups and debilitating symptoms. Outbreak frequency is unpredictable. Common symptoms include dysuria, cold sores, blisters, muscle aches, pain, headaches, and fevers. More severe conditions caused by herpes are eye disease, encephalitis and meningitis. There is no cure or successful vaccine for HSV-1 available. The range of effects this virus has on the immune system throughout a lifetime is not fully understood. HSV-1 has developed many strategies to impair intracellular sorting and processing of antigens to evade immune response. It is possible that the long term effects of HSV-1 contribute to immunosuppression and neurological disorders. This paper aims to analyze the relationship between HSV-1 infection, Rab7 expression, and F-actin polymerization in polarized M1 and M2 macrophages.

Macrophages are a major factor in launching the immune response and serve as the first line of defense against viral infection. In reaction to HSV-1 infection both the innate and adaptive immune responses become triggered. Macrophages are leukocytes differentiated from monocytes that work to phagocytose any foreign materials they
recognize. These cells then utilize their endocytic pathway to present antigens on their outer surface & release cascades of cytokine signals to attract other immune cells. There are multiple kinds of specialized macrophages in the body. Based on polarization, macrophages can be differentiated into either an M1 or M2 sub-type. These diverse phenotypes work in different ways to supplement the immune system response. RAW 264.7 murine macrophages are polarized into both M1 and M2 phenotypes in this study to compare levels of Rab7 expression and F-actin polymerization over time.

Rab7 expression is a GTP-binding protein that functions in regulating late endosomal maturation and endo-lysosomal trafficking (Wang et al., 2010). It also participates in endosome sorting and directing trafficking of cargos along components of the cell cytoskeleton (Hyttinen et al., 2012). This protein is part of the Ras oncogene superfamily (Rojas et al., 2012). Mutations in Rab7 expression cause cellular traffic disorders which may lead to neuropathy, lipid metabolism disease, or cancer (Wandinger-Ness et al., 2014). Viral entry into cells is understood to be mediated by endocytic pathways. HSV-1 may utilize Rab7 expression endosomes to deliver its capsids. The effect of HSV-1 on Rab7 expression levels over a 24 hour time period in M0, M1, and M2 macrophages remains unknown.

Actin is an important protein component in the cytoskeleton of eukaryotic cells that forms microfilaments. Actin is classified into either G-actin or F-actin polymerization depending on its structure. G-actin is found as a single monomer while F-actin
polymerization is a linear structure composed of two monomers (Miranda-Saksena et al., 2018). Both of these proteins function to control significant processes such as cell motility, endocytosis, intracellular trafficking, cell polarity, and cell signaling (Gordon-Alonso et al., 2013). HSV-1 has been shown to induce a continuous decrease in cellular F-actin polymerization levels during later stages of infection (Xiang, Yangfei et al., 2012). This study will evaluate the levels of F-actin polymerization in M0, M1, and M2 macrophages infected with HSV-1 at 2, 4, 6, and 24 hour time points. Both Rab7 expression and F-actin polymerization are involved in endocytosis and intracellular trafficking therefore the focus of this paper is to better understand their relationship in HSV-1 infection.

Suppressors of cytokine signaling (SOCS) are proteins that negatively regulate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway which is involved in the process of immunity (Shunsuke, et al., 2017). There are various proteins in the SOCS family that play a role in adaptive immunity by regulating inflammation. Cytokines are essential in the functioning of macrophages and the immune response. The effects of SOCS1 and SOCS3 on Rab7 expression and F-actin polymerization levels in M0, M1, and M2 polarized macrophages uninfected and infected with HSV-1 are examined in this study.
HERPES SIMPLEX VIRUS 1:

HSV-1 belongs to the Herpesviridae family (Laine et al., 2015). The virus has a linear double-stranded DNA genome that is about 152 kbp in length (Smith et al., 2014). The genome is packaged into an icosahedral capsid which is surrounded by a membrane envelope. Viral entry can occur via direct membrane fusion with cells or by endocytosis. Fusion entails binding of the viral envelope glycoproteins to specific receptors on the host cell (Almutairi 2016). Endocytosis can be clathrin-mediated, caveolar-mediated, or involve micropinocytosis (Mercer et al., 2010). Entering a host cell by endocytosis is more beneficial to the virus because it is said to delay immune response by avoiding leaving viral traces on the plasma membrane (Chang et al., 2016). In endocytosis the virus travels along with the cell’s built-in transport mechanism across the plasma membrane and is provided access to intracellular organelles which allow it to sense any environmental fluctuations (Schelhaas 2010). Actin-associated myosin motors and rearrangement of the actin cytoskeleton allow the trafficking of virions in both entry and exit from host cells (Clement et al., 2006). Once inside the cell the capsid is transported along components of the cytoskeleton, including actin, to the nucleus where the viral DNA is released. The virus must depend on host cell machinery to achieve infection. Rab GTPases are important regulators of vesicular transport and are known to intersect in the
viral replication cycle. Investigating the relationship of Rab7 expression in HSV-1 infection poses possible insight into future therapy.

**Rab7 expression:**

More than 60 human small GTPase Rab proteins have been discovered that control various aspects of membrane trafficking in eukaryotic cells (Girard, 2013). Rab7 expression more specifically regulates late endosome maturation, endosome trafficking or sorting, and endosome fusion to lysosomes. Endosome sorting includes recycling to the plasma membrane such as in exocytosis and transferring material to the trans-Golgi network. Harley and colleagues (2001) concluded that endosomes are key organelles in HSV exocytosis. As a GTPase, Rab7 expression is activated by a guanine nucleotide exchange factor (GEF) which converts the inactive GDP bound form to an active GTP form (Hyttinen et al., 2013). Early endosome and phagosome activity is regulated by a different protein, Rab5, until it is released and Rab7 expression is recruited. This conversion is a highly coordinated process because it should not occur unless the endosome has accumulated enough cargo for degradation. Rab7 expression serves as a late endosome marker which associates with HSV-1 before it enters the cytoplasm (Spearman 2017). Caillet and fellows (2011) conducted experiments on HIV-1 and reported that the most profound inhibition on replication and infectivity was seen secondary to Rab7 expression knockdown. The effect of HSV-1 infection on the levels of Rab7 expression over time has not yet been identified.
Macrophages:

Macrophages (M0s) have the potential to differentiate into M1 and M2 phenotypes. This polarization occurs when cells interact with particular stimuli. The M1 cell type is referred to as “classical” because it releases pro-inflammatory cytokines to help fight infection. The M2 cell type is known as “alternative” because it releases anti-inflammatory cytokines to promote tissue repair and prevent damage caused by inflammatory conditions. M2 cells are said to exhibit a much weaker antigen-presenting capability compared to M1 cells however they show increased phagocytosis receptors expression which may promote M1 responses (Zdrenghea et al., 2014). Imbalance of macrophage polarization has been seen in many diseases. This polarization is a highly regulated process in the macrophages. M1 is stimulated by microbial products, like LPS, or pro-inflammatory cytokines such as IFN-γ while M2 is stimulated by anti-inflammatory cytokines like interleukin-4 (IL-4) and interleukin-10 (IL-10) (Wang et al., 2014). These cytokines have been shown to affect the endocytic pathway and impact phagosome maturation (Barry et al., 2011). During the immune response many intracellular trafficking routes are activated in immune cells. When phagocytosis is upregulated during infection more phagosomes become generated inside the macrophage therefore more degradative compartments are needed to eliminate the pathogenic agents (Pei et al., 2012). Roles of M1 and M2 macrophages are not well understood in relation to Rab7 expression levels and HSV-1 infection.
Figure 1: Role of Rab7 expression in late endosome maturation: Schematic representation of a eukaryotic cell with indicated specific Rab7 expression regulating steps of membrane trafficking.

Figure 2: Polarization of RAW 264.7 M0 macrophages into M1 and M2 phenotypes.
Cytokines and LPS:

Cytokines are signaling proteins that regulate cellular development, function, and overall existence. Macrophages release various cytokine signals to transmit information to other cells in order to properly fight infections and regulate the immune system. Interferon gamma (IFN-γ) is known as a macrophage-activating factor that induces diverse antimicrobial activities and inflammation. This cytokine regulates stimulation of phagosome maturation, facilitates phagosomal acidification, and modulates intracellular membrane trafficking in macrophages (Pei et al., 2012). IL-4 and IL-10 promote M2 cell activation and inhibit activation of M1 cells (Ishii et al., 2009). The anti-inflammatory effects of these cytokines enhance proteolytic activity of phagosomes within macrophages (Pei et al., 2012). In vitro macrophages are able to transition between phenotypes continuously in response to cytokines (Davis et al., 2013). Lipopolysaccharides (LPS) are found in gram-negative bacteria and are known to produce a pro-inflammatory effect on macrophages. LPS increases expression of Rab7 expression and promotes lysosomal degradation (Pei et al., 2012).

F-actin polymerization:

F-actin polymerization is a fundamental component of the cellular cytoskeleton. Actin has been considered one of the first components of a host cell to be disrupted to facilitate viral entry (Delorme-Axford et al., 2011). Research has implicated that actin is crucial for viral replication during various stages of the viral life cycle “including
binding, entry, nuclear localization, genomic transcription, reverse transcription, assembly, and egress/dissemination” (Spear et al., 2014). It has been shown that HSV-1 infection first increases F-actin polymerization expression to allow viral transport then decreases F-actin polymerization assembly at later stages of infection when checked at 6 and 24 hour time points (Xiang et al., 2012). Although there is evidence of actin depolymerization in cells we do not fully understand its mechanism at this time. Rab7 expression also seems to have direct roles on actin microfilaments due to its involvement in cell trafficking. Greene and Gao (2009) reported that Kaposi’s sarcoma-associated herpesvirus (KSHV) particles were colocalized with markers of early and recycling endosomes and lysosomes as well as with actin filaments at early time points of infection. Per Hutagalung and Novick (2011) Rab proteins recruit effectors that are critical for vesicle movement along actin-based cytoskeletal structures.

**Suppressors of Cytokine Signaling - SOCS1 & SOCS3:**

SOCS are known to inhibit cytokines and negatively regulate the JAK/STAT signaling pathway (Shunsuke, et al., 2017). The JAK/STAT pathway is used to transduce signals for development and homeostasis in mammals (Rawlings et al., 2004). The SOCS family consists of 8 proteins that suppress responses to many microbial products like LPS as well as interleukins (Stevenson et al., 2010). SOCS1 more specifically reduces the signaling of IFN-γ (Hashimoto et al., 2011). Researchers suggest that SOCS1 and SOCS3 may also control chemotaxis and adhesion ultimately assisting with localizing immune
cells to sites of inflammation (Stevenson et al., 2010). SOCS1 expression can aid HSV-1 in evading the immune response (Bigley, 2014).
MATERIALS AND METHODS

Cell Line:

The RAW264.7 murine macrophage cell line utilized in this experiment was obtained from the American Type Culture Collection (ATCC, Manassas, VA). These macrophages were procured from tumors of adult male BALB/c mice infected with the Abelson murine leukemia virus. Cells were contained inside of vented BioLite flasks (Fisher Scientific, Pittsburgh, PA.) and cultured in a media consisting of Dulbecco’s Modified Eagle Medium (DMEM; Hyclone), 10% fetal bovine serum (FBS) (heat-sensitive) (from Fisher Scientific) and 10 mM HEPES buffer. These macrophages were incubated at 37° in 100% humidified air with 5% carbon dioxide. When reaching 70% confluency cells were then scraped from the flask with a cell scraper (Fisher Scientific) and split. Cell splitting occurred two to three times weekly as needed. Infection of HSV-1 (Syn 17+) (originally supplied by Dr. Nancy Sawtell, Children’s Hospital Medical Center, Cincinnati, OH) was applied with 0.1 multiplicity of infection (MOI).

Polarization Induction:

In order to induce a M1 phenotype the RAW 264.7 murine macrophages were treated with lipopolysaccharide (LPS) (100 ng/mL) (from Chondrex) and IFN-γ(20 ng/mL) (from Peprotech) after reaching an observably confluency of at least 70%. Two variations of a M2 phenotype were produced by treating appropriately confluent cells
with either IL-4 (20 ng/mL) or IL-10 (20 ng/mL). Macrophages were polarized for periods of 2 hours, 4 hours, 6 hours, and 24 hours.

**Cell Viability:**

RAW 264.7 murine macrophages were grown to an observable confluency of about 70% before being split and appropriately seeded into experimental 12-well plates. Treatments were then applied to cells including IFN-γ + LPS, IL-4, IL-10, SOCS1 peptide (35 μM/mL) and SOCS3 peptide (35 μM/mL) with or without a 0.1 multiplicity of infection of HSV-1. Cells were incubated for 24 hours. Untreated cells served as the control “M0.” Cells were then obtained from the 12-well plates using cell scrapers and were centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was aspirated and the cell pellet was re-suspended in 1 mL of 10% DMEM medium. Trypan blue stain (Fisher Scientific) was applied to a portion of the cells at a 1:2 ratio. A hemocytometer was filled with 10μL and viewed under the microscope at 10x magnification.

**Table 1: Cell Viability Calculations**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Cell Viability</td>
<td>([\text{Viable cells / Total cells (live+dead)}] \times 100)</td>
</tr>
<tr>
<td>Viable Cells per mL</td>
<td>Average # viable cells ( \times ) Dilution Factor ( \times 10000 )</td>
</tr>
</tbody>
</table>
Table 2: Cell Treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (M0)</td>
<td>No Treatment</td>
</tr>
<tr>
<td>LPS</td>
<td>100 ng/mL</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>20 ng/mL</td>
</tr>
<tr>
<td>IL-4</td>
<td>20 ng/mL</td>
</tr>
<tr>
<td>IL-10</td>
<td>20 ng/mL</td>
</tr>
<tr>
<td>SOCS1</td>
<td>35 μM/mL</td>
</tr>
<tr>
<td>SOCS3</td>
<td>35 μM/mL</td>
</tr>
<tr>
<td>HSV-1</td>
<td>MOI=0.1 mL</td>
</tr>
</tbody>
</table>

Immunofluorescent Staining:

RAW 264.7 murine macrophages were seeded into 12-well removable silicone chamber slides (Ibidi USA, Fitchburg, WI). Each well was seeded with about 5,000 cells and incubated for 24 hours to allow cell adhesion to the bottom of the plate. Appropriate treatments were then added to their designated wells based on concentrations seen in Table 2. Cells were incubated for 2 hours, 4 hours, 6 hours, or 24 hours. Wells were aspirated after each time point and then washed three times with chilled phosphate buffer saline (PBS). Cells were then fixed for 15 minutes with 4% paraformaldehyde at room
temperature. Wells were washed with PBS three times and cells were permeabilized with 0.01% saponin for 10 minutes at room temperature. Wells were washed three times with PBS and cells were blocked to prevent non-specific binding at room temperature using 10% goat for 1 hour. Again wells were washed three times with PBS. An appropriately diluted Anti-RAB7 EXPRESSION (primary antibody) was then added to the cells & incubated overnight at 4°C. Wells were washed with PBS three times. The lights were then turned off in order to apply the secondary antibody for Rab7 expression (goat anti-mouse IgG) for 1 hour in the dark. Wells were washed three times with PBS then Texas Red-Phalloidin X was added to cells for 2 hours in the dark. Cells were rinsed with PBS three times then wells were aspirated via micropipette, silicone wells were removed, and the remaining glass slide was left in the dark to dry completely for about 10 minutes. Once dry, a drop of hard-set mounting medium, Vectashield (H-1400) from Vector Laboratories, was used to mount the stained cells. Lastly a cover slip was applied to the microscope slide. Cells were viewed on an ACCU-SCOPE EXC-350 microscope at 50x magnification with a scope LED fluorescence illuminator adding an additional 0.5x magnification for the pictures that were taken.
Table 3: Antibodies and Stains utilized in immunofluorescence experiments

<table>
<thead>
<tr>
<th>Antibody/Stain</th>
<th>Concentration/Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-RAB7 EXPRESSION (Primary RAB7 EXPRESSION Antibody)</td>
<td>1 μg/mL</td>
<td>Abcam, San Francisco, CA</td>
</tr>
<tr>
<td>Goat anti-mouse IgG (H&amp;L) (Secondary RAB7 EXPRESSION Antibody)</td>
<td>1:500</td>
<td>Abcam, San Francisco, CA</td>
</tr>
<tr>
<td>Texas Red-Phalloidin</td>
<td>1:40</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
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</table>

Table 4: Variation of Cell Types Observed: note that all cell types were tested with and without HSV-1 infection.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>Cells with no treatment</td>
</tr>
<tr>
<td>M0S1</td>
<td>Cells + SOCS1</td>
</tr>
<tr>
<td>M0S3</td>
<td>Cells + SOCS3</td>
</tr>
<tr>
<td>M1</td>
<td>Cells + LPS + IFN-γ</td>
</tr>
<tr>
<td>M1S1</td>
<td>Cells + LPS + IFN-γ + SOCS1</td>
</tr>
<tr>
<td>M1S3</td>
<td>Cells + LPS + IFN-γ + SOCS3</td>
</tr>
<tr>
<td>M2</td>
<td>Cells + IL-4 or IL-10</td>
</tr>
<tr>
<td>M2S1</td>
<td>Cells + IL-4 or IL-10 + SOCS1</td>
</tr>
<tr>
<td>M2S3</td>
<td>Cells + IL-4 or IL-10 + SOCS3</td>
</tr>
</tbody>
</table>
Image Analysis:

Image J software (National Institutes of Health, [http://imagej.nih.gov/ij/](http://imagej.nih.gov/ij/)) was used to process and analyze all fluorescent cell images taken. In order to quantify the region of interest (qROI) the images were opened in Image J and first converted to black and white by changing the “type” to an 8-bit image. Next the background was subtracted from images. This enabled the measuring of image intensity by removing the pixel information from the continuous background. The image threshold was then isolated to control the exclusion region. Lastly, the image particles can be analyzed. Image J counts the cells, measures the intensity in each image, and divides the total area of intensity by the number of cells to quantify the final qROI (total area/number of counted cells).

Statistical Analysis:

All experiments were done in triplicate. Data from cell viability and immunofluorescence were analyzed using SigmaPlot 14.0 software. One-Way ANOVA tests were ran to compare collected data and calculate statistically significant results.
RESULTS

Cell viability declined in uninfected and HSV-1 infected M1 polarized RAW 264.7 murine macrophages after 24 hours. Both with and without SOCS1 and SOCS3 treatment the M1 polarized RAW 264.7 murine macrophages that were not infected with virus showed decreased viability (p≤0.001) in cultures as seen in Figure 3A. The HSV-1 infected cells yielded these same results (p≤0.001) pictured in Figure 3B.

Morphological changes occurred in M1 polarized RAW 264.7 murine macrophages exposed to SOCS1 and SOCS3 after 24 hours. Uninfected M1, M1S1, and M1S3 macrophages became elongated, flattened, and showed internal vacuoles at the 24 hour observation period as seen in Figure 7B and Figure 21B. At this same time HSV-1 infected M1V, M1S1V, and M1S3V cells showed both rounded and elongated morphologies (Figure 7B, Figure 21B). At 2 hours, 4 hours and 6 hours all cell types with and without HSV-1 infection presented with a rounded morphology.

Rab7 expression levels in RAW 264.7 murine macrophages polarized into M1 and M2 phenotypes exposed to HSV-1, SOCS1, and SOCS3 after 2, 4, 6, and 24 hour time points (Figure 4 - 16). The levels of the Rab7 expression was determined by calculating the qualified region of interest (qROI) found by ImageJ and dividing it by the number of cells in each image analyzed for all cell treatments. RAW 264.7 unpolarized macrophages that did not receive any treatment were used as a control (M0).
Rab7 expression levels increased in HSV-1 infected and uninfected unpolarized M0 macrophages from 2 hours to 24 hours. After 2 hours no significance was noted between uninfected unpolarized M0 and HSV-1 infected M0 cells (M0V) regarding the levels of Rab7 expression (Figure 4A). Uninfected M0 cells had increased Rab7 levels at 4 hours compared to HSV-1 infected M0V (p≤0.001), but no significance was present at the 6 hour or 24 hour time points (Figures 5A, 6A, 7A). The Rab7 expression increased (p≤0.001) overtime in both HSV-1 infected and uninfected unpolarized M0 cells from 2 hours to 24 hours (Figures 4A, 5A, 6A, 7A).

SOCS3 decreased Rab7 expression levels in M0 macrophages at 24 hours. The level of Rab7 expression was seen to decrease in uninfected M0 cells treated with SOCS3 (M0S3) (p≤0.001) compared to M0 and M0S1 values (Figure 8A). The level of Rab7 expression was similar in uninfected M0 and M0 treated with SOCS1 (M0S1) macrophages at 24 hours (Figure 8A).

HSV-1 infection decreased Rab7 expression levels in SOCS1 treated M0 cells at 24 hours. Uninfected M0S1 had significantly higher Rab7 expression levels than in HSV-1 infected M0S1 macrophages (M0S1V) at 24 hours (p≤0.001). There was no significant change in Rab7 expression levels between uninfected and virus infected M0 or M0S3 samples (Figure 8A).

Following INF-γ and LPS treatment, both uninfected and HSV-1 infected M1 macrophages showed decreased Rab7 expression from 2 hours to 24 hours. The
Rab7 expression levels of both uninfected and HSV-1 infected polarized M1 cells decreased over 24 hours (p≤0.001) (Figures 4A, 5A, 6A, 7A). Rab7 expression in uninfected M1 macrophages was initially higher than M0 at 2 hours (p≤0.001) (Figure 4A) but became lower than M0 over 4 hours (p≤0.001) (Figure 5A), 6 hours (p≤0.001) (Figure 6A), and 24 hours (p≤0.001) (Figure 7A). HSV-1 infected M1V cells had similar Rab7 expression levels to M0V at 2 hours (Figure 4A), 4 hours (Figure 5A), and 6 hours (Figure 6A) which decreased lower than M0V by 24 hours (p≤0.001) (Figure 7A).

At 2 hours uninfected M1 macrophages showed higher Rab7 expression levels than with virus infection. The expression level of Rab7 in M1 macrophages was higher than HSV-1 infected M1V Rab7 levels at 2 hours (p≤0.01) (Figure 4A). At the 4 hour, 6 hour, and 24 hour time points HSV-1 infection had no significant effect on M1 Rab7 expression levels (Figure 5A, Figure 6A, Figure 7A).

SOCS1 and SOCS3 peptide treatments decreased Rab7 expression in M1 macrophages over 24 hours. Rab7 expression in both virus infected and uninfected M1S1 decreased at 24 hours (p≤0.001) (Figure 7A). HSV-1 infection did not significantly change Rab7 expression in SOCS1 or SOCS3 treated M1 macrophages at 2 hours (Figure 4A) or at 4 hours (Figure 5A). At 6 hours, uninfected M1S1 showed higher Rab7 expression levels than HSV-1 infected M1S1V macrophages (p≤0.05) (Figure 6A). At 24 hours HSV-1 infected M1S3V had higher Rab7 levels than uninfected M1S3 cells (p≤0.05) (Figure 7A). Virus-infected M1S1V showed more Rab7 expression than virus-
infected M1S3V at the 2 hour time point (p≤0.001) (Figure 4A). No other significance was shown between SOCS1 and SOCS3 treated cells at the other times analyzed (Figure 5A, Figure 6A, Figure 7A)

Both HSV-1 infected and uninfected IL-4 polarized M2 macrophages showed increases in Rab7 expression levels over 24 hours. The Rab7 expression levels in uninfected M2 (IL-4) polarized macrophages increased over 24 hours (Figures 9A, 10A, 11A, 12A). The Rab7 expression in virus infected M2 (IL-4V) cells increased from 2 hours to 24 hours (Figures 9A, 10A, 11A, 12A). At both 4 hour (p≤0.01) and 6 hour (p≤0.05) time points Rab7 expression was significantly higher in virus infected M2 (IL-4V) macrophages than in uninfected M2 (IL-4) cells (Figures 10A, Figure 11A). There were no expression differences between HSV-1 infected cells and uninfected cells at 2 hours or at 24 hours (Figure 9A, Figure 12A).

At 24 hours, uninfected and HSV-1 infected IL-4 polarized M2 macrophages exhibited similar Rab7 expression levels to those of uninfected and virus-infected M0 cells. At the 4 hour time point M2 (IL-4V) macrophage Rab7 expression was higher than that of M0V (p≤0.01) (Figure 10A). Uninfected M2 (IL-4) cells showed lower Rab7 expression levels than uninfected M0 macrophages at 4 hours and 6 hours (p≤0.01) (Figure 10A, Figure 11A). After 24 hours both virus-infected M2 (IL-4V) and uninfected M2 (IL-4) macrophages had similar Rab7 expression levels to M0 and M0V macrophages with no significant differences (Figure 12A).
SOCS1 and SOCS3 peptide treatments caused decreased Rab7 expression in uninfected and HSV-1 infected M2 IL-4 polarized macrophages over 24 hours. At 24 hours the Rab7 expression of HSV-1 infected and uninfected M2 (IL-4) cells treated with SOCS1 and with SOCS3 was much decreased from the Rab7 expression levels of M2 (IL-4) macrophages (p≤0.001) (Figure 12A). At 6 hours and 24 hours SOCS peptide treated uninfected M2 (IL-4) cells showed lower Rab7 expression levels than M0 macrophages (p≤0.001) (Figure 11A, Figure 12A). Comparing uninfected to HSV-1 infected cells there was no significance at any time points for SOCS treated M2 (IL-4) samples (Figure 9A, Figure 10A, Figure 11A, Figure 12A.) Rab7 expression levels in SOCS1 and SOCS3 peptide mimetics treated cells remained similar at all times.

Uninfected and HSV-1 infected IL-10 polarized M2 macrophages exhibited a slight increase in Rab7 expression levels from 2 hours to 24 hours. Rab7 expression in uninfected M2 (IL-10) macrophages slightly increased after 24 hours as did Rab7 expression in HSV-1 infected M2 (IL-10V) cells (Figure 13A, Figure 14A, Figure 15A, Figure 16A). There was no significance in Rab7 expression levels between virus-infected and uninfected M2 (IL-10) cells. At the 2 hour, 4 hour, and 6 hour time points uninfected M2 (IL-10) and HSV-1 infected M2 (IL-10V) Rab7 levels remained similar to those of uninfected M0 and HSV-1 infected M0V macrophages (Figure 13A, Figure 14A, Figure 15A). At 24 hours uninfected M2 (IL-10) and HSV-1 infected M2 (IL-10V) Rab7 expression protein dropped below the levels shown in M0 and M0V macrophages (p≤0.001) (Figure 16A).
SOCS1 and SOCS3 peptide mimetics caused decreased Rab7 expression in HSV-1 infected and uninfected IL-10 polarized M2 macrophages by 24 hours. After 24 hours the Rab7 expression in uninfected M2 (IL-10S1) and M2 (IL-10S3) cells was significantly lower compared to Rab7 expression in M2 (IL-10) macrophages at 24 hours (p≤0.001) (Figure 16A). Rab7 expression data is similar for HSV-1 infected SOCS peptide treated M2 (IL-10V) macrophages vs. the HSV-1 infected M2 (IL-10V) sample (p≤0.001). At the 2 hour time point uninfected M2 (IL-10S1) and HSV-1 infected M2 (IL-10S1V) showed higher Rab7 expression levels than M0 (p≤0.05), M0V (p≤0.001), and M2 (IL-10) cells (p≤0.05) (Figure 13A). At 4 hours Rab7 expression in uninfected M2 (IL-10S1) was lower than M0 (p≤0.01) while uninfected M2 (IL-10S3) was lower than M0 (p≤0.001) and M2 (IL-10) macrophages (p≤0.01) (Figure 14A). Uninfected M2 (IL-10S1) had higher Rab7 expression levels than HSV-1 infected M2 (IL-10S1V) at 6 hours (p≤0.05) (Figure 15A) but there was no significance between HSV-1 infected and uninfected cells at other times. After 24 hours both virus-infected and uninfected SOCS peptide treated M2 (IL-10S1) and M2 (IL-10S3) showed lower Rab7 expression levels (p≤0.001) than uninfected M2 (IL-10) and M0 cells (Figure 16A).

F-actin polymerization levels in RAW 264.7 murine macrophages polarized into M1 and M2 phenotypes exposed to HSV-1, SOCS1, and SOCS3 after 2, 4, 6, and 24 hour time points. The quantity of F-actin polymerization levels was determined by calculating the qualified region of interest (qROI) found by ImageJ and dividing it by the
number of cells in each image analyzed for all cell treatments. RAW 264.7 macrophages that did not receive any treatment were used as a control (M0) (Figures 17-29).

**HSV-1 infection decreased F-actin polymerization levels in M0 and SOCS1 and SOCS3 peptide treated M0 macrophages (M0S1 and M0S3).** The F-actin polymerization levels in HSV-1 infected M0V macrophages were decreased at 24 hours compared to its uninfected counterparts (p≤0.001) (Figure 21A). F-actin polymerization levels increased from 2 hours to 24 hours in uninfected M0 macrophages (Figure 17A, 18A, 19A, 21A). HSV-1 infected M0V F-actin polymerization levels did not significantly change as time passed. Uninfected M0 macrophage F-actin polymerization levels remained significantly higher than HSV-1 infected M0V at 2 hours (p≤0.05), 4 hours (p≤0.001), 6 hours (p≤0.001), and 24 hours (p≤0.001) (Figure 17A, 18A, 19A, 21A). SOCS1 and SOCS3 peptide mimetics treatment didn’t change uninfected M0 F-actin polymerization levels. Virus infection of M0S1V and M0S3V revealed lower F-actin polymerization levels compared to the uninfected M0S1 and M0S3 samples at 24 hours (p≤0.001) (Figure 20A).

**By 24 hours uninfected and HSV-1 infected M1 polarized macrophages exhibited decreased F-actin polymerization.** F-actin polymerization levels remained lower in uninfected M1 polarized cells than in uninfected M0 cells at 2 hours (p≤0.01), 4 hours (p≤0.001), 6 hours (p≤0.05), and 24 hours (p≤0.001) (Figure 17A, Figure 18A, Figure 19A, Figure 21A). F-actin polymerization decreased over time in HSV-1 infected M1V
cells by 24 hours (Figure 17A, Figure 18A, Figure 19A, Figure 21A). At 2 hours the F-actin polymerization level was higher in HSV-1 infected M1V cells than in uninfected M1 cells (p≤0.001) (Figure 17A). Then at 4 hours (p≤0.001), 6 hours (p≤0.001), and 24 hours (p≤0.01) the uninfected M1 cells have increased F-actin polymerization levels compared to HSV-1 infected M1V macrophages (Figure 18A, Figure 19A, Figure 21A).

**HSV-1 infected M1 polarized macrophages treated with SOCS3 peptide showed lower F-actin polymerization levels at 2 hour, 4 hour, and 24 hour time points.**

HSV-1 infection decreased F-actin polymerization levels in M1S3V compared to uninfected M1S3 at 2 hours (p≤0.05), 4 hours (p≤0.001), and 24 hours (p≤0.05) (Figure 17A, Figure 18A, Figure 19A, Figure 21A). Uninfected M1S1 cells showed higher F-actin polymerization levels than HSV-1 infected M1S1V at 2 hours (p≤0.01) and 6 hours (p≤0.001) (Figure 17A, Figure 19A). At 24 hours there was no significance in F-actin polymerization levels between uninfected M1S1 and HSV-1 infected M1S1V macrophages (Figure 21A). At 2 hours, 6 hours, and 24 hours there was no change in F-actin polymerization levels between M1 and M1 treated with SOCS1 or SOCS3 (Figure 17A, Figure 19A, Figure 21A). During the 4 hour time period, F-actin polymerization levels in uninfected M1S1 cells was significantly lower than in uninfected M1 macrophages (p≤0.001) (Figure 18A).

**F-actin polymerization levels in IL-4 polarized M2 macrophages were decreased by HSV-1 infection at 24 hours.** The F-actin polymerization levels of uninfected M2 (IL-4)
cells was higher than F-actin polymerization levels in virus infected M2 (IL-4) macrophages at 24 hours (p≤0.001) (Figure 21A). Uninfected M2 (IL-4) cells had increased F-actin polymerization levels over time (Figure 22A, Figure 23A, Figure 24A, Figure 25A). At 2 hours, there was no difference in F-actin polymerization levels between uninfected M2 (IL-4) or virus infected M2 (IL-4V) cells compared with F-actin polymerization levels in uninfected M0 and HSV-1 infected M0V macrophages (Figure 22A). After 4 hours F-actin polymerization levels in uninfected M2 (IL-4) cells dropped below that of M0 (p≤0.05) but virus infected M2 (IL-4V) cells had similar F-actin polymerization levels to infected M0V macrophages (Figure 23A). There were no significant changes in F-actin polymerization at 6 hours and 24 hours for uninfected M0 vs. M2 (IL-4) cells or M0V vs. M2 (IL-4V) cells (Figure 24A, Figure 25A).

**SOCS1 and SOCS3 peptide treatments decreased F-actin polymerization levels at 24 hours in uninfected M2 (IL-4) polarized macrophages.** At 24 hours uninfected SOCS peptide treated M2 (IL-4S1) and M2 (IL-4S3) both showed lower F-actin polymerization levels than did uninfected M0 and M2 (IL-4) macrophages (p≤0.001) (Figure 25A). At 2 hours, F-actin polymerization levels of uninfected SOCS1 peptide treated M2 (IL-4S1) was higher than uninfected SOCS3 peptide treated M2 (IL-4S3) cells (p≤0.001) (Figure 22A). F-actin polymerization was higher in HSV-1 infected SOCS3 peptide treated M2 (IL-4S3V) cells than in uninfected SOCS3 peptide treated M2 (IL-4S3) cells at both 2 hours (p≤0.001) and 4 hours (p≤0.01) (Figure 22A, Figure 23A). At 6 hours and 24 hours
there was no significance in F-actin polymerization levels comparing virus-infected SOCS1 or SOCS3 peptide treated macrophages with their uninfected counterparts (Figure 24A, Figure 25A).

**Uninfected M2 (IL-10) polarized macrophages showed lower F-actin polymerization levels at 2 hours, 4 hours, and 24 hours compared to M0.** At 24 hours, F-actin polymerization was lower in uninfected M2 (IL-10) macrophages than in M0 cells ($p \leq 0.001$) (Figure 29A). F-actin polymerization levels in uninfected M2 (IL-10) macrophages were significantly lower at 2 hours ($p \leq 0.05$) and 4 hours ($p \leq 0.001$) but not at 6 hours compared to uninfected M0 cells (Figure 26A, Figure 27A, Figure 28A, Figure 29A). F-actin polymerization in HSV-1 infected M2 (IL-10V) showed no significance compared to F-actin polymerization levels of uninfected M0 cells (MOV) at any time point. At 24 hours, virus infection increased F-actin polymerization levels ($p \leq 0.01$) in M2 (IL-10V) compared to the uninfected M2 (IL-10) cells (Figure 29A).

**HSV-1 infection decreased F-actin polymerization levels in M2 (IL-10) polarized macrophages treated with SOCS1 or SOCS3 peptides.** Uninfected M2 (IL-10) cells treated with SOCS1 peptide (M2 IL-10S1) showed significantly higher F-actin polymerization levels compared to virus-infected M2 (IL-10V) cells treated with SOCS1 (M2 IL-10S1V) at 4 hours ($p \leq 0.01$), 6 hours ($p \leq 0.01$), and 24 hours ($p \leq 0.001$) (Figure 27A, Figure 28A, Figure 29A). Uninfected M2 (IL-10) macrophages treated with SOCS3 peptide (M2 IL-10S3) also exhibited significantly higher F-actin polymerization levels
compared to uninfected SOCS3 treated M2 (IL-10) macrophages at 6 hours ($p \leq 0.01$) and 24 hours ($p \leq 0.05$) (Figure 28A, Figure 29A).
Figure 3: Cell viability of RAW264.7 murine macrophages after 24 hour exposure to M1, M2, and SOCS treatments: (A) Macrophages without HSV-1 infection B) Macrophages infected with HSV-1. Each bar represents mean value ± standard error (SE) of three separate experiments. ***, p ≤ 0.001.
Figure 4: Rab7 expression levels in RAW 264.7 un-polarized M0 and M1 polarized with and without SOCS 1 or 3 (M1S1/M1S3) after 2 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 5: Rab7 expression levels in un-polarized M0 and M1 polarized with and without SOCS 1 or 3 (M1S1/M1S3) after 4 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
**Figure 6:** Rab7 expression levels in RAW 264.7 un-polarized M0 and M1 polarized with and without SOCS 1 or 3 (M1S1/M1S3) after 6 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 7: Rab7 expression levels in RAW 264.7 un-polarized M0 and M1 polarized with and without SOCS 1 or 3 (M1S1/M1S3) after 24 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 8: Rab7 expression levels in RAW 264.7 un-polarized M0 with and without SOCS 1 or 3 (M0S1/M0S3) after 24 hours in both uninfected and HSV-1 infected cells.

A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 9: Rab7 expression levels in RAW 264.7 un-polarized M0 and IL-4 polarized M2 with and without SOCS 1 or 3 after 2 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 10: Rab7 expression levels in RAW 264.7 un-polarized M0 and IL-4 polarized M2 with and without SOCS 1 or 3 after 4 hours in both uninfected and HSV-1 infected cells.

A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50μm).
Figure 11: Rab7 expression levels in RAW 264.7 un-polarized M0 and IL-4 polarized M2 with and without SOCS 1 or 3 after 6 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured a 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
**Figure 12:** Rab7 expression levels in RAW 264.7 un-polarized M0 and IL-4 polarized M2 with and without SOCS 1 or 3 after 24 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 13: Rab7 expression levels in RAW 264.7 un-polarized M and IL-10 polarized M2 with and without SOCS 1 or 3 after 2 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 14: Rab7 expression levels in RAW 264.7 un-polarized M0 and IL-10 polarized M2 with and without SOCS 1 or 3 after 4 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
**Figure 15:** Rab7 expression levels in RAW 264.7 unpolarized M0 and IL-10 polarized M2 with and without SOCS 1 or 3 after 6 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using anti-Rab7 antibody. (Scale bar= 50 μm).
Figure 16: Rab7 expression levels in RAW 264.7 un-polarized M0 and IL-10 polarized M2 with and without SOCS 1 or 3 after 24 hours in both uninfected and HSV-1 infected cells. A) Quantification of Rab7 expression levels. Each value represents the mean ± standard error (SE) of three separate experiments. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Rab7 antibody. (Scale bar= 50 μm.)
**Figure 17:** F-actin polymerization levels in RAW 264.7 un-polarized M0 and polarized M1 with and without SOCS 1 or 3 (M1S1/M1S3) after 2 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤0.01. ***, p≤0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
**Figure 18:** F-actin polymerization levels in RAW 264.7 un-polarized M0 and polarized M1 with SOCS 1 or 3 (M1S1/M1S3) after 4 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50μm).
**Figure 19:** F-actin polymerization levels in RAW 264.7 un-polarized M0 and M1 polarized with and without SOCS 1 or 3 (M1S1/M1S3) after 6 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments, *p ≤ 0.05, ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
**Figure 20:** F-actin polymerization levels in RAW 264.7 un-polarized M0 with and without SOCS 1 or 3 (M0S1/M0S3) after 24 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
**Figure 21**: F-actin polymerization levels in RAW 264.7 un-polarized M0 and polarized M1 with and without SOCS 1 or 3 (M1S1/M1S3) after 24 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
Figure 22: F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-4 polarized with and without SOCS 1 or 3 after 2 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
Figure 23: F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-4 polarized with and without SOCS 1 or 3 after 4 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
Figure 24: F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-4 polarized M2 with and without SOCS 1 or 3 after 6 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
**Figure 25:** F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-4 polarized M2 with and without SOCS 1 or 3 after 24 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
Figure 26: F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-10 polarized M2 with and without SOCS 1 or 3 after 2 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
A. **F-actin Polymerization Levels in M2 (IL-10) Cells at 4H**

![Graph showing F-actin polymerization levels in M0 and IL-10 polarized M2 cells with and without SOCS 1 or 3 after 4 hours in both uninfected and HSV-1 infected cells. Each value represents the mean ± standard error (SE) of three separate experiments. **, p ≤ 0.01. ***, p ≤ 0.001.](image)

B. Uninfected Cells vs HSV-1 Infected Cells

![Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).](image)

**Figure 27:** F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-10 polarized M2 with and without SOCS 1 or 3 after 4 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
Figure 28: F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-10 polarized M2 with and without SOCS 1 or 3 after 6 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50 μm).
**Figure 29:** F-actin polymerization levels in RAW 264.7 un-polarized M0 and IL-10 polarized M2 with and without SOCS 1 or 3 after 24 hours in both uninfected and HSV-1 infected cells. A) Quantification of F-actin polymerization levels. Each value represents the mean ± standard error (SE) of three separate experiments. *, p ≤ 0.05. **, p ≤ 0.01. ***, p ≤ 0.001. B) Immunofluorescence images captured at 500x oil magnification using Texas red-Phalloidin. (Scale bar= 50μm).
DISCUSSION

RAW 264.7 murine macrophages were polarized to M1 and M2 phenotypes, infected with HSV-1, treated with SOCS peptide mimetics, and then analyzed for Rab7 expression and F-actin polymerization over 2, 4, 6, and 24 hour time periods. Cell viability and morphology were examined as well. As hypothesized, polarization treatment affected cell viability more than virus infection. Generally, HSV-1 infection did not significantly alter cell viability. However, a significant decrease in cell viability was noted in both virus infected and uninfected polarized M1, M1S1, and M1S3 macrophages (p≤0.001). ROS, NO, and TNF-α production contributed to the loss in cell viability of M1 polarized macrophages (AlSharif, 2009). There was no significant difference in cell viability between the polarized uninfected M1 and HSV-1 infected M1 subtypes.

Cell morphology changes were observed mainly at the 24 hour time point in both the uninfected and HSV-1 infected M1 polarized RAW 264.7 macrophages as hypothesized. The uninfected polarized M1 phenotypes (M1, M1S1, M1S3) appeared elongated, flattened, and presented with vacuoles as seen in Figure 7B and Figure 21B. HSV-1 infected M1 macrophages displayed both rounded and elongated shapes. Alterations taking place in the actin cytoskeleton during the virus life cycle may explain the partial resistance to morphological elongation (Reichard, 2012). At the 2 hour, 4 hour, and 6 hour time points all other cell types presented with a rounded morphology.
Immunofluorescent staining revealed that Rab7 expression increased significantly (p≤0.001) from 2 hours to 24 hours in unpolarized M0, IL-4 polarized M2, and IL-10 polarized M2 macrophages as hypothesized. In contrast, M1 polarized macrophages showed significant decreases (p≤0.001) in Rab7 expression over the 24 hours which wasn’t expected. As Rab7 expression is a marker for the maturation of endosomes it can be considered relative to endocytic performance. These results relate to those of Edin and colleagues (2013) who tested the endocytic capacity of M1 and M2 macrophage populations and found that M1 macrophages exhibited a lower endocytic ability (about 20%) compared to M2 macrophages (about 50-100%). Similar Rab7 expression values occurred in HSV-1 infected and uninfected macrophage phenotypes suggesting that HSV-1 infection does not interfere or contribute to endocytic activity in unpolarized M0, M1 polarized, IL-4 polarized M2 or IL-10 polarized M2 cells at 2, 4, 6, or 24 hours. This result does not support the hypothesis that HSV-1 infection would increase Rab7 expression. The virus may fuse with the late endosomal vesicle containing Rab7 permitting eventual exocytosis and not lysosomal fusion and degradation. Although Rab7 expression has been found to control vital roles in the endocytic pathway its specific expression levels have not previously been related to how HSV-1 exploits endocytosis/exocytosis in macrophages. Further research on the relationship between Rab7 and HSV-1 is required to better understand how the virus propagates while escaping the killing mechanism of the macrophage.
As hypothesized, both SOCS1 and SOCS3 peptide treatments decreased Rab7 expression (p≤0.001) in IL-4 polarized M2 and IL-10 polarized M2 macrophages over 24 hours. This occurred in both uninfected and HSV-1 infected phenotypes. SOCS peptides negatively regulate the JAK/STAT signaling pathway which is involved with receptor signaling in clathrin-mediated endocytosis (Chen et al., 2017). Decreased Rab7 expression following SOCS peptide treatment suggests that endocytosis becomes impaired. Uninfected and HSV-1 infected polarized M1 macrophages did not exhibit changes in Rab7 expression levels when treated with SOCS peptides. In unpolarized uninfected M0 cells only SOCS3 peptide caused a decrease in Rab7 expression at 24 hours (p≤0.001). Both SOCS1 and SOCS3 peptide treatments caused decreased Rab7 expression in HSV-1 infected unpolarized M0 macrophages at the 24 hour observation period (p≤0.001).

F-actin polymerization levels were also measured by immunofluorescent staining from 2 to 24 hours after polarization and/or HSV-1 infection. At 24 hour observation period, HSV-1 infection caused a decrease in F-actin polymerization in unpolarized M0 (p≤0.01), M1 polarized (p≤0.01), and IL-4 polarized M2 cells (p≤0.001). This was predicted to occur in all macrophage phenotypes. HSV-1 infection has been shown to increase F-actin polymerization expression in early stages of infection and then decrease F-actin polymerization at later stages, but the mechanism of assembly/disassembly or turnover is unresolved (Xiang et al., 2012). Both HSV-1 infected and uninfected M1 polarized macrophages exhibited lower F-actin polymerization levels compared to
unpolarized M0 cells at 24 hours (p≤0.001). SOCS1 and SOCS3 peptide treatment on HSV-1 infected unpolarized M0 and polarized M2 (IL-10V) macrophages reduced F-actin polymerization expression by 24 hours. SOCS peptide treatment also caused decreases in F-actin polymerization levels in uninfected polarized M2 (IL-4) cells at 24 hours. Stevenson and colleagues (2010), noted that SOCS1 and SOCS3 enhance focal adhesion kinase (FAK) expression which increases cell adhesion ultimately reducing F-actin polymerization.
FUTURE STUDIES

In future studies, flow cytometry could be used to quantify the changes in expression of Rab7 and F-actin polymerization. Immunofluorescence was useful in observing cell morphology of the macrophages secondary to polarization; however the enormous amount of data obtained from taking numerous pictures of each cell treatment may have contributed possible error in the data collected. Co-localization of Rab7 and HSV-1 in the endosomes of the various uninfected and infected M0, M1, and M2 macrophage phenotypes would reveal possible interaction distinctions. Co-localization of Rab7 expression and F-actin polymerization could be performed as well. The mechanisms and features of endocytosis whereby the SOCS1 and SOCS3 peptides caused a decrease in Rab7 expression and in F-actin polymerization could be explored. One such future study could determine whether anti-SOCS1 and anti-SOCS3 antibodies would enhance clathrin coated-pit formation on macrophages. Additionally, there are Rab adaptor proteins which exist and add more complexity to virus-host trafficking interactions. These Rab adaptor proteins should be further studied as potential targets for anti-viral therapy (Spearman et al., 2017) in the macrophage phenotypes used in the present study. Rab7 expression and F-actin polymerization in HSV-1 infected resident macrophage cell types such as Kupffer cells, microglial cells, and Langerhans cells should be studied for comparison with the results of the present study using a macrophage cell line. Pei et al. (2012) noted that the direct degradation of specific Rab proteins provides an effective strategy for pathogens to control intracellular transport.
Although inhibiting or blocking normal Rab expression has been linked to various diseases developing a particular Rab inhibitor could be useful in anti-HSV-1 therapy (Raza et al., 2018). Overall the mechanisms and implications of the Rab7 protein in its roles in endocytosis and viral infection remain unresolved. Future research has the potential to contribute to a better understanding of Rab7 for anti-viral strategies.
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