THE EFFECTS OF HSV-1 CHALLENGE ON POLARIZED MURINE MACROPHAGES: AN IN VITRO MODEL USING THE J774A.1 MURINE MACROPHAGE CELL LINE

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

Master of Science.

By

ADAM REICHARD

B.S., Wright State University, 2010

2012

Wright State University

Nancy J. Bigley, Ph.D.
Thesis Director

Committee on Final Examination

Nancy J. Bigley, Ph.D.
Professor of Microbiology and Immunology

Barbara E. Hull, Ph.D.
Director of Microbiology and Immunology Program, College of Science and Mathematics

Barbara E. Hull, Ph.D.
Professor of Biological Sciences

Julian Gomez-Cambronero, Ph.D.
Professor, Department of Biochemistry & Molecular Biology

Andrew T Hsu, Ph.D.
Dean, Graduate School
Reichard, Adam. M.S. Department of Microbiology and Immunology, Wright State University, 2012. The Effects of HSV-1 Challenge on Polarized Murine Macrophages: An In Vitro Model Using the J774A.1 Murine Macrophage Cell Line.

In our current study we examined the effects of HSV-1 challenge on J774A.1 macrophages polarized to either a proinflammatory (M1) or anti-inflammatory (M2) phenotype. Polarized J774A.1 macrophages were characterized using CD14-CD86 and SOCS1-SOCS3 expression levels. SOCS proteins are a family of proteins that are capable of inhibiting cytokine-signaling pathways. HSV-1 up regulates expression of SOCS1 protein levels in infected cells, inhibiting the ability of infected cells to produce proinflammatory products (Nowoslawski Akhtar and Benveniste, 2011). This study shows that signals within the microenvironment play a greater role in macrophage polarization, and SOCS1-SOCS3 expression levels, than does HSV-1 challenge. M1 macrophages showed morphological changes following polarization, a significant decrease in cell viability, a two-fold increase in the number of CD14⁺-CD86⁺ cells, similar levels of SOCS1 expression, and a 11-fold decrease in SOCS3 expression when compared to control cells. M2 macrophages also exhibited morphological changes, a slight decrease in cell viability, a 26.0% decrease in the number of CD14⁺-CD86⁺ cells, and SOCS1-SOCS3 expression levels similar to that of control cells. Following HSV-1 challenge (0.1 MOI), the majority of M1 macrophages and M2 macrophages appeared rounded, possibly due to disruption of actin filaments. Virus-infected M1 macrophages
showed a slight decrease in cell viability when compared to uninfected M1 macrophages. Additionally, the number of CD14^-CD86^ cells of both M1 and M2 phenotypes decreased. M1 macrophages exhibited a 39.9% decrease, while M2 macrophages exhibited a 13.2% decrease. SOCS1 expression levels remained relatively unchanged in virus-infected M1 macrophages, while SOCS3 expression levels increased by 30.4% at 24 hours after infection. Increase in SOCS3 levels is hypothesized to be a protective response of infected M1 macrophages due to the release of high levels of proinflammatory molecules. Alternatively, the SOCS1-SOCS3 ratio remained relatively unchanged in the anti-inflammatory phenotype.
Table of Contents

INTRODUCTION.............................................................................................................1

LITERATURE BACKGROUND AND PRELIMINARY OBSERVATIONS..............5

HSV-1.........................................................................................................................5

Macrophage Function and Polarization.................................................................6

Cluster of Differentiation Markers.........................................................................8

Cell Viability.............................................................................................................8

SOCS Proteins.........................................................................................................9

MATERIALS AND METHODS.......................................................................................10

Cell Lines................................................................................................................10

Polarization Treatment..............................................................................................10

Cell Viability.............................................................................................................11

TNF-α Neutralization...............................................................................................11

Immunofluorescent Staining......................................................................................12

Flow Cytometry........................................................................................................14

Virus Challenge........................................................................................................15

Statistical Analysis...................................................................................................16

RESULTS......................................................................................................................17

DISCUSSION..............................................................................................................24

FIGURES....................................................................................................................28
REFERENCES........................................................................................................61
List of Figures and Tables

Figure 1: J774A.1 Macrophage Morphology and Actin Arrangement Following Polarization..........................................................28

Figure 2: Immunofluorescent Images of M1 Macrophages Stained with anti-CD14, anti-CD86, and Phalloidin.........................................................30

Figure 3: Immunofluorescent Images of M2 Macrophages Stained with anti-CD14, anti-CD86, and Phalloidin.................................................................31

Figure 4: Immunofluorescent Images of Control Macrophages Stained with anti-CD14, anti-CD86, and Phalloidin..................................................................................32

Figure 5: Flow Cytometry Analysis of CD14-CD86 Expression Levels in M1, M2, and Control Macrophages..........................................................33

Figure 6: CD14-CD86 Expression Level Averages for M1, M2, and Control Macrophages..........................................................................................34

Figure 7: Cell Viability of Polarized and Control Macrophages..........................................................35

Figure 8: Immunofluorescent Images of M1 Macrophages Stained with anti-SOCS1 and anti-SOCS3 Antibodies..................................................................................36

Figure 9: Immunofluorescent Images of M2 Macrophages Stained with anti-SOCS1 and anti-SOCS3 Antibodies..................................................................................37

Figure 10: Immunofluorescent Images of Control Macrophages Stained with anti-SOCS1 and anti-SOCS3 Antibodies..................................................................................38
Figure 11: Flow Cytometry Analysis of SOCS1-SOCS3 Expression Levels in M1 Macrophages..................................................................................................................39

Figure 12: Flow Cytometry Analysis of SOCS1-SOCS3 Expression Levels in M2 Macrophages..................................................................................................................40

Figure 13: Flow Cytometry Analysis of SOCS1-SOCS3 Expression Level in Control Macrophages...........................................................................................................41

Figure 14: SOCS1-SOCS3 Ratios for M1, M2, and Control Macrophages............42

Figure 15: Virus-Infected J774A.1 Macrophage Morphology and Actin Arrangement Following Polarization....................................................................................................43

Figure 16: Immunofluorescent Images of Virus-Infected M1 Macrophages Stained with anti-CD14, anti-CD86, and Phalloidin.................................................................44

Figure 17: Immunofluorescent Images of Virus-Infected M2 Macrophages Stained with anti-CD14, anti-CD86, and Phalloidin.................................................................45

Figure 18: Immunofluorescent Images of Virus-Infected Control Macrophages Stained with anti-CD14, anti-CD86, and Phalloidin.................................................................46

Figure 19: Virus-Infected J774A.1 Macrophage Flow Cytometry Analysis of CD14-CD86 Expression Levels in M1, M2, and Control Macrophages..........................47

Figure 20: CD14-CD86 Expression Level Averages in Virus-Infected M1, M2, and Control Macrophages.........................................................................................48

Figure 21: CD14-CD86 Expression Level Averages in Uninfected M1, M2, and Control Macrophages and Their Virus-Infected Counterparts.................................49
List of Abbreviations

BSA = Bovine serum albumin
CD14 = Cluster of differentiation 14
CD86 = Cluster of differentiation 86
CD206 = Cluster of differentiation 206
DMEM = Dulbecco's Modified Eagle's Medium
FCS = Fetal calf serum
HSV-1 = Herpes simplex virus type 1
IFN-γ = Interferon-gamma
IL-4 = Interleukin-4
IL-13 = Interleukin-13
iNOS = Inducible nitric oxide synthase
KIR = Kinase inhibitory region
LPS = Lipopolysaccharide
MOI = Multiplicity of infection
PBS = Phosphate buffered saline
PFU = Plaque forming units
SOCS1 = Suppressor of cytokine signaling-1
SOCS3 = Suppressor of cytokine signaling-3
TNF-α = Tumor necrosis factor-alpha
Acknowledgement

I would like to thank Dr. Nancy Bigley for all the support she has provided me over the past two years. Her guidance has been invaluable and I am vastly grateful for her contribution to my work. I would also like to thank my program director, Dr. Barbara Hull. I am very appreciative of the suggestions and guidance she provided during the duration of my project. I would like to thank Dr. Julian Gomez-Cambronero for his suggestions and contribution to my research. I would like to thank the Sigma Xi Scientific Research Society for their financial contribution to my work. Lastly, I would like to thank my parents for supporting my decision to continue my education.
Dedication

I would like to dedicate my thesis project to my amazing wife for her unconditional love and never ending support. She is my inspiration and has provided me with constant encouragement while in graduate school.
Introduction

Herpes simplex virus type-1 (HSV-1) is a dsDNA virus that currently affects approximately 70-80% of adults within the United States (Roizman et al., 2007; Dakvist et al., 1995; Miller et al., 1998; Stock et al., 2001). Under normal conditions, a latent infection is established and maintained within the host. If the host immune system is compromised, the virus can be reactivated, resulting in a lytic infection (Cunningham et al., 2006; Diefenbach et al., 2008; Koelle and Corey, 2008; Roizman et al., 2007). Lytic infections clinically manifest as mild cutaneous disease. At a lesser frequency HSV-1 reactivation can result in infection of the corneal epithelium and possibly lead to blindness (Cheng et al., 2000).

The host immune response to HSV-1 infection is very complex and involves cells of both the innate and adaptive immune system. The innate immune response to HSV-1 infection is comprised of three main cell types: natural killer cells (NK), macrophages, and γ/δ T cells. These cells are recruited to the site of infection and activated when infected keratinocytes release high levels of cytokines. This release of cytokines activates innate immune cells that attempt to control the infection by killing infected cells and inhibiting virus replication (Cunningham et al., 2006; Mikloska et al., 1998). Even though the microenvironment during HSV-1 infection is incredibly complex, it is well established that macrophages play a pivotal role in controlling HSV-1 replication. Macrophages are capable of inhibiting virus replication within themselves when infected.
Additionally, macrophages can inhibit the viral activity of extracellular particles, and possess the ability to target and destroy virus-infected cells, slowing virus replication in infected neighboring cells (Wu and Morahan, 1992). In this study the J774A.1 murine macrophage cell line was selected due to the important role macrophages play in controlling HSV-1 infections.

Macrophages are considered "professional" phagocytic cells, meaning, they express a wide variety of cell surface receptors allowing them to recognize signals not normally found within the host. Signals present within the microenvironment can alter macrophage function and lead to multiple effector subpopulations (Murray and Wynn, 2011). This ability to alter function is known as macrophage "polarization". The two polarized macrophage populations we examined in this study are known as M1 and M2 macrophages; however, there are multiple M2 like subtypes that are currently known. M1 macrophages are a proinflammatory, "classically" activated, population that secrete high amounts of proinflammatory cytokines, such as inducible nitric oxide synthases (iNOS) and tumor necrosis factor-α (TNF-α), following activation by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS). M2 macrophages are activated by interleukin-4 (IL-4) or interleukin-13 (IL-13), and are considered anti-inflammatory due to the molecules they release, such as interleukin-10 (IL-10), that lead to tissue remodeling and angiogenesis (Junliang et al., 2010; Kigerl et al., 2009; Wang et al., 2010).

In our preliminary studies we found that polarization treatment leads to morphological changes. Following IFN-γ and LPS treatment, M1 macrophages appeared
flattened, were irregularly shaped, and contained many visible intracellular vacuoles. M1 treatment also led to a decrease in cell viability, possibly due to IFN-γ/TNF-α toxicity (Kyoungho et al., 2001). M2 treatment induced cells with an elongated morphology, similar to that seen in control macrophages cultures in the majority of cells following IL-4 treatment. Untreated control macrophages appeared as round or elongated in culture.

In order to identify accurately the M1 and M2 phenotype following treatment, it was necessary to characterize markers that accurately discriminated between the two phenotypes. Cluster of differentiation marker 14 (CD14) and cluster of differentiation 86 (CD86) were expressed at different levels in the two phenotypes, making CD14 and CD86 effective markers for identification of the M1 and M2 subpopulations. M1 macrophages showed a significantly higher number of CD14⁺-CD86⁺ cells when compared to control cells, while M2 macrophages showed a significant decrease in the number of CD14⁺-CD86⁺ cells.

Suppressor of cytokine signaling (SOCS) proteins are frequently manipulated by viruses to maintain an infection within the host (Nowoslawski Akhtar and Benveniste, 2011). The function of the different SOCS proteins is to inhibit the cytokine-signaling pathway, thereby influencing the inflammatory response (Nowoslawski Akhtar and Benveniste, 2011). SOCS proteins can be quickly up regulated in macrophages, making SOCS protein expression levels a target of observation in our study (Whyte et al., 2011). Our goal was to determine the effects HSV-1 challenge plays on morphology, CD14-CD86 expression, cell viability, and SOCS protein levels in M1 and M2 macrophages.
Hypothesis: The cytokines/signals present within the microenvironment play a greater role in macrophage polarization, than infection with HSV-1.
Literature Background and Preliminary Observations

HSV-1

Herpes simplex virus is a dsDNA virus that undergoes replication within the cell nucleus of the host (Roizman et al., 2007). HSV-1 infections are found throughout the world, and under normal circumstances, only infect humans (Whitley and Roizman, 2001). It is estimated that HSV-1 affects approximately 70-80% of adults (Dakvist et al., 1995; Miller et al., 1998; Stock et al., 2001), the virus remains latent until reactivation leads to cutaneous or mucocutaneous disease, which is most often mild (Arduino and Porter, 2006). At a lesser frequency HSV-1 can infect the cornea resulting in corneal scarring, and when high infectious doses are used, can lead to fatal encephalopathy in mice (Cheng et al., 2000).

HSV-1 gains entry into cells of the mucosal membrane by attaching to the cell surface by way of glycoproteins gB, gC, gD, gH, and gL. Two additional glycoproteins, gE and gI, facilitate HSV-1 infection of neighboring cells (Rajcani et al., 2000). Following infection of the mucosal membrane, sensory nerve fibers retrogradely transport virus to the neuronal cell body of the trigeminal, or dorsal root ganglion where a latent infection is established and maintained. The virus can periodically reactivate and anterogradely travel to the original site of infection, leading to cutaneous disease (Cunningham et al., 2006; Diefenbach et al., 2008; Koelle and Corey, 2008; Roizman et al., 2007).
The microenvironment during HSV-1 infections involves many cell types that are part of a complex immune response to control virus replication and destroy infected cells. Upon infection keratinocytes secrete large amounts of cytokines that lead to the recruitment and activation of NK cells, macrophages, and γ/δ T cells (Cunningham et al., 2006; Mikloska et al., 1998).

Even though the microenvironment influences many cell types, macrophages play a pivotal role in controlling virus replication during HSV-1 infections (Staats et al., 1991; Johnson, 1964). One possible mechanism is the ability of a macrophage to inhibit virus replication within itself, and the second mechanism involves the ability of a macrophage to inhibit the activity of extracellular virus particles, destroy virus-infected cells, and slow virus replication in infected neighboring cells (Wu and Morahan, 1992). In the present study the J774A.1 murine macrophage cell line was selected due to the vital role macrophages play during HSV-1 infection.

**Macrophage Function and Polarization**

Haematopoietic stem cells, which are located in bone marrow, differentiate into monocytes and are released into circulation. Monocytes differentiate to macrophages and dendritic cells (DC) once they pass through the endothelium and take up residence within tissues. Therefore, the main role of monocytes is to replenish macrophages and DCs that reside in tissue. Macrophages are "professional" phagocytic cells that express a wide variety of surface receptors recognizing signals that are not normally expressed by tissues
within the host, making them a vital component of the host immune system. Macrophages are widely distributed within the body and alter their function depending on signals present in the microenvironment (Murray and Wynn, 2011). This ability to differentiate into different macrophage subpopulations is known as macrophage "polarization".

Macrophages exist as multiple subpopulations within the host, and depending on which signals are present, can respond in either a proinflammatory or anti-inflammatory manner. When treated with IFN-γ and LPS, macrophages respond by producing proinflammatory molecules such as iNOS and TNF-α, and are termed M1 macrophages (Junliang et al., 2010; Kigerl et al., 2009; Wang et al., 2010). Preliminary experiments with IFN-γ/LPS in this study showed that M1 macrophages exhibited morphological differences when compared to control cells. The M1 macrophage response is necessary to control virus replication, however, a consequence of inflammation is host tissue damage. In regards to corneal HSV-1 infections, inflammation is responsible for corneal scarring and can lead to blindness.

When macrophages are treated with IL-4 or IL-13, they respond by releasing anti-inflammatory molecules that lead to tissue remodeling and angiogenesis. These anti-inflammatory macrophages are known as the M2 macrophage subpopulation (Junliang et al., 2010; Kigerl et al., 2009; Wang et al., 2010). Additional types of M2-like macrophages exist. Ligation of the TGF-β receptor, IL-10 receptor, or FC gamma receptors in the presence of LPS, has been shown to lead to multiple M2 like
subpopulations (Martinez et al., 2008; Gordan and Martinez, 2010; Mosser and Edwards, 2008). The balance between proinflammatory and anti-inflammatory macrophage populations is important clinically because different macrophage subtypes have been shown to exacerbate certain disease processes (Junliang et al., 2010; Cheng et al., 2000).

**Cluster of Differentiation Markers**

In our study it was necessary to establish markers that differentiated between the M1 and M2 subtypes. In order to do this we looked at differences in expression levels of two specific cell surface receptors found on macrophages, cluster of differentiation 14 (CD14) and cluster of differentiation 86 (CD86). CD14 has been shown to form a receptor complex with TLR4; this receptor complex binds LPS with high affinity, resulting in a signaling cascade and the activation of multiple transcription factors (Dobrovolskaia and Vogel, 2002). CD86 (B7-2) is a co-stimulatory molecule found on the surface of antigen presenting cells. Co-stimulation of naive T cells with CD86 leads to T cell activation and survival (Sugamura et al., 2004).

**Cell Viability**

Cell viability experiments showed that there was a decrease in the number of viable cells following M1 treatment. Since M1 macrophages secrete high levels of TNF-α (Junliang et al., 2010; Kigerl et al., 2009; Wang et al., 2010); this decrease in cell viability was attributed to potential IFN-γ/TNF-α toxicity. Kyoungho et al. (2001), found
that IFN-γ and TNF-α together led to death of pancreatic islet cells through activation of the classical caspase-dependent apoptotic pathway. However, IFN-γ or TNF-α alone did not lead to apoptosis, implying both are required for toxicity. In order to prove that the presence of IFN-γ and TNF-α in culture led to a decrease in cell viability, anti-TNF-α neutralizing antibodies were added to M1 cell cultures to protect cells from the toxic effects of IFN-γ and TNF-α. Only minimal protection occurred in these 24-hour cultures. Since the action of anti-TNF-α antibodies takes place slowly (18-24 hours), further exploration of this decrease in cell viability is necessary.

**SOCS Proteins**

Suppressor of cytokine signaling (SOCS) proteins have been shown to be a frequent target for viral manipulation (Nowoslawski Akhtar and Benveniste, 2011). Eight members of the SOCS protein family have been identified; all of them can be rapidly expressed in macrophages (Whyte et al., 2011). The main function of SOCS proteins is to limit the inflammatory response while still maintaining a sufficient immune response to clear pathogens. Therefore, regulation of SOCS proteins is crucial in ensuring the immune response is sufficient enough to clear pathogens, while avoiding excessive host tissue damage. SOCS1 and SOCS3 possess a kinase inhibitory region (KIR), the main function of which is to inhibit kinase activity, and ultimately inhibit signaling thorough the JAK/STAT pathway (Nowoslawski Akhtar and Benveniste, 2011).
Materials and Methods

Cell Lines

The J774A.1 murine macrophage cell line (ATCC) is a reticulum cell sarcoma, adherent macrophage cell line derived from an adult female BALB/cN mouse. J774A.1 cells were propagated in 25 cm² vented cap cell culture flasks and incubated at 37°C and 5% CO₂ using a water-jacketed incubator. Cells were cultured in a medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal bovine calf serum (FCS). Cells were subcultured at a subcultivation ratio of 1:6, two to three times weekly. Cell culture flasks, growth medium, and fetal bovine calf serum were purchased from Fisher Scientific.

Polarization Treatment

J774A.1 macrophages were grown to approximately 50% confluency, at which time the polarization treatment was administered. To induce the M1 phenotype J774A.1 macrophages were treated with IFN-γ (20 ng/mL) and LPS (100 ng/mL) for 24 hours; IL-4 (20 ng/mL) was used to induce the M2 phenotype. After 24 hours cells were removed from the cell culture flasks using a cell scraper so they could be further analyzed. The murine cytokines (IFN-γ and IL-4) were purchased from Peprotech; LPS was purchased from Chondrex.
Cell Viability

J774A.1 macrophages were grown to approximately 50% confluency, at which time either IFN-γ/LPS (M1) or IL-4 (M2) were added with or without virus. Untreated cells were used as a control. After 24 hours cells were removed from the cell culture flasks using a cell scraper. Cells were then centrifuged at 4°C for 5 minutes to obtain a cell pellet. The supernatant was aspirated following centrifugation and the cell pellet was resuspended in 1 mL of complete growth medium. Trypan blue (Fisher Scientific) was used to determine viability, and a hemocytometer was used to obtain cell counts.

TNF-α Neutralization

Protocol 1: J774A.1 macrophages were grown to approximately 50% confluency, at which time IFN-γ and LPS were added for 24 hours simultaneously with anti-TNF-α neutralizing antibodies. After 24 hours cells were removed using a cell scraper and centrifuged at 4°C for 5 minutes to obtain a cell pellet. The supernatant was aspirated and 1 mL of complete growth medium was used to resuspend the cell pellet. Trypan blue was used to determine viability and a hemocytometer was used to obtain counts of visible cells.

Protocol 2: J774A.1 macrophages were grown to approximately 50% confluency, at which time IFN-γ and LPS was added for 24 hours. After 24 hours the supernatant was collected and separated into two 15 mL centrifuge tubes. Anti-TNF-α neutralizing
antibodies were added to one of the 15 mL centrifuge tubes, both 15 mL centrifuge tubes were then placed in the incubator (37°C and 5% CO₂) for 24 hours. After 24 hours the supernatant was added to untreated cells for an additional 24 hours, after which time the cells were collected using a cell scraper. Cells were then centrifuged at 4°C for 5 minutes in order to obtain a cell pellet. After centrifugation the supernatant was aspirated and the cell pellet was resuspended using 1 mL of complete growth medium. Trypan blue was used to determine cell viability and a hemocytometer was used to obtain counts of viable cells.

**Immunofluorescent Staining**

Cells were grown in slide chambers (Fisher Scientific) to approximately 50% confluency, at which time either IFN-γ and LPS (M1) or IL-4 (M2) were added for 24 hours with and without virus. Untreated cells were used as an experimental control. Following polarization treatment, cells were rinsed with 1% bovine serum albumin (BSA) suspended in phosphate buffered saline (PBS). Cells were then fixed using 4% Paraformaldehyde/PBS (15 minutes at room temperature) and permeabilized using ice-cold acetone (10 minutes at -20°C). Blocking buffer (5% serum from species of fluorochrome-conjugated primary antibody or fluorochrome-conjugated secondary antibody, 3% BSA, suspended in PBS) was added for 1 hour at room temperature. Following blocking, antibodies were diluted in blocking buffer and added per recommended concentration from distributor. Slide chambers were then incubated
overnight at 4°C. For fluorochrome-conjugated primary antibodies (CD14, CD86) and Phalloidin, cells were rinsed following incubation and a cover slip was added using VectaShield hard set mounting medium (VectaShield from Vector Laboratories). For primary antibodies not fluorochrome-conjugated (SOCS1 and SOCS3), cells were rinsed following incubation, at which time a fluorochrome-conjugated secondary antibody was added for 1 hour (1:50 dilution) at room temperature. A coverslip was then added using VectaShield hard set mounting medium. Fluorescence microscopy was used to analyze slides. Anti-CD14 and anti-CD86 antibodies were purchased from BioLegend; anti-SOCS1 and anti-SOCS3 antibodies were a gift from Dr. Howard Johnson (University of Florida); FITC-conjugated secondary antibody was purchased from Invitrogen; Texas Red-Phalloidin X was purchased from Life Technologies. *Between each step cells were rinsed three times for three minutes using 1% BSA/PBS.

<table>
<thead>
<tr>
<th>Antibody/Stain</th>
<th>Concentration/Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC anti-mouse CD14</td>
<td>0.25 µg/million cells</td>
</tr>
<tr>
<td>Brilliant Violet 421 anti-mouse CD86</td>
<td>5 µL/million cells</td>
</tr>
<tr>
<td>Texas Red-Phalloidin X</td>
<td>5 µL/million cells</td>
</tr>
<tr>
<td>anti-mouse SOCS1 (rabbit) and anti-mouse SOCS3 (rabbit)</td>
<td>10 µg/million cells</td>
</tr>
<tr>
<td>FITC anti-rabbit secondary (goat)</td>
<td>1:50</td>
</tr>
</tbody>
</table>
Flow Cytometry

Cells were grown to approximately 50% confluency in cell culture flasks, at which point M1 or M2 treatment was administered with or without virus. After 24 hours of treatment, cells were removed using a cell scraper and centrifuged at 4°C for 5 minutes in order to obtain a cell pellet. After centrifugation the supernatant was aspirated and the cell pellet was resuspended using 1 mL of complete growth medium. A hemocytometer was used to obtain viable cell counts; one million cells were used for each sample.

Cell surface staining (CD14 and CD86): Cells were rinsed three times with 1% BSA/PBS. For cell surface markers no permeabilization step was required. Blocking buffer (5% mouse serum/3% BSA/PBS) was added to cells for 45 minutes at room temperature. Following blocking, antibodies were diluted in blocking buffer and added to cells per concentrations suggested by distributor for 45 minutes at 4°C. After antibody incubation, cells were fixed using 4% Paraformaldehyde/PBS for 15 minutes at room temperature. Cells were then rinsed three times and resuspended in 250 µL of ice cold PBS with 0.5% sodium azide. Samples were then analyzed using an Accuri C6 flow cytometer.

Intracellular staining (SOCS1 and SOCS3): Cells were rinsed three times with 1% BSA/PBS. After rinsing cells were fixed with 4% Paraformaldehyde/PBS for 15 minutes at room temperature. Cells were then rinsed three times. Next, cells were permeabilized using 0.2% Triton-X/PBS for 15 minutes at room temperature. Cells were then rinsed
three times. Blocking buffer (5% goat serum/3% BSA/PBS) was added to cells for 1 hour at room temperature. Following blocking, SOCS1 and SOCS3 antibodies were suspended in blocking buffer at a concentration of 10 µg/million cells and added to cells for 45 minutes at 4°C. Cells were then rinsed three times. After rinsing, FITC-conjugated secondary antibody was diluted in blocking buffer (1:50 dilution) and added to cells for 45 minutes at 4°C. Cells were then rinsed three times and suspended in ice cold PBS with 0.5% sodium azide. Samples were analyzed using an Accuri C6 flow cytometer.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration/Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC anti-mouse CD14</td>
<td>0.25 µg/million cells</td>
</tr>
<tr>
<td>PE/Cy5 anti-mouse CD86</td>
<td>0.25 µg/million cells</td>
</tr>
<tr>
<td>anti-mouse SOCS1 (rabbit) and anti-mouse SOCS3 (rabbit)</td>
<td>10 µg/million cells</td>
</tr>
<tr>
<td>FITC anti-rabbit secondary (goat)</td>
<td>1:50</td>
</tr>
</tbody>
</table>

**Virus Challenge**

Cells were grown to approximately 50% confluency in cell culture flasks. Cells were then removed using a cell scraper and centrifuged at 1500 revolutions per minute (4°C) for 5 minutes. After centrifugation the supernatant was aspirated and the cell pellet was resuspended using 1 mL of complete growth medium. Packed cell volume (PCV) cell counting tubes (MidSci) were used to obtain cells counts so multiplicity of infection
(MOI) could be accurately calculated. Cells were added to cell culture flasks; M1 and M2 treatments were then administered simultaneously with 0.1 MOI of HSV-1. After 24 hours cells were collected so they could be further analyzed.

**Statistical Significance**

Statistical significance was calculated using a paired t-test (SigmaPlot 12.0); all experiments were completed at least twice.
Results

Polarized Macrophages Exhibited Morphological Changes When Compared to Control Macrophages

Following treatment with IFN-γ and LPS for 24 hours, M1 macrophages appeared flattened, were irregularly shaped, and contained visible intracellular vacuoles (Fig. 1). The M2 phenotype exhibited an elongated cellular morphology following treatment with IL-4 (Fig. 1) when compared to control cells, which included cells with both a rounded and elongated morphology (Fig. 1). The morphological changes exhibited by M1 and M2 macrophages made it possible to differentiate between the two phenotypes based on morphology alone.

CD86 is Up Regulated in Uninfected M1 Macrophages and Down Regulated in Uninfected M2 Macrophages

CD86 is up regulated in macrophages treated with IFN-γ and LPS, making it an accurate identifier of the M1 phenotype (Fig. 2, Fig. 5, and Fig. 6) when used in conjunction with CD14 expression. Additionally, CD14-CD86 is down regulated following IL-4 treatment, allowing for identification of the M2 phenotype (Fig. 3, Fig. 5, and Fig. 6) (Kigerl et al., 2009). Therefore, an increase in the number of CD14⁻-CD86⁺ cells was used to identify the M1 phenotype, while a decrease in the number of CD14⁺-
CD86$^+$ cells was used to identify the M2 phenotype. Untreated cells were used as an experimental control for CD14$^-$-CD86$^+$ expression (Fig. 4, Fig. 5, and Fig. 6).

**Uninfected M1 Macrophages Exhibited a Decrease in Cell Viability Following Treatment with IFN-γ and LPS for 24 Hours**

Cell viability experiments showed a decrease in the number of viable cells following treatment with IFN-γ and LPS for 24 hours (Fig. 7). M1 macrophages showed a 31.0% decrease in the number of viable cells when compared to control cells. M2 macrophages exhibited on a 9.0% decrease, following IL-4 treatment for 24 hours, when compared to control cells. The decrease in M1 macrophage cell viability is due to potential toxicity from secreted pro-inflammatory products.

**M1 Macrophages Showed High Expression Levels of SOCS1 in Comparison to SOCS3, while Both M2 and Control Macrophages Expressed Higher Levels of SOCS3 in Comparison to SOCS1**

Following polarization, M1 macrophages expressed higher levels of SOCS1 when compared to SOCS3, resulting in a 6.1:1 SOCS1-SOCS3 ratio (Fig. 8, Fig. 11, and Fig. 14), while M2 macrophages expressed more SOCS3 than SOCS1 resulting in a SOCS1-SOCS3 ratio of 1:2.2 (Fig. 9, Fig. 12, and Fig. 14). Control macrophages expressed SOCS proteins similarly to that of M2 macrophages with a SOCS1-SOCS3 ratio of 1:1.9 (Fig. 10, Fig. 13, and Fig. 14).
Virus Challenge Leads to Morphological Changes in Polarized and Control Macrophages

Uninfected control macrophages included both round and elongated cells. Uninfected M1 macrophages were flattened, irregularly shaped, and contained visible intracellular vacuoles, while M2 macrophages exhibited an elongated morphology. Following challenge with 0.1 MOI of HSV-1, M1 macrophages appeared rounded (Fig. 15). Similar morphological changes were observed in M2 macrophages and control macrophages when virus was introduced to cultures (Fig. 15). Therefore we concluded that viral challenge leads to a rounded morphology in virus-infected polarized and control macrophages. Consequently, virus induced morphological changes made it difficult to differentiate between phenotypes based on morphology alone.

HSV-1 Infection Decreases Expression of CD14 and CD86 in M1, M2, and Control Macrophages

CD 14 and CD 86 were expressed by both M1 and M2 macrophages, but at varying levels, allowing both CD14 and CD86 to be used as markers to discriminate between the two phenotypes. Before virus challenge, M1 macrophages showed an increase (41.2%) in the number of CD14^+^-CD86^+^ cells when compared to control cells. Conversely, M2 macrophages exhibited a decrease (26.9%) in the number of CD14^+^-CD86^+^ cells. This allowed us to correlate an increase in CD14^+^-CD86^+^ expression to the
M1 phenotype and a decrease to the M2 phenotype. Following challenge with 0.1 MOI of HSV-1, overall expression of CD14 and CD86 was decreased. Immunofluorescent images of virus-infected M1 macrophages stained with anti-CD86 antibodies showed an increase in CD86 expression in comparison to virus-infected control cells (Fig. 16 and Fig. 18). M2 macrophages stained with anti-CD86 antibodies did not show any observable differences when compared to virus-infected control cells (Fig. 17 and Fig. 18). Flow cytometric analysis of virus-infected M1 macrophages showed an increase in the number of CD14\(^+\)-CD86\(^+\) cells when compared to virus-infected control cells (Fig. 19 and Fig. 20), while the M2 phenotype showed a decrease (Fig. 19 and Fig. 20). When compared to their uninfected counterparts, CD14-CD86 expression in virus-infected M1, M2, and control cells was decreased. Virus-infected M1 macrophages showed a 39.4% decrease (p-value=0.001) and virus-infected M2 macrophages showed a decrease of 13.2% (p-value=0.039) (Fig. 21). Virus-infected control macrophages also showed a significant decrease of 27.8% (p-value=0.022) (Fig. 21). These results suggest that HSV-1 infection decreases the ability of polarized and control macrophages to express both CD14 and CD86. While CD14 and CD86 may not play a direct role in the innate immune response to HSV-1 infection, CD14-CD86 expression is an indicator of a macrophages ability to mount an effective proinflammatory immune response. CD86 is an important surface molecule that stimulates naive T cells and leads to their maturation and activation. T cells play an important role in the adaptive immune response to HSV-1.
infection, signifying the importance of CD86 expression levels on the surface of macrophages found at the infection site.

**Following IFN-γ and LPS, Virus-Infected Macrophages Experience a Significant Decrease in Cell Viability**

Following IFN-γ and LPS treatment, virus-infected M1 macrophages exhibited a decrease in the number of viable cells when compared to virus-infected control cells (Fig. 22). This decrease in cell viability may be due to potential IFN-γ/TNF-α toxicity. In order to demonstrate this we added anti-TNF-α neutralizing antibodies to culture at the same time point as M1 polarization treatment and virus treatment. However, only a slight, statistically insignificant, increase resulted. It is well documented that M1 macrophages secrete high levels of TNF-α. In our study we found that M1 polarization can occur as quickly as 12 hours, leading to TNF-α production. The anti-TNF-α neutralizing antibodies require 24 hours to effectively neutralize TNF-α. This time frame does not allow the neutralizing antibody enough time to neutralize newly synthesized TNF-α, rendering the anti-TNF-α neutralizing antibody ineffective. Therefore, more work needs to be completed. Nevertheless, there was a statistically significant decrease in viability in virus-infected M1 macrophages when compared to virus-infected control cells by an unknown mechanism.
Virus Challenge Up Regulates SOCS3 Expression in M1 Macrophages, and SOCS1 Expression in Control Cells

Immunofluorescent images showed that virus-infected M1 macrophages expressed comparable levels of SOCS1 and SOCS3, while virus-infected M2 macrophages expressed higher levels of SOCS3 compared to SOCS1 (Fig. 23 and Fig. 24). Virus-infected control cells showed an increase in SOCS1 expression compared to uninfected control cells, while SOCS3 was decreased in control cells following virus infection (Fig. 25). Flow cytometric analysis of virus-infected M1 macrophages suggested that virus challenge leads to up regulation of SOCS3 (Fig. 26). Following virus challenge, M1 macrophages exhibited a SOCS1-SOCS3 ratio of 1:1.1, compared to a SOCS1-SOCS3 ratio of 6.1:1 in uninfected M1 macrophages (Fig. 29 and Table 1). These data suggest the microenvironment signals that lead to M1 polarization, play a greater role in polarization and activation of the M1 subset than does challenge with HSV-1. SOCS expression levels appeared relatively unchanged in virus-infected M2 macrophages when compared to uninfected M2 macrophages (Fig. 27). Both samples expressed significantly higher levels of SOCS3 compared to SOCS1. Virus-infected M2 macrophages exhibited a SOCS1-SOCS3 ratio of 1:1.8, while uninfected M2 macrophages cells exhibited a ratio of 1:2.2, once again signifying the importance of microenvironment signals (Fig. 29 and Table 1). Virus-infected control cells showed high expression levels of SOCS1 when compared to SOCS3 (Fig. 28). By contrast, the SOCS1-
SOCS3 ratio in virus-infected control cells was 3.8:1, while uninfected control cells exhibited a ratio of 1:1.9 (Fig. 29 and Table 1).
Discussion

In this study J774A.1 macrophages were polarized to either the M1 or M2 phenotype. M1 macrophages were flattened, irregularly shaped, and contained visible intracellular vacuoles. Uninfected M1 macrophages were more adherent when compared to uninfected M2 and uninfected control macrophages. Uninfected M2 macrophages developed into elongated cells, which more closely resembled uninfected control cells, which included both elongated and rounded cells. Following challenge with virus, the majority of cells in M1, M2, and control cultures were rounded, making it difficult to differentiate based on morphology. These data suggest virus infection leads to a rounded morphology in polarized and control cells, making it difficult to identify the M1 and M2 phenotypes based on morphology. This rounded morphology is possibly due to disruption of the actin cytoskeleton. HSV-1 infection leads to actin cytoskeleton disruption. Disruption of the actin cytoskeleton is required for the HSV-1 to replicate effectively (Ying et al., 2010).

Following M1 treatment, a significant decrease in cell viability occurred, this was not observed following M2 treatment. The decrease in M1 cell viability may stem from TNF-α production. It has been documented that the combination of IFN-γ and TNF-α in culture is toxic to cells (Kyoungho et al., 2001). This would explain why IFN-γ treated cells secreting TNF-α, would have a decrease in viability. M1 macrophages may continuously secrete TNF-α while being stimulated with IFN-γ and LPS, not allowing
enough time for the neutralizing antibodies to bind and inhibit function of newly synthesized TNF-α in culture. Additionally, the effectiveness of anti-TNF-α neutralizing antibodies relies on TNF-α not being degraded within the 24-hour time point.

CD14 and CD86 expression were used as markers to identify the M1 and M2 phenotype. Using immunofluorescent imaging and flow cytometric analysis, a significant increase in the number of cells expressing both CD14 and CD86 was observed, while M2 macrophages showed a significant decrease in expression of both molecules. However, following viral challenge, there was an overall decrease in CD14 and CD86 expression in all three experimental groups. Therefore, HSV-1 infection decreases the ability of M1, M2, and control cells to express both CD14 and CD86. This decrease made it difficult to identify M1 and M2 phenotypes based on the criteria set before virus infection. However, while viral challenge led to an overall decrease in CD14 and CD86 expression, the trend was maintained. Virus-infected M1 macrophages still showed an increase in CD14-CD86 expression when compared to virus-infected control cells; similar to what was seen in uninfected M1 macrophages. Additionally, M2 macrophages showed a decrease in CD14-CD86 expression when not treated, or treated, with HSV-1. It appears the signals within the microenvironment play a key role in CD14-CD86 expression, and HSV-1 infection decreases total expression.

The unpolarized control macrophage may represent the initial subtype of the macrophage lineage present at the HSV-1 infection site. Note that SOCS1 expression levels increased from 30.5% to 83.7% at 24 hours after HSV-1 infection. There was also
a decrease in SOCS3 expression levels from 57.7% to 22.1%. The increase in SOCS1 expression levels inhibits the ability of this macrophage subtype to produce pro-inflammatory products through disruption of the JAK-STAT signaling pathway, while the decrease in SOCS3 expression suggests that SOCS3 may have been compromised following HSV-1 infection. Once polarized to the M1 phenotype, the macrophages were resistant to the increase in SOCS1, however, there was an increase in SOCS3 expression levels. This increase in SOCS3 expression could be a protective mechanism to protect the cells from pro-inflammatory secretory products. There was essentially no difference in SOCS1 and SOCS3 expression levels in virus-infected and uninfected M2 macrophages reflecting their anti-inflammatory function. From these observations it is suggested that the series of events occurring at the initial site of HSV-1 infection lead to the production of the M1 pro-inflammatory macrophage subtype following IFN-γ production by NK cells and γ/δ T cells (Cunningham et al., 2006; Mikloska et al., 1998). Resistance to SOCS1 manipulation through HSV-1 infection is necessary in maintaining an effective immune response against the virus.

Together our data show the importance of microenvironment signals during HSV-1 infections. During infection, large amounts of IFN-γ, produced by NK cells and γ/δ T cells, would be present causing polarization to the M1 phenotype, which is necessary to control virus replication. In this study, introducing HSV-1 to M1 cultures caused a decrease in CD14 and CD86 expression; but significant increase in SOCS3 expression. However, SOCS1 plays the greater role in inhibiting IFN-γ signaling and
dampening the inflammatory response. M2 macrophages showed a significant decrease in total CD14-CD86 expression following virus treatment, while SOCS expression levels remained relatively unchanged. The biggest observable change came in virus-infected control cells, which exhibited significant up regulation of SOCS1. These data suggest that microenvironment signals inhibit the ability of HSV-1 to up regulate SOCS1 and decrease the effectiveness of macrophages to control virus replication (Fig. 30).

In future studies it would be beneficial to study the secretory products of the M1 and M2 phenotypes. This would provide additional markers for identification. Also, when studying cell viability, it appears necessary to collect the supernatant and add both anti-TNF-α and anti-IFN-γ neutralizing antibodies to inhibit production of TNF-α once the supernatant is reintroduced back to culture, therefore, additional work needs to be completed on the decrease in cell viability following M1 treatment. Also, the development of a system that would allow for quick identification of the two phenotypes would be vastly beneficial. This could be accomplished by inserting the iNOS promoter (M1 marker) and the Arginase I promoter (M2 marker) into a plasmid engineered to produce either GFP or RFP when the promoter is activated. This would allow for time-lapse studies to be completed, which could verify how quickly polarization can occur, and how long polarization can be maintained.
Figure 1. J774A.1 macrophages treated with: Top, IFN-γ and LPS Middle, IL-4 Bottom, or untreated. Right, Texas-Red Phalloidin X stain showing actin arrangement of each macrophage experimental group. M1 macrophages appeared flattened, were irregularly
shaped, and contained visible intracellular vacuoles. M2 macrophages exhibited an elongated morphology that was similar to the morphology of control cells.
Figure 2. J774A.1 macrophages treated with IFN-γ and LPS for 24 hours and: A, stained with anti-CD14 FITC-conjugated antibodies B, stained with anti-CD86 Brilliant Violet 421-conjugated antibodies C, stained with Texas-Red Phalloidin X D, and CD14, CD86, and Phalloidin merged image. M1 macrophages showed an increase in CD86 expression when compared to control cells. (Images captured at 400X magnification, scale bar = 50µm)
Figure 3. J774A.1 macrophages treated with IL-4 for 24 hours and: A, stained with anti-CD14 FITC-conjugated antibodies B, stained with anti-CD86 Brilliant Violet 421-conjugated antibodies C, stained with Texas-Red Phalloidin X D, and CD14, CD86, and Phalloidin merged image. M2 macrophages showed a decrease in CD86 expression when compared to control cells. (Images captured at 400X magnification, scale bar = 50µm)
Figure 4. J774A.1 macrophages untreated for 24 hours and: A, stained with anti-CD14 FITC-conjugated antibodies B, stained with anti-CD86 Brilliant Violet 421-conjugated antibodies C, stained with Texas-Red Phalloidin X D, CD14, CD86, and Phalloidin merged image. (Images captured at 400X magnification, scale bar = 50µm)
Figure 5. Flow cytometry analysis of CD14-CD86 expression levels in M1, M2, and control macrophages. Negative isotype control CD14 and CD86 antibodies were used to set quadrants. M1 macrophages had a significant increase (41.2%) in the number of CD14⁺-CD86⁺ cells when compared to control cells, while M2 macrophages had a decrease (26.0%) in the number of CD14⁺-CD86⁺ cells, however, the decrease was statistically insignificant.
Figure 6. Percentage averages of CD14$^+$-CD86$^+$ cells. There was an increase (41.2%, p-value=0.024) in the number of CD14$^+$-CD86$^+$ M1 macrophages when compared to the control cells. There was a decrease (26.9%, p-value=0.100) in the number of CD14$^+$-CD86$^+$ M2 macrophages when compared to control cells, however, the results were statistically insignificant. (*=p-value<0.05)
Figure 7. Percentage of viable cells following polarization treatment. M1 macrophages showed a decrease (31.0%, p-value<0.001) in cell viability following IFN-γ/LPS treatment for 24 hours. M2 macrophages showed a slight decrease (9.0%, p-value=0.004) in cell viability following treatment with IL-4 for 24 hours. (*=p-value<0.05, **=p-value<0.001)
Figure 8. SOCS1 and SOCS3 expression levels in M1 macrophages: stained with A, anti-SOCS1 antibodies B, anti-SOCS3 antibodies C, anti-SOCS1 antibodies/Phalloidin merged image D, and anti-SOCS3 antibodies/Phalloidin merged image. M1 macrophages expressed higher levels of SOCS1 when compared to SOCS3. (Images captured at 400X magnification, scale bar = 50μm)
Figure 9. SOCS1 and SOCS3 expression of M2 macrophages: stained with A, anti-SOCS1 antibodies B, anti-SOCS3 antibodies C, anti-SOCS1 antibodies/Phalloidin merged image D, and anti-SOCS3 antibodies/Phalloidin merged image. M2 macrophages expressed higher levels of SOCS3 when compared to SOCS1. (Images captured at 400X magnification, scale bar = 50µm)
Figure 10. SOCS1 and SOCS3 expression of control macrophages: stained with A, anti-SOCS1 antibodies B, anti-SOCS3 antibodies C, anti-SOCS1 antibodies/Phalloidin merged image D, and anti-SOCS3 antibodies/Phalloidin merge. Control macrophages expressed higher levels of SOCS3 when compared to SOCS1. (Images captured at 400X magnification, scale bar = 50µm)
Figure 11. Flow cytometry analysis of: \textbf{Left}, SOCS1 \textbf{Right}, and SOCS3 expression levels in M1 macrophages at 24 hours. M1 macrophages expressed higher levels of SOCS1 when compared to SOCS3 with a SOCS1/SOCS3 ratio of 6.1:1.
Figure 12. Flow cytometry analysis of: **Left**, SOCS1 **Right**, and SOCS3 expression levels in M2 macrophages at 24 hours. M2 macrophages expressed higher levels of SOCS3 when compared to SOCS1 with a SOCS1/SOCS3 ratio of 1:2.2.
Figure 13. Flow cytometry analysis of: Left, SOCS1 Right, and SOCS3 expression levels in control macrophages at 24 hours. Control macrophages expressed higher levels of SOCS3 when compared to SOCS1 with a SOCS1/SOCS3 ratio of 1:1.9.
Figure 14. SOCS1-SOCS3 ratio for M1, M2, and control macrophages. Control macrophages SOCS expression levels were similar to M2 macrophage SOCS expression levels, with both expressing higher levels of SOCS3 when compared to SOCS1. M1 macrophages expressed higher amounts of SOCS1 when compared to SOCS3.
Figure 15. Virus-infected (0.1 MOI) J774A.1 macrophages treated with: Top, IFN-γ and LPS Middle, IL-4 Bottom, or uninfected. Right, Texas-Red Phalloidin X stain showing actin arrangement of each macrophage experimental group. Following virus challenge, cells in all three experimental groups exhibited a rounded morphology.
Figure 16. Virus-infected (0.1 MOI) J774A.1 macrophages treated with IFN-γ and LPS for 24 hours and: A, stained with anti-CD14 FITC-conjugated antibodies B, stained with anti-CD86 Brilliant Violet 421-conjugated antibodies C, stained with Texas-Red Phalloidin X D, and CD14, CD86, and Phalloidin merged image. M1 macrophages showed an increase in CD86 expression when compared to virus-infected control cells. (Images captured at 400X magnification, scale bar = 50µm)
Figure 17. Virus-infected (0.1 MOI) J774A.1 macrophages treated with IL-4 for 24 hours and: A, stained with anti-CD14 FITC-conjugated antibodies B, stained with anti-CD86 Brilliant Violet 421-conjugated antibodies C, stained with Texas-Red Phalloidin X D, and CD14, CD86, and Phalloidin merged image. There was no observable difference in CD86 expression when compared to virus-infected control cells. (Images captured at 400X magnification, scale bar = 50µm)
Figure 18. J774A.1 macrophages infected with 0.1 MOI of HSV-1 for 24 hours and: A, stained with anti-CD14 FITC-conjugated antibodies B, stained with anti-CD86 Brilliant Violet 421-conjugated antibodies C, stained with Texas-Red Phalloidin X D, and CD14, CD86, and Phalloidin merged image. (Images captured at 400X magnification, scale bar = 50µm)
**Figure 19.** Flow cytometry analysis of CD14-CD86 expression in virus-infected (0.1 MOI) M1, M2, and control macrophages. Negative isotype control CD14 and CD86 antibodies were used to set quadrants. There was a 29.6% increase in CD14-CD86 expression in virus-infected M1 macrophages when compared to virus-infected control cells, and an 11.4% decrease in CD14-CD86 expression in virus-infected M2 macrophages, however, the results were statistically insignificant.
Figure 20. Average percentages of CD14<sup>+</sup>-CD86<sup>+</sup> cells in virus-infected M1, M2, and control macrophages. There was an increase (29.6%, p-value=0.055) in the number of CD14<sup>+</sup>-CD86<sup>+</sup> virus-infected M1 macrophages when compared to virus-infected control cells. There was a decrease (11.4%, p-value=0.158) in the number of CD14<sup>+</sup>-CD86<sup>+</sup> virus-infected M2 macrophages when compared to virus-infected control cells, however, the results were statistically insignificant.
Figure 21. Average percentages of CD14\(^+\)-CD86\(^+\) cells for uninfected and virus-infected M1, M2, and control macrophages. There was a decrease (39.4%, p-value=0.001) in the number of CD14\(^+\)-CD86\(^+\) virus-infected M1 macrophages when compared to uninfected M1 macrophages. There was a decrease (13.2%, p-value=0.039) in the number of CD14\(^+\)-CD86\(^+\) virus-infected M2 macrophages when compared to uninfected M2 macrophages. Additionally, virus-infected control cells experienced a decrease in the number of CD14\(^+\)-CD86\(^+\) cells when compared to control cells not challenged with virus. These results suggest that HSV-1 infection decreases CD14 and CD86 expression in M1, M2, and control macrophages. (*=p-value<0.05)
Figure 22. Percentage of virus-infected viable cells following polarization treatment.

Virus-infected M1 macrophages showed a decrease (31.0%, p-value=0.003) in cell viability following IFN-γ/LPS treatment for 24 hours. M2 macrophages cell viability values were statistically insignificant from virus-infected control cells. (*=p-value<0.05)
Figure 23. SOCS1 and SOCS3 expression levels in virus-infected M1 macrophages: stained with A, anti-SOCS1 antibodies B, anti-SOCS3 antibodies C, anti-SOCS1 antibodies/Phalloidin merged image D, and anti-SOCS3 antibodies/Phalloidin merged image. Virus-infected M1 macrophages expressed comparable levels of SOCS1 when compared to SOCS3. (Images captured at 400X magnification, scale bar = 50µm)
Figure 24. SOCS1 and SOCS3 expression levels in virus-infected M2 macrophages: stained with A, anti-SOCS1 antibodies B, anti-SOCS3 antibodies C, anti-SOCS1 antibodies/Phalloidin merged image D, and anti-SOCS3 antibodies/Phalloidin merged image. Virus-infected M2 macrophages expressed higher levels of SOCS3 when compared to SOCS1. (Images captured at 400X magnification, scale bar = 50µm)
Figure 25. SOCS1 and SOCS3 expression levels in virus-infected control macrophages: stained with A, anti-SOCS1 antibodies B, anti-SOCS3 antibodies C, anti-SOCS1 antibodies/Phalloidin merged image D, and anti-SOCS3 antibodies/Phalloidin merged image. Virus-infected control macrophages expressed higher levels of SOCS1 when compared to SOCS3. (Images captured at 400X magnification, scale bar = 50µm)
Figure 26. Flow cytometry analysis of: **Left**, SOCS1 **Right**, and SOCS3 expression levels in virus-infected M1 macrophages at 24 hours. Virus-infected M1 macrophages expressed comparable levels of SOCS1 when compared to SOCS3 with a SOCS1/SOCS3 ratio of 1:1.1.
Figure 27. Flow cytometry analysis of: **Left**, SOCS1, and SOCS3 expression levels in virus-infected M2 macrophages at 24 hours. Virus-infected M2 macrophages expressed higher levels of SOCS3 when compared to SOCS1 with a SOCS1/SOCS3 ratio of 1:1.8.
Figure 28. Flow cytometry analysis of: Left, SOCS1 Right, and SOCS3 expression levels in virus-infected control macrophages at 24 hours. Virus-infected control macrophages expressed higher levels of SOCS1 when compared to SOCS3 with a SOCS1/SOCS3 ratio of 3.8:1.
**Figure 29.** SOCS1-SOCS3 ratio for virus-infected M1, M2, and control macrophages. Virus-infected control macrophages expressed higher levels of SOCS1 when compared to SOCS3. Virus-infected M1 macrophages expressed comparable levels of SOCS1 and SOCS3; while virus-infected M2 macrophages expressed higher levels of SOCS3 when compared to SOCS1.
Figure 30. Diagram outlining the effects of HSV-1 on polarized J774A.1 macrophages.

Viral challenge led morphological changes in M1, M2, and control macrophages. Additionally, HSV-1 infection decreased the ability of all cell types to express CD14 and CD86. In control cells, virus challenge leads to increased expression of SOCS1 when compared to uninfected control cells. M1 macrophages expressed higher levels of SOCS3 following virus infection.
Table 1. Averages of SOCS expression levels in M1, M2, and control Macrophages before and following virus treatment. SOCS3 was increased in M1 macrophages following challenge with virus. SOCS expression levels remained relatively unchanged in

<table>
<thead>
<tr>
<th></th>
<th>SOCS1</th>
<th>SOCS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 No Virus</td>
<td>34.7%</td>
<td>5.7%</td>
</tr>
<tr>
<td>M1 Virus</td>
<td>33.6%</td>
<td>36.1%</td>
</tr>
<tr>
<td>M2 No Virus</td>
<td>37.3%</td>
<td>81.5%</td>
</tr>
<tr>
<td>M2 Virus</td>
<td>47.6%</td>
<td>85.4%</td>
</tr>
<tr>
<td>Control No Virus</td>
<td>30.5%</td>
<td>57.7%</td>
</tr>
<tr>
<td>Control Virus</td>
<td>83.7%</td>
<td>22.1%</td>
</tr>
</tbody>
</table>
the M2 phenotype, and in virus-infected control cells, SOCS1 was significantly increased.
References


recurrent herpes simplex lesions: do herpes simplex virus infected keratinocytes contribute to their production? Journal of Infectious Diseases. 177: 827-838.


