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SOCS1/SOCS3 Expression and Virus Replication of DENV2 and HSV-1 in Cytokine-Polarized Subsets of RAW 264.7 Macrophages

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SOCS1/SOCS3 Expression and Virus Replication of DENV2 and HSV-1 in Cytokine-Polarized Subsets of RAW 264.7 Macrophages

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

By

NAGARJUNA REDDY CHEEMARLA
B.Tech., Jawaharlal Nehru Technological University, 2010

2014
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY NAGARJUNA REDDY CHEEMARLA ENTITLED SOCS1/SOCS3 Expression and Virus Replication of DENV2 and HSV-1 in Cytokine-Polarized Subsets of RAW 264.7 Macrophages BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Cheemarla, Nagarjuna Reddy. M.S. Department of Microbiology and Immunology, Wright State University, 2013. SOCS1/SOCS3 Expression and Cytokine Characterization in HSV-1 and DENV2 Infected Macrophage Subsets.

Cytokine activation of macrophages leads to “macrophage polarization” The M1 subset is polarized by exposure to lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) and M2 polarized cells develop after exposure to interleukin-4 (IL-4) or IL-13. In this study, the RAW 264.7 murine macrophage cell line was used to study the effect herpes simplex virus – Type I (HSV-1) and Dengue virus serotype-2 (DENV2) infections have on cultures of unpolarized (M0) or polarized subsets. The macrophage subsets were characterized using cluster of differentiation markers CD14 and CD86. Uninfected M1 macrophages showed up regulation in expression of the co-stimulatory factor B7.2 (CD86) compared to expression by M0 or M2 macrophages. M1 macrophages exhibited distinct morphological and viability features including presence of vacuoles, strong adherence, enlargement of cell body and significant decrease in cell viability at 24 hours following exposure to LPS and IFN-γ. Infection with either HSV-1 or DENV-2 induced a down regulation in expression of CD86 by M1 cells and an up-regulation of expression in M0 and M21 cells. Immunofluorescence analysis using flow cytometry showed a decrease in CD86 expression levels in only M1 cells by 24 hours following infection with either infected with HSV-1 or DENV2. Virus-infected M1 macrophages showed further decreases in cell viability compared to viabilities of infected M0 or M2 macrophages. In
unpolarized and polarized macrophages in this study, SOCS 3 expression was highest in M0 and M2 cell prior to and after infection with either HSV-1 or DENV2. In these same cell populations higher cell viabilities were observed at 12 and 24 hours after infection as well as higher viral titers by 24 hours post infection. In contrast, the M1 macrophage populations showed lesser increases in SOCS 3 expression, markedly decreased cell viabilities and 2.8fold decrease in viral titers by 24 hour after infection with HSV-1. These observations suggest that the ameliorating effect of SOCS3 on inflammation in the M0 and M2 macrophages permitted both a slower decline in cell viabilities and enhanced viral replication in these isolated cell populations. Cells converted to the M1 phenotype rapidly diminished viral replication and cell viability.
HYPOTHESIS

The hypothesis of this study was that the M1 subpopulation of murine RAW 264.7 cells in comparisons with the M0 and M2 subpopulations would exhibit significant decreases in cell viability but greater ability to control viral replication by 24 hours following infection either HSV-1 or DENV2; and these difference could be related to relative expression of suppressors of cytokine signaling 1 and 3 (SOCS1 and SOCS3).
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LIST OF ABBREVIATIONS

WBC= White Blood Cells

LPS= Lipopolysaccharide

IFN-γ= Interferon gamma

NO= Nitric Oxide

HVEM= Herpes Virus Entry Mediator

GAGs= Glycosaminoglycans

GM-CSF= Granulocyte Macrophage Colony Stimulating Factor

CD= Cluster of Differentiation

SOCS= Suppressor of Cytokine Signaling

ADE= Antibody Dependent Enhancement
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INTRODUCTION

Dengue virus is a positive single-stranded RNA virus that belongs to the Flaviviridae family of viruses (Rodenhuis-Zybert et al., 2010). Dengue viral infection occurs in sylvatic cycles. The sylvatic cycle refers to the population of pathogen’s life span spent cycling between wild animals and the vector. Humans are a dead-end host of this virus life cycle (Fernandes et al., 1999).

Dengue virus initiates both innate and adaptive immune responses. Dengue virus non-structural (NS) proteins have the ability to inhibit innate immune response during infection. These non-structural proteins inhibit the innate responses at two-levels:

1) Inhibition of interferon signaling by blocking signal transducer:
   
   NS4, a membrane protein associated with endoplasmic reticulum, inhibits the phosphorylation of STAT1 after induction by type I interferons (IFN) alpha, beta. Consequently, immune response are blocked and interferon stimulated genes (ISG) are not expressed. With dengue virus infection, Tyk2 kinase activity decreases resulting in a decrease in STAT1 phosphorylation (Ho et al., 2005).
   
   Also, NS2A and NS4A cofactors take part in STAT inhibition (Muñoz-Jordan et al., 2003). NS5A is a 105KDa protein that inhibits activation of STAT2 and is cleaved by a protease. This cleaved NS5A binds to STAT2 via an E3 ligase association, which degrades STAT2 (Mazzon et al., 2009; Morrison et al., 2012).
2) Inhibition of Type I interferon response:

NS2B3 is a protease complex core that consists of partly both NS2B and NS3 proteins (Yusof et al., 2000). This complex inhibits the production of IFN-β by interfering with the activity of IFN-β promoter by inhibiting phosphorylation of IRF3 (Rodriguez-Madoz et al., 2010; Yu et al., 2012).

Herpes simplex virus infection also triggers the immune regulation pathways in the host. Although primary infection by HSV is usually undetectable, the virus persists in a latent infection within the host and can be stimulated by a secondary infection at a later point. The virus remains in the trigeminal ganglia during latency and show minimal virus gene expression (Rautemaa et al., 2002). The immune responses triggered by HSV infection mainly involve three cells: macrophages, natural killer cells (NK) and γ/δ T cells. These cells are recruited to the site of infection by chemotactic cytokines. These cells then prevent viral replication and eliminate infected cells (Cunningham et al., 2006; Mikloska et al., 1998).

Macrophages are “phagocytic” cells and express a variety of surface receptors that are activated by many signals. Both HSV and DENV infection involves recruitment of macrophages to the site of infection upon cytokine signaling. DENV has affinity for FcγR expressing cells such as dendritic cells and macrophages. Macrophages play a crucial role in controlling viral replication. They can themselves inhibit viral replication when infected. They signal extracellular viral proteins for degradation via proteasome and
ubiquitylation and kill virus-infected cells attenuating viral replication (Wu and Morahan, 1992). At the time of viral infection signaling molecules such as cytokines are produced that act as signals to activate the receptor thus altering the structure and functions of macrophages by characterizing them into different subpopulations (Murray and Wynn, 2011). Cytokine activation of macrophage subpopulations is referred to as “macrophage polarization”. Unstimulated macrophages are called M0 macrophages. M1 macrophages are produced by signal such as bacterial lipopolysaccharides (LPS) and interferon-γ and produce pro-inflammatory cytokines such as iNOS, TNF-α and IL-12. They are mobilized by infectious agents and hence promote inflammation. A variety of cytokine trigger polarization to M2 macrophages especially IL-4 and IL-13. M2 macrophages on the other hand produce anti-inflammatory cytokines such as IL-10, Arg-1 and IL-4 that are involved in wound healing and tissue repair (Junliang et al., 2010; Kigerl et al., 2009; Wang et al., 2010). The different macrophage subpopulations express variations in cluster of differentiation markers (CD markers) on the cell surface. The CD markers essential for differentiating M1 and M2 macrophage subpopulations are CD14 and CD86. CD14 acts as a co-receptor along with TLR4 and detects LPS. CD86 is expressed on antigen presenting cells and is involved in B cell stimulation, antigen presentation to activate T cell signaling and survival (Sugamura et al., 2004).

Another factor that influences viral infection and immune responses in the host is Suppressor of cytokine signaling (SOCS) proteins. Under normal conditions, SOCS proteins act in an anti-inflammatory manner involve ubiquitylation and degradation of
inflammatory proteins while maintaining antipathogenic functions (Kubo et al., 2003). When the host cells are infected by viruses, these SOCS proteins become the targets for viral evasion. The virus implements robust mechanisms to “hijack” SOCS functions to promote virus survival and replication in the host. SOCS proteins inhibit antiviral pathways thus allowing the viral proteins to be taken up by the cells, subjecting these proteins to ubiquitylation, facilitates viral trafficking, progeny virus assembly and increase in the copy number of virions (Lisa and Benveniste, 2011).

The overall process of viral infection, macrophage polarization, surface receptor expression and SOCS protein function varies from macrophage to macrophage and different viruses that infect. The current study shows these differences in RAW 264.7 macrophages upon infection with either DENV or HSV. And the variation in responses between J774.1 macrophages from earlier findings and RAW264.7 macrophages is discussed.
LITERATURE REVIEW AND PRELIMINARY DATA

Dengue Virus and Infection

Dengue virus (DENV) belongs to the Flaviviridae family of viruses. DENV infection is an arthropod-borne disease. There are 4 serotypes of DENV, including serotypes- 1 through -4 are transmitted from person to person by Aedes aegypti and Aedes albopictus mosquitos (Gubler and Kuno, 1997). Dengue is a Center for disease control (CDC) category A infectious disease. Dengue disease presents in three clinical forms: Dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Over 2.5 billion people are living in areas at risk of Dengue infection [WHO factsheet 2012]. The number of deaths by Dengue Hemorrhagic fever has increased rapidly in the past decade. Approximately more than 50 million cases take place each year, associated with more than 25,000 fatalities, especially in children (WHO factsheet 2002). Still there is neither a vaccine nor a specific treatment for Dengue.

This Flavivirus is composed of three structural proteins or glycoproteins: the C protein, which forms the nucleocapsid containing viral genome, and proteins M and E, which are inserted into the lipid membrane surrounding the nucleocapsid. The genome of DENV is composed of a single-stranded RNA of ~11Kb. The E protein is expressed on the surface and is responsible for binding and entry of virus into the cell. It is also the neutralization antigen and is responsible for variability of the four major serotypes DENV 1-4 (Kuhn et al., 2002). Dengue infection occurs in three stages: 1) mosquito injects the
dengue virus into the human body and enters the dendritic cells (specialized cells present in most tissues); 2) Dengue virus targets primary monocytes/macrophages in spleen, bone marrow and glands; 3) entering the monocytes and lymphatic system, allows the dengue virus to enter the circulatory system and spread throughout the body.

**Macrophages and Dengue Infection**

When a person is infected for the first time with DENV, both humoral and cellular immunity develop into action. The humoral response produces many neutralizing antibodies necessary to prevent the virus from fusing with cell membrane. The cellular response involves activation of T lymphocytes by the dendritic cells carrying the dengue virus, which help fight off primary infection and development of serotype-specific memory B cells. These memory cells have the ability to fight off any future infections by the same serotype (Ho et al., 2001).

In vivo, the predominant site of DENV infection is the cells of mononuclear phagocyte lineage (Scott et al., 1980; Halstead, 1989). In case of a secondary infection by a different serotype, the memory B cells produce antibodies that accelerate the process of dengue infection and onset of hemorrhagic fever by increasing viral uptake (Dimmock, 2007). The prevailing theory is that, the non-neutralizing antibodies of a different serotype tries to fight off the new serotype, but actually cross-react and form a complex with the virus. This complex is internalized by the macrophages with the help of Fc receptors and the virus multiplies rapidly in these infected macrophages, thus speeding up
the process of infection (Fig 1). In case of dengue infection, there is increased release of pro-inflammatory cytokines. This increased release and accumulation of cytokines result in vascular permeabilization and hence plasma leakage, a symptom of DHF (Ho et al., 2001). This type of immune system response to a secondary infection is called antibody dependent enhancement (ADE). Due to plasma leakage occurs internal bleeding and thrombosis results. After continuous release of platelets and increasing bleeding, disseminated intravascular coagulation occurs. This results in hemorrhage and a “glitch” in the circulatory system resulting in shock and ultimately death of a person (WHO, 1999).

**Macrophage Function and Activation**

Macrophages are phagocytic cells that are produced by differentiation of monocytes in the tissues.

Two types of T-helper CD4+ cell responses are induced by antigen presenting cells, namely Th1 and Th2 respectively. The Th1 response is characterized by activation of bactericidal activities of macrophages and release of pro-inflammatory cytokines such as IFN-γ. Th2 responses on the other hand, are characterized by the release of anti-inflammatory cytokine such as interleukin-4 (IL-4) and activation of B cells to produce neutralizing antibodies. The CD4+ Th cell differentiation is analogous to the macrophage polarization pathways that are designated M1 and M2 (David, Mosser and Justin, 2008). M1 macrophages are activated by LPS and IFN-γ and known as immune effector cells
that act against microbes, digest affected cells and produce many lymphokines. M1 macrophages produce pro-inflammatory cytokines such as IL-1β, IL-12 and TNF-α, as well as reactive oxygen species, NO and mediate resistance to tumors (Mantovani et al., 2004; Goerdt et al., 1999; Mantovani et al., 2005; Mills et al., 2000). While M2 macrophages produce anti-inflammatory cytokines such as IL-10, contribute to Th2 responses, promotes tissue repair, induce proliferation, collagen production, and enhances phagocytosis and parasite elimination (Fadok et al., 1998; Munder et al., 1998; Porcheray et al., 2005). The M2 macrophages are classified into M2a, M2b and M2c depending upon specific factors of stimulation or activation. M2a are stimulated by IL-4, M2b by TLR and IL-1ra and M2c by IL-10 (Mantovani et al., 2004). M2 macrophages are thought to be tumor- associated macrophages and produce high IL-10 and low IL-12 unlike M1 macrophages that produce high IL-12 and low IL-10 (Galdiero et al., 2013). The substrate for both the pathways is the same, an amino acid called arginine. Nitrous oxide synthase (NOS) induced by LPS and IFN-γ produces HO-Arginine and then NO. While the Th2 cytokines convert arginine into ornithine and eventually polyamines and proline (Classen et al., 2009).
Figure 1. Two-loop model of antibody dependent enhancement. DENV-subneutralization antibody complex ligates with the FcγR and induces suppression of innate immune responses. This is done by two different pathways:

1) Negative regulators of PPR, DAK, Atg5 to Atg12, SARM and TANK are upregulated, that abolish RLR and TLRs and its signaling pathway, resulting in decreased type 1 interferon receptor and pro-inflammatory cytokine production, which suppresses IFN–mediated antiviral responses

2) IL-10 that activates SOCS system is subject to early activation, suppressing the JAK/STAT signaling pathway and also the IFN-signaling pathway.

These two loops switch off the intracellular antiviral responses in DENV-infected cells under the influence of ADE conditions, resulting in viral replication and increase in copy number of virions. (Adapted from Ubol and Halstead, 2010)
Herpes Simplex Virus and Infection

Herpes simplex virus belongs to the *Herpesviridae* family (Ryan and Ray, 2004). HSV contains a double-stranded, linear DNA genome enclosed in a protein capsid wrapped by an envelope. HSV enters the cells with the help of glycoproteins gC and gD, the former helps in attaching to the host cells and the latter helps in entry into the host cells via interactions with one of the three cell surface receptors namely HVEM, nectin-1 and 3-O sulfated heparan sulfate. When gD binds to HVEM, gD changes its conformation and interacts with gH and gL which forms a complex. Another glycoprotein gB interacts with gH/gL complex to form a virus capsid entry pore by interfering with the GAGs in the cell wall of the host cells (Subramanian and Geraghty, 2007). After viral entry into the cell, the viral genome is transmitted by microtubules into the host genome by entry into the nucleus through a nuclear entry pore (Newcomb et al., 2007).

HSV blocks the transporter associated with antigen processing (TAP) through release of ICP-47. The viral ICP-47 on the other hand inhibits TAP, thus inhibiting the activation of CTLs and allowing the virus to stay active for a longer period (Mojadadi et al., 2009).

Cluster of Differentiation (CD) Markers

CD markers are a critical factor to differentiate M1 macrophages from M2. This is done by studying the number cells expressing the cell surface receptors cluster of
differentiation 14 (CD14) and CD86. CD14 is essential as it acts as a co-receptor along with Toll-like receptor 4 (TLR4) and MD-2 and detects bacterial LPS. It is known to bind to LPS with the help of LPS binding protein (LBP) and is involved in the activation of many transcription factors. CD86, also known as B7-2 is expressed on antigen presenting cells that is involved in B cell co-stimulation, T cell activation and survival. CD86 also acts as a counter-receptor for CD28 (Sugamura et al., 2004). Some of the earlier findings in our lab suggest that CD14⁺CD86⁺ cells are higher in M1 polarized macrophages than the control macrophages. While cells infected with HSV-1 and polarized to M1 or M2 phenotype show a decrease in the percent of CD14-CD86 positive cells. This study is to show the effect of HSV1, DENV2 infection and cytokine induced polarization on RAW264.7 macrophages and their CD markers CD14 and CD86.

**SOCS Proteins**

Suppressor of cytokine signaling (SOCS) are proteins that inhibit cytokine signaling pathways. They are involved in both innate and adaptive immunity and regulate the macrophages and dendritic cells and play an important role in T cell development and differentiation (Yoshimura, 2007). There are eight subtypes of SOCS proteins (SOCS1-SOCS 7 and CIS) that have a –SH2 domain and C-terminal SOCS box (Fig 2).

The expression of SOCS1-3 and CIS is stimulated by various cytokines and these are known to regulate pathways such as JAK/STAT pathway (Fig 3). These SOCS proteins are known to implement one or more of the three mechanisms: 1) binding to
phosphotyrosines through SH2 domain resulting in N-terminal inactivation of JAK; 2) blocking receptor binding sites of STAT; 3) by SOCS box-targeting bound proteins to ubiquitylation.
**Figure 2. Structure of SOCS proteins.** SOCS proteins contains three domains namely, kinase inhibitory region (KIR), SH2 domain and the SOCS box. KIR is involved in inhibiting the phosphorylation and activation of JAK by acting as a pseudosubstrate. SH2 domain binds to phosphotyrosine residues and causes N-terminal inactivation of JAK. The SOCS box is a crucial component that signals that target proteins for degradation by ubiquitylation upon interaction with complex comprising of Elongin B, Elongin C, RBX2, Cullin-5 and E2 ligase. (Adapted from Yoshimura et al., 2007)
Figure 3. Suppression of TLR and Interleukin pathways by SOCS. LPS activates TLR4 that transmits signals through adaptor proteins MyD88, MyD88-adaptor-like protein (MAL), TIR domain-containing adaptor inducing interferon (TRIF) and TRAM. Then TRAF6 is activated that activates NF-κB and MAPK pathways thus activating pro-inflammatory cytokines. TRIF and TRAM activate JAK2/STAT5 pathway that produces IL-6 and also activates TRIF/IRF3 pathway that produces Type I interferons. These Type I interferons activate the JAK/STAT1 pathway producing CD40, iNOS and IFN-inducible genes. The IL-1R signals MyD88 to activate TRAF6, TAK1 and ultimately produce pro-inflammatory cytokines by NF-κB and MAPK pathways. Phosphorylated MAL interacts with SOCS1 resulting in MAL polyubiquitylation and degradation. SOCS3 inhibits the association between TRAF6 and TAK1 thus inhibiting NF-κB dependent transcription. (Adapted from Yoshimura et al., 2007)
SOCS1 is stimulated by cytokines IL2, EPO, IFN-γ and GM-CSF. It acts as a negative regulator of IFN-γ and JAK-STAT pathway as it acts through a negative feedback loop to inhibit the JAK-STAT pathway (Minamoto et al., 1997).

SOCS3 proteins are induced by cytokines such as IL6, IL10 and IFN-γ. They inhibit the activity of JAK2-kinase (Minamoto et al., 1997). Both SOCS1 and SOCS3 encode the STAT-induced STAT inhibitor (SSI) gene. It is also shown that SOCS1 is expressed more in IL-4 induced M2 macrophages than pro-inflammatory M1 macrophages (Whyte et al., 2011). The SOCS proteins mainly SOCS1 and SOCS3 additionally have a Kinase inhibitory region (KIR) upstream of the central SH2 domain that acts as a pseudosubstrate. SOCS1 inhibits the catalytic activity of JAK by binding to the activation loop through its KIR and SH2 domains (Yoshimura, 2007).

From Reichard’s study, we know that SOCS1 expression is enhanced in HSV-1 infected M1 macrophages more than in HSV1 infected M2 macrophages. This study involves studying the effect of cytokine-induced polarization to RAW 264.7 cells infected with HSV-1 and DENV2. The SOCS1/SOCS3 expression ratio in polarized macrophages and virus treated polarized macrophages were significantly different with SOCS1/SOCS3 ratio being higher in M1 polarized uninfected cells, while the ratio is higher in control cells with virus infected polarized macrophages.
MATERIALS AND METHODS

Cell Lines

The RAW 264.7 murine macrophage cell line (ATCC: TIB 71) is established from a tumor induced by Abelson murine leukemia virus in male BALB/c mouse are adherent in nature. RAW 264.7 macrophages were cultured in 100mm x 20mm cell culture treated petri dishes (BD Biosciences) and incubated at 37°C and 5% CO₂ in a humidified incubator. Cells were cultured and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (HyClone) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Fisher brand) and antibiotics (0.1% gentamycin (100000U/mL) and 1% pencillin-streptomycin (10000U/mL Penicillin and 10000U/mL Streptomycin)). Cells were sub-cultured every two days at a ratio of 1:6. Vero 76 cells (CCL-81, ATCC) are African green monkey kidney epithelial cells and were used in this study for propagating viruses and calculating their titers.

Polarization Treatment

RAW 264.7 macrophages at approximately 50% confluence were polarized to either M1 or M2 phenotypes by respective cytokine treatments. M1 phenotype was induced using 20 ng/ml of IFNγ (Peprotech) and 100ng/ml of LPS (Chondrex) and M2 phenotype was induced using 20 ng/ml of IL-4 (Peprotech). Cytokine treatment for both...
the phenotypes continued for 24 hours after which the cells were collected using a
dissociation reagent (Cell Stripper by Gibco) and used for further analysis.

**Virus Culture and Quantification**

Herpes Simplex Virus-1 strain Syn 17+ initially obtained from Dr. Nancy Sawtell,
Children's Hospital Medical Center, Cincinnati, OH was propagated on confluent monolayers of Vero cells. After 4-5 days post infection or when CPE was evident, the cells were spun down, supernatant was aliquoted and stored at -80°C. Virus was quantified by infecting Vero cell monolayers with different dilutions of virus and plaque forming units were counted to calculate volume required for 0.1 multiplicity of infection (MOI).

Propagation of DENV serotype 2 (DENV-2) (Dr. Eric M. Vela, Battelle Research Center) was done on Vero 76 cells. Briefly, Vero 76 cells grown in 100 mm petri dishes to a confluence of approximately 85% at 37 °C, were infected with DENV-2 for 5-6 days or until CPE was evident. Cells were then scraped and centrifuged at 1500 rpm to eliminate cell debris. The supernatant was aliquoted and stored at −80°C until use. Dengue virus titers were determined by plaque assay in confluent monolayers of Vero 76 cells grown in 6-well plates. Vero 76 cells were inoculated with ten-fold serial dilutions when monolayers reached 80 to 90% confluence. After 2 h of viral adsorption, the monolayers were overlaid with 2 ml of Opti-MEM GlutaMAX (Life Technologies Inc.) containing 2% Methyl-cellulose (Acros Organics) and 0.5% fetal bovine serum. The
cultures were incubated at 37 °C for 5-6 days and then counted for plaque formation after fixation with 4% paraformaldehyde and staining with 1% crystal violet solution (Sigma). Additionally viral titers were also determined using flow cytometry (Fig 4) as described in F Medina and Juan F. Medina et al, 2012. Briefly, Vero cells grown in 24-well plates at a density of 1.25 x 10^5 per well one day prior to infection were infected with ten-fold serial dilutions of DENV2 diluted in Opti-MEM. After incubating one hour with gentle rocking every 15 minutes, the supernatant was aspirated and overlaid with 1ml of 2% methyl cellulose and incubated at 37 °C for 24 hours. Next day, infected monolayers were trypsinized, fixed, blocked for unspecific binding and then stained with DENV2 monoclonal antibody 4G2 (Millipore). These antibodies are directed to the E protein of the dengue virus and are capable of specifically neutralizing DENV2 serotype. Because these antibodies are not fluorescent tagged, the cells were stained with FITC conjugated secondary antibodies (Biolegend) and analyzed using Accuri C6 flow cytometer. Final viral titers were calculated as infectious units (IU)/ml.

**Cell Viability**

RAW 264.7 cells were sub cultured at a ratio of 1:6 and incubated for 12 or 24 hours. At this time, the flasks were approximately 40-50% confluent. They were then treated with either IFN-γ/LPS for M1 or IL-4 for M2 phenotype polarization with or without virus (HSV-1 or DENV2). After 12 or 24 hours of treatment, cells were detached from the cell culture flask using a dissociation reagent. Trypan blue staining was used to
determine the cell viability using a hemocytometer. Cell Stripper and Trypan Blue were purchased from Fisher Scientific.

**Immunofluorescent Staining**

RAW 264.7 cells were grown on cover slips (Fisher Scientific) to approximately 40% confluence. Cells were then treated with IFN-γ and LPS for M1 or IL-4 for M2 with or without virus for 24 hours. Following treatment, growth medium was aspirated and cells were rinsed three times with 1% bovine serum albumin (BSA) suspended in phosphate buffered saline (PBS). Cells were then fixed using 4% Paraformaldehyde in PBS for 15 minutes and permeabilized using Acetone for 10 minutes at -20°C (for intracellular staining). Cells were blocked for non-specific binding using blocking buffer (5% serum from species of fluorochrome-conjugated secondary antibody and 3% BSA suspended in PBS) for one hour. After blocking, fluorochrome-conjugated antibodies and phalloidin were added at recommended concentrations to the cover slip and incubated overnight in the dark at 4°C. Following incubation, cells were rinsed three times with 1% BSA in PBS. The cover slips were then mounted on glass slides using a drop of VectaShield fluorescence mounting medium. Slides were then analyzed for antibody tagged fluorescence using the Olympus Epi-fluorescence Spot Scope. Anti-CD14 (0.25μg/10^6 cells), anti-CD86 (0.25μg/10^6 cells) and isotype control antibodies for respective antibodies were purchased from BioLegend; Texas Red-Phalloidin X
(3μl/million cells) was purchased from Life Technologies (Gaithersburg, MD) and VectaShield mounting medium was purchased from Fisher Scientific.

**Flow cytometry Analysis**

RAW 264.7 cells were grown in 150mm x 15mm petri dishes (BD Biosciences) to approximately 50% confluence and then treated with IFN-γ and LPS for M1 or IL-4 for M2 with or without virus for 24 hours. Following treatment, cells were detached and counted using a hemocytometer by trypan blue exclusion method. Cells were then rinsed three times with 1% bovine serum albumin (BSA) suspended in phosphate buffered saline (PBS). Blocked for non-specific binding using blocking buffer (5% serum from species of fluorochrome-conjugated secondary antibody or normal goat serum (NGS) and 3% BSA suspended in PBS) for one hour. After blocking, fluorochrome-conjugated antibodies and were diluted in blocking buffer and added at recommended concentrations to the cell pellet and incubated in the dark for 1 hour. Following incubation, cells were rinsed three times with 1% BSA in PBS and pellet re-suspended in sodium azide solution and analyzed for antibody tagged fluorescence using the Accuri C6 flow cytometer. Anti-CD14 (0.25μg/10^6 cells), anti-CD86 (0.25μg/10^6 cells) and isotype control antibodies for respective antibodies were purchased from BioLegend.
**Western Blotting**

Control and polarized cell (uninfected or infected with HSV-1 or DENV-2) lysates were prepared and suspended in special lysis buffer (SLB) (1mM HEPES, 0.1% Triton X-100) containing protease inhibitors aprotonin (2mg/ml) and leupeptin (5mg/ml). Protein concentration was determined using BIORAD protein estimation method and samples separated by SDS-PAGE using 4-20% gels (Pierce Thermo Scientific) and western blotted onto polyvinylidine difluoride membranes (Thermo Scientific). Membranes were blocked with 5% non-fat milk in PBS-Tween 20 (PBST) and probed with rabbit anti-mouse SOCS1 and SOCS3 antibodies. Actin probing was used as a control. Goat anti-rabbit conjugated secondary antibodies (Cell Signaling, Boston, MA) were used to detect primary antibody binding by adding Luminol (Peroxidase substrate) purchased from Pierce Thermo Scientific. SOCS antibodies were kindly donated by Dr. Howard M. Johnson from the University of Florida, Gainesville.

**Calculation of Viral Titers in HSV-1 Infected Macrophage Subsets**

Viral titers were calculated in HSV-1 infected macrophage subsets 24 hours post infection. Briefly, supernatants of infected subsets (M0, M1 and M2) were collected 24 hours post infection and various dilutions of supernatants were prepared to infect confluent Vero cell monolayers for 2 hours. Virus supernatants were aspirated after 2 hours and Vero cells overlaid with 1% methyl cellulose in OptiMEM. Plates were stained
with 0.5% crystal violet upon visualization of plaques. Virus yield in each experimental group was calculated in terms of PFU/ml.

**Statistical Analysis**

Sigma Plot 12.0 software was used for calculating statistical significance and graphical representations. Analysis conditions were compared using t-test, and \( P \) values less than 0.05 were considered statistically significant. All experiments were conducted in triplicates.
RESULTS

**Uninfected Control and Cytokine Polarized Macrophages**

**M1 Macrophages Exhibited Morphological Changes Compared to Control or M2 Macrophages**

Macrophages were either unpolarized (control) or polarized with LPS+IFN-γ or IL-4 into M1 or M2 phenotypes respectively. M1 macrophages appeared to be enlarged, contained intracellular vacuoles, strongly adherent and stellate following treatment with LPS and IFN-γ for 24 hours (Fig. 7). These changes in M1 macrophages were observed as early as 12 hours following treatment (Fig. 5). M2 macrophages appeared similar to control macrophages without any distinct morphological changes (Fig. 6).

**CD Marker Profiling of Polarized and Control Macrophages**

Immunofluorescence analysis showed upregulation of CD86 (B7-2) in M1 macrophages (Fig 9) compared to control macrophages (Fig 8). M2 macrophages (Fig 10) exhibited very little or no CD86 expression. CD14 expression was found to be similar among polarized and control cells, hence it was not characterized as a distinct marker for particular phenotype.

Flow cytometry analysis showed upregulation of CD86 expression in M1 macrophages compared to control cells. Flow cytometry analysis of CD markers on uninfected polarized macrophages show that M1 macrophages exhibit upregulation of
CD14⁺-CD86⁺ expression (33.37%, P-value< 0.001) when compared to control macrophages. Conversely, M2 macrophages showed insignificant changes in CD14⁺-CD86⁺ expression (1.14%, P-value= 0.319) when compared to untreated control (Fig 11 and 12).

**Cell Viability Studies Following Polarization**

Cells were either unpolarized or polarized with cytokines for 12 or 24 hours. Cell viability assay was performed after 12 or 24 hours by Trypan Blue assay. Both M1 (73.76%, P-value< 0.001) and M2 (47.41%, P-value= 0.001) macrophages exhibited significant decrease in cell viability 12hrs post-cytokine treatment (Fig 13). M1 macrophages exhibited significant decrease (~85%, P-value <0.001) in cell viability following polarization with LPS and IFN-γ compared to control at 24hours post cytokine treatment. Slight significant decrease in viability was observed in M2 macrophages (~15%, P-value= 0.012) following IL-4 treatment for 24 hours (Fig. 14).

**Control and Polarized Macrophages Infected with HSV-1 or DENV2**

**Morphological Changes Post-infection with HSV-1 or DENV2 and Polarization**

M1 macrophages appeared enlarged, strongly adherent and vacuolated post-infection similar to uninfected M1 macrophages after 12 (Fig. 5) and 24hours (Fig. 15 and 22) of infection (HSV-1 or DENV2) and cytokine treatment. Some M2 macrophages post-infection with HSV-1 for 24 hours appeared highly enlarged (Fig. 16) and all the M1
macrophages appeared densely vacuolated. M2 macrophages were also similar in morphology to uninfected macrophages. Overall morphological changes were similar before and after DENV2 infection (Fig. 22).

**CD Marker Profiling in Control and Polarized Macrophages Following HSV-1 or DENV2 Infection**

Immunofluorescence analysis of CD markers showed downregulation of CD86 expression in M1 macrophages (Fig. 18) and upregulation of CD86 expression in control (Fig 17) and M2 (Fig. 19) upon HSV-1 infection. In case of DENV2 infection, CD86 expression is downregulated in M1 (Fig. 24) and upregulated in M2 (Fig 25) macrophages. CD86 expression was downregulated upon HSV-1 or DENV2 infection in M1 macrophages compared to infected control (Fig 17 and Fig 23) and M2 as well as uninfected macrophages. CD86 expression was upregulated in M2 macrophages post-infection. CD14 expression was similar in both HSV-1 or DENV2 infected cells. But CD14 expression was further downregulated in M1 macrophages infected with DENV2 compared to HSV-1 infected or uninfected M1 macrophages.

Flow cytometry analysis of CD markers on HSV-1 infected polarized macrophages show that M1 macrophages exhibit upregulated expression (28.34%, P-value= 0.017) of CD14⁺-CD86⁺ in comparison with control. On the other hand, CD14⁻-CD86⁻ expression changed insignificantly in M2 macrophages (3.04%, P-value= 0.145) (Fig. 20 and 21). In the case of DENV2 infected macrophages, CD14+-CD86+
expression was upregulated in M1 phenotype (36.57%, P-value= 0.013) compared to control (Fig. 26 and 27). Also, there was a minor increase in CD14+CD86+ HSV-1 M1 macrophages compared to uninfected M1 macrophages. CD14+CD86+ expression decreased slightly in DENV2 infected M2 macrophages (3.7%, P-value= 0.098) compared to control. Overall CD14+CD86+ expression was downregulated by about 7 times in DENV2 infected macrophage phenotypes (control and M2) compared to uninfected or HSV-1 infected control and M2 macrophages.

**Cell Viability**

Cell viability studies by Trypan Blue assay showed further decrease in infected macrophage populations compared to uninfected macrophages. Cell viability of HSV-1 infected M1 macrophages decreased significantly (43.61%, P-value<0.001) while that of M2 macrophages was insignificant (5%, P-value= 0.116) after 12 hours of infection and cytokine treatment (Fig. 13). DENV2 infected M1 (39.05%, P-value= 0.001) and M2 (3.11%, P-value= 0.219) macrophages also showed the same changes in cell viability as HSV-1 infected macrophage populations after 12 hours of virus and cytokine treatment. HSV-1 infected M1 macrophages decreased by ~80% (P-value<0.001) and insignificant difference in M2 (P-value=0.293) compared to control (Fig. 12). Cell viability of DENV2 infected M1 macrophages decreased significantly (~70%, P-value< 0.001) after 24 hours of infection and cytokine-treatment. Also the cell viability of DENV2 infected M2 macrophages changed insignificantly (P-value=0.054). The overall cell viability among
the macrophage subpopulations decreased significantly in both uninfected and infected M1 macrophages after 24 hours (Fig. 14).

**SOCS Expression in Uninfected and HSV-1 or DENV2 Infected Macrophages**

SOCS1 and SOCS3 expressions were determined by western blotting. Macrophages were either unpolarized or cytokine-polarized and uninfected or infected for 12 (Fig. 28 and 29) or 24 hours (Fig. 30 and 31). At 12 hours post cytokine polarization and virus infection in M1 macrophages in each of the experimental groups compared with the respective M0 cells, SOCS1 expression was significantly lower in uninfected (P-value= 0.043), HSV-1 infected (P-value=0.001) and DENV2 infected (P-value<0.001). In M2 macrophages in each of the above groupings compared with respective M0 cells, SOCS1 expression in HSV-1-infected cells decreased (P-value= 0.038); there was no significant variation in DENV2- infected M2 macrophages (P-value> 0.05).

At 24hours after cytokine polarization and virus infection in M1 macrophages in each of the experimental groups compared with the respective M0 cells, SOCS3 expression was significantly lower in HSV-1 infected (P-value< 0.001) and significantly higher in uninfected (P-value<0.001), however, an insignificant change in DENV2 infected (P-value>0.05) cells. In M2 macrophages in each of the experimental groups compared with the respective M0 cells, SOCS3 expression was significantly higher in uninfected (P-value=0.004) and HSV-1 infected (P-value=0.014) and significantly lower
in DENV2 infected (P-value=0.009). The overall expression of SOCS3 was found to be significantly higher than the expression of SOCS1 after 24 hours of cytokine treatment.

The SOCS1/SOCS3 ratio at 12 hours after infection with HSV-1 was significantly higher (P-value= 0.004) in M1 cells than that seen for the uninfected M1 cells- while this ratio in DENV2-infected M1 cells was significantly lower (P-value= 0.002). At this same time, the SOCS1/SOCS3 ratios of HSV-1 or DENV2-infected M2 cells showed no difference from uninfected M2 cells.

By 24 hours after infection the SOCS1/SOCS3 ratio of HSV-infected M1 cells to that of uninfected M1 macrophages was significantly higher (P-value= 0.015) while SOCS1/SOC3 ratio was no higher in DENV2-infected cells than that seen in uninfected M1 cells. At 24 hours post infection, SOCS1/SOCS3 ratio of HSV-1-infected M2 cells was the same as that of uninfected M2 cells. SOC1/SOCS3 ratio was significantly higher (P-value<0.001) in DENV2-infected macrophages than in uninfected M2 cells.

**Virus Replication in Polarized Macrophages**

Viral titers were measured by standard plaque assay (Fig. 34). Titer for HSV-1 in M1 macrophages was 3-fold lower than in M0 and 3.5-fold less than titer of virus in M2 cell cultures.
DISCUSSION

In this study we found that macrophages infected with HSV-1 and DENV2 along with cytokine induced polarization show differences in the expression of CD14 and CD86 cell surface markers after 24 hours. In macrophages subjected to polarization with LPS+IFN-γ for development of the M1 subpopulation and with IL-4 for polarization of M2 cells, changes in the properties and morphology of the cells after 12 and 24 hours were observed. M1 cells were large, elongated, vacuolated and strongly adherent. On the other hand, the morphology of M2 macrophages was similar to control M0 cells in that the cells were round or elongated and stellate. At these same times, cell viability of the M1 macrophages was markedly decreased compared to control and M2 macrophages. The results from HSV-1 plaque assay show a 3 – 3.5 fold decrease in viral titers suggesting decrease in viral uptake and replication by M1 macrophages. This decrease may reflect production of pro-inflammatory cytokines and cytotoxic molecules leading to cell death and impaired virus replication.

There is a decrease in cell viability of M1 macrophages, while not in case of M2 macrophages. This decrease in M1 cell viability can be attributed to the production of cytotoxic molecules such as the reactive oxygen-intermediates, nitric oxide and TNF-α (Naha et al., 2010; Kyoungho et al., 2001). We know that M1 macrophages are known to be associated with pro-inflammatory cytokines such as TNF-α and other cytotoxic compounds such as inducible nitric oxide species (iNOS) and reactive oxygen species...
(ROS) (Schachtele et al., 2010). In future experiments, nitric oxide concentrations in the culture can be detected by Griess reagent and the culture can be treated with a blocking agent for iNOS (or NO inhibitors) to see if the cell viability increases. Also, TNF-α can be neutralized by the help of anti-TNF-α antibodies which requires more than 24 hours for action on TNF-α.

Cell-surface markers CD14 (LPS co-receptor) and CD86 (B7-2 co-stimulatory molecule) expressions differed between polarized subsets of macrophages. It was observed that expression of CD14 was uniformly higher in all the macrophage subtypes as opposed to higher expression only in M1 macrophages (Gordon and Taylor, 2005). This might be attributed to the property of RAW264.7 cells being virus transformed and belonging to monocyte-macrophage derived lineage of cells (Raschke et al., 1978). Also it has been found in a recent study that the RAW 264.7 cells release leukemia virus in the culture (Hartley et al., 2008). During M1 polarization, bacterial LPS acts as a ligand interacting with and activating CD14 and TLR4 on macrophage surface and IFN-γ along with LPS upregulates expression of CD86 for the antigen-presenting function of macrophages (Tapping et al., 2000; Kitchens, 2000).

Similar to the observations of Reichard (2013), CD14-CD86 expression was higher in M1 macrophages prior to and after virus infection. These results suggest that HSV-1 or DENV2 infection affects the ability of unpolarized or polarized RAW 264.7 macrophages to express CD14 and CD86. CD14 can implement an innate immune
response and CD86 can mount an adaptive immune response against the virus by promoting pro-inflammatory immune responses. CD86 is involved in B cell stimulation, T-cell activation and maturation (Chen et al., 1994). T cells play a major role in adaptive immunity against HSV-1 infection but not significantly against DENV2 infection signifying the expression of CD86 on M2 macrophages (Johnson et al., 2008).

Suppressors of cytokine signaling (SOCS) proteins are antagonists of cytokine signaling. Under normal conditions, SOCS proteins are anti-inflammatory in nature and subject inflammatory proteins to proteasomal degradation by ubiquitylation (Yoshimura, 2003). They inhibit cytokine production thus inhibiting immune system defense against infection by inhibiting the JAK/STAT pathway (Cooney, 2002). But in case of a viral infection, viral proteins have ability to hijack SOCS functions making them the targets for viral evasion in the host. SOCS proteins inhibit antiviral immune responses thus allowing viral invasion and replication (Nowoslawski et al., 2010 In this study the overall SOCS3 expression was higher than SOCS1 expression. SOCS3 expression in M1 macrophages is high suggesting the that viral infection activates SOCS proteins in macrophages thus inhibiting the production of pro-inflammatory cytokines such as TNF-α, IL-12, etc. and other cytotoxic agents such as iNOS and ROS by inhibiting either MAPK pathway or IFNAR signaling cascade respectively. Thus, inhibition of pro-inflammatory immune responses allows virus survival in the host. Studies by Qin et al., 2012 have shown that SOCS3 represses M1 macrophage polarization and thus inhibits
pro-inflammatory immune responses. We determined that M1 cell viability decreased further post-infection and that SOCS3 was upregulated in DENV2 infected M1 cells compared to control and M2 macrophages this is in correlation with a recent study that showed that SOCS3 is essential in regulating macrophage polarization showing SOCS deletion induced resistance to LPS induced endotoxic shock. Another finding from this study showed enhanced IL-4 plus IL-13 induced STAT6 phosphorylation in SOCS3 knockout macrophages (Spence et al., 2013).

In future studies, the effect of SOCS proteins upon infection can be studied with the help of specific SOCS antagonists, on how they inhibit SOCS thus allowing release of pro-inflammatory cytokines that are effective against viral infections. Some specific SOCS1 antagonists such as small peptide antagonist that corresponds to the activation loop of Jak2 can be used in future studies and SOCS expression can be observed post-infection and polarization of macrophages (Johnson et al., 2010). In the present study, change in SOCS1 expression and not SOCS3 expression after 12 hours of treatment stems from increasing concentrations of pro-inflammatory cytokines and ROS or iNOS produced by IFNAR pathway may be inhibited by increasing SOCS1 (Song et al., 1998). But 24 hours after cytokine treatment with or without infection, SOCS1 expression is suppressed with an increase in SOCS3 activity. We assume this might be due to inter-regulation of SOCS1 by SOCS3 and vice versa. Also increase in SOCS3 directly affects the MAPK pathway to inhibit pro-inflammatory cytokine production showing an
ameliorating effect on rapid decrease in cell viability and delayed viral elimination. Increase in SOCS1/SOCS3 ratio after 24 hours of cytokine polarization and virus challenge in M1 macrophages in HSV-1 infected cells suggests SOCS3 may have compromised following infection resulting in inhibition of JAK/STAT as well as IFNAR pathway of pro-inflammatory cytokines, ROS and iNOS production.
Figure 4. DENV2 Plaque assay. A) DENV2 plaque assay performed using crystal violet staining of infected Vero cells after 5 days of incubation. (PFU= $6.9 \times 10^{10}$) B) Flow cytometry analysis using FITC-4G2 mAb (DENV2 specific) monoclonal antibody for quantification of infected cells after 24 hours of incubation. (FACS infectious units/ml=$7.3 \times 10^{10}$)
Figure 5. Morphological changes exhibited by RAW 264.7 macrophage sub-populations 12 hours post cytokine treatment with or without infection. M1 macrophages (uninfected or infected) exhibited cytosolic vacuoles as early as 12 hours post LPS and IFN-γ treatment. IL-4 treated macrophages (M2) showed no visible changes in morphology as compared to control macrophages.
Figure 6. RAW 264.7 macrophages: Top, Untreated control cells Middle, IFN-γ and LPS treated (M1) and Bottom, IL-4 treated (M2). Right, Respective experimental groups phalloidin stained for actin arrangement. M1 macrophages appeared enlarged, irregularly shaped, strongly adherent, and highly vacuolated. M2 macrophages exhibited morphology similar to that of control cells.
Figure 7. LPS + IFN-γ treated RAW 264.7 macrophages: **Left**, Field view of M1 macrophages. **Right**, Enlarged view showing visible vacuolated M1 macrophages.
Figure 8. RAW 264.7 macrophages untreated for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. M1 macrophages showed slight CD86 expression (Scale bar =50μm).
Figure 9. RAW 264.7 macrophages treated with LPS and IFNg for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. M1 macrophages showed increased CD86 expression compared to control macrophages (Scale bar =50μm).
Figure 10. RAW 264.7 macrophages treated with IL4 for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. M2 macrophages showed very low or no CD86 expression compared to control and M1 macrophages (Scale bar =50μm).
Figure 11. 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. Flow cytometry analysis of CD markers on uninfected polarized macrophages show that M1 macrophages exhibit upregulation of CD14+CD86+ expression (33.37%, P-value< 0.001) when compared to control macrophages. Conversely, M2 macrophages showed insignificant changes in CD14+CD86+ expression (1.14%, P-value= 0.319) when compared to untreated control.
**Figure 12.** CD14⁺-CD86⁺ expression profile of uninfected control, M1 and M2 macrophages. CD14⁺-CD86⁺ expression is significantly higher in M1 macrophages ($P$-value< 0.001), while the difference in CD14⁺-CD86⁺ expression in M2 macrophages is insignificant ($P$-value> 0.05).
Figure 13. Cell viability studies 12 hours post cytokine treatment with or without infection. Cell viability of HSV-1 (43.61%, P-value < 0.001) infected M1 macrophages decreased significantly while that of M2 macrophages was insignificant (5%, P-value = 0.116) after 12 hours of infection and cytokine treatment. DENV2 infected M1 (39.05%, P-value = 0.001) and M2 (3.11%, P-value = 0.219) macrophages also showed the same changes in cell viability as HSV-1 infected macrophage populations after 12 hours of DENV2 infection and cytokine treatment. Cell viability of uninfected M1 (73.76%, P-value < 0.001) and M2 (47.41%, P-value = 0.001) macrophages decreased significantly compared to control.
Figure 14. Cell viability studies 24 hours post cytokine treatment with or without infection. Cell viability of DENV2 infected M1 macrophages decreased significantly (~70%, P-value < 0.001) after 24 hours of infection and cytokine-treatment. Also the cell viability of DENV infected M2 macrophages changed insignificantly (P-value = 0.054). The overall cell viability among the macrophage subpopulations decreased significantly in both uninfected and infected M1 macrophages after 24 hours. HSV-1 infected M1 macrophages decreased by ~80% (P-value < 0.001) and insignificant difference in M2 (P-value = 0.293) compared to control. Uninfected M1 macrophages showed highly significant (96%, P-value < 0.001) decrease in viability compared to control.
Figure 15. HSV-1 infected RAW 264.7 macrophages: Top, Unpolarized control cells Middle, IFN-γ and LPS treated (M1) and Bottom, IL-4 treated (M2). Right, Respective experimental groups phalloidin stained for actin arrangement. Few M2 macrophages appeared enlarged compared to control as well as uninfected control and M2 cells.
Figure 16. HSV-1 infected M2 polarized RAW 264.7 macrophages.
Figure 17. RAW 264.7 infected with HSV-1 for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. Control macrophages showed increased CD86 expression compared to uninfected control macrophages (Scale bar =50μm).
Figure 18. RAW 264.7 macrophages infected with HSV-1 and treated with LPS and IFN-γ for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. M1 macrophages showed decrease in CD86 expression compared to uninfected M1 macrophages (Scale bar = 50μm).
Figure 19. RAW 264.7 macrophages infected with HSV-1 and treated with IL-4 for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. M2 macrophages showed increase in CD86 expression compared to uninfected M2 macrophages (Scale bar =50μm).
Figure 20. Flow cytometry analysis of CD14-CD86 expression levels in HSV-1 infected M1, M2, and control macrophages. Negative isotype control CD14 and CD86 antibodies were used to set quadrants. CD14-CD86 expression in M1 macrophages was significantly upregulated (28.34%, P-value= 0.017) compared to control macrophages. On the other hand, CD14-CD86 expression changed insignificantly in M2 macrophages (3.04%, P-value= 0.145). CD14-CD86 expression levels increased in all three experimental groups when compared to respective uninfected groups. (P-value<0.05 is significant)
Figure 21. CD14⁺/CD86⁺ expression profile of HSV-1 infected control, M1 and M2 macrophages. CD14⁺/CD86⁺ expression is significantly higher in M1 macrophages (P-value = 0.017), while the difference in CD14⁺/CD86⁺ expression in M2 macrophages is insignificant (P-value > 0.05).
Figure 22. DENV2 infected RAW 264.7 macrophages: **Top,** Unpolarized control cells **Middle,** IFN-γ and LPS treated (M1) and **Bottom,** IL-4 treated (M2). **Right,** Respective experimental groups phalloidin stained for actin arrangement.
Figure 23. RAW 264.7 macrophages infected with DENV2 for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. Control macrophages showed expression patterns similar to uninfected control macrophages (Scale bar =50μm).
**Figure 24.** RAW 264.7 macrophages infected with DENV2 and treated with LPS and IFNγ for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. M1 macrophages showed decrease in CD86 expression compared to uninfected M1 macrophages (Scale bar =50μm).
Figure 25. RAW 264.7 macrophages infected with DENV2 and treated with IL4 for 24 hours and: A, Actin stained with Texas-Red Phalloidin X for B, stained with FITC conjugated anti-CD14 antibodies C, stained with Brilliant violet conjugated anti-CD86 antibodies D, CD14, CD86, and Phalloidin merged image. M2 macrophages showed increase in CD86 expression compared to uninfected M2 macrophages (Scale bar =50μm).
Figure 26. Flow cytometry analysis of CD14-CD86 expression levels in DENV2 infected M1, M2, and control macrophages. Negative isotype control CD14 and CD86 antibodies were used to set quadrants. CD14⁺-CD86⁺ expression levels in all three experimental groups when compared to respective uninfected groups. M1 macrophages had a significant increase (36.57%, P-value= 0.013) in the number of CD14⁺-CD86⁺ cells when compared to control cells and a slight increase in the number of CD14⁺-CD86⁺ cells when compared to uninfected M1 cells. While there was slight significant decrease (3.7%, P-value= 0.098) in CD14⁺-CD86⁺ expression M2 macrophages. (P-value< 0.05 is significant)
Figure 27. CD14⁺-CD86⁺ expression profile of DENV2 infected control, M1 and M2 macrophages. CD14⁺-CD86⁺ expression is significantly higher in M1 macrophages (P-value=0.01), while the difference in CD14⁺-CD86⁺ expression in M2 macrophages is insignificant (P=value> 0.05).
Figure 28. SOCS1 expression was analyzed by western blotting. Lysates were prepared from RAW 264.7 cells 12 hours after exposure to cytokine polarization and virus infection and western blotted. In M1 macrophages in each of the above groupings compared with the respective M0 cells, SOCS1 expression was significantly lower in uninfected (P-value= 0.043), HSV-1 infected (P-value=0.001) and DENV2 infected (P-value<0.001). In M2 macrophages in each of the above groupings compared with respective M0 cells, SOCS1 expression in HSV-1-infected cells decreased (P-value= 0.038); there was no significant variation in DENV2- infected M2 macrophages (P-value> 0.05). (P-value<0.05 is significant) (P-values were calculated individually for each experimental group)
Figure 29. SOCS3 expression was analyzed by western blotting. Lysates were prepared from RAW 264.7 cells 12 hours after exposure to cytokine polarization and virus infection and western blotted. In M1 macrophages in each of the above groupings compared with the respective M0 cells, SOCS3 expression was significantly lower in HSV-1 infected (P-value<0.001) and significantly higher in uninfected (P-value<0.001), however, an insignificant change in DENV2 infected (P-value>0.05) cells. In M2 macrophages in each of the above groupings compared with the respective M0 cells, SOCS3 expression was significantly higher in uninfected (P-value=0.004) and HSV-1 infected (P-value=0.014) and significantly lower in DENV2 infected (P-value=0.009). (P-value<0.05 is significant) (P-values were calculated individually for each experimental group)
Figure 30. SOCS1 expression was analyzed by western blotting. Lysates were prepared from RAW 264.7 cells 24 hours after exposure to cytokine polarization and virus infection and western blotted. In M1 macrophages in each of the above groupings compared with the respective M0 cells, SOCS1 expression was significantly lower in uninfected (P-value=0.003) and DENV2 infected (P-value=0.002), however, change was insignificant in HSV-1 infected (P-value>0.05). In M2 macrophages in each of the above groupings compared with respective M0 cells, SOCS1 expression in HSV-1-infected and DENV2 infected cells was insignificant (P-value>0.05), however, significantly higher in uninfected (P-value=0.038). (P-value<0.05 is significant) (P-values were calculated individually for each experimental group)
Figure 31. SOCS3 expression was analyzed by western blotting. Lysates were prepared from RAW 264.7 cells 24 hours after exposure to cytokine polarization and virus infection and western blotted. In M1 macrophages in each of the above groupings compared with the respective M0 cells, SOCS3 expression was significantly lower in uninfected (P-value=0.001), HSV-1 infected (P-value<0.001) and DENV2 infected (P-value=0.016) M1 macrophages. In M2 macrophages in each of the above groupings compared with the respective M0 cells, SOCS3 expression was significantly higher in uninfected (P-value=0.002) and DENV2 infected (P-value=0.006) and significantly lower in HSV-1 infected (P-value=0.002). (P-value<0.05 is significant) (P-values were calculated individually for each experimental group)
Figure 32. The ratio of SOCS1 and SOCS3 were plotted for different experimental groups at 12 hours after exposure to cytokine polarization and virus infection as shown above. In M1 macrophages in each of the above groupings compared with the respective M0 cells, the ratio of SOCS1/SOCS3 was significantly lower in DENV2 infected cells (P-value= 0.003), however, change was insignificant in uninfected and HSV-1 infected cells (P-value>0.05). In M2 macrophages the ratio of SOCS1/SOCS3 was significantly lower in HSV-1 infected (P-value= 0.011), however, change was insignificant in uninfected and DENV2 infected cells (P-value>0.05). (P-value<0.05 is significant) (P-values were calculated individually for each experimental group)
Figure 33. The ratio of SOCS1 and SOCS3 were plotted for different experimental groups at 24 hours after exposure to cytokine polarization and virus infection as shown above. In M1 macrophages in each of the above groupings compared with the respective M0 cells, the ratio of SOCS1/SOCS3 was significantly lower in uninfected (P-value= 0.001) and DENV2 infected cells (P-value= 0.013), however, there was a significant increase in HSV-1 infected cells (P-value=0.02). In M2 macrophages the ratio of SOCS1/SOCS3 was significantly higher in HSV-1 infected (P-value= 0.003), however, change was insignificant in uninfected cells (P-value>0.05) and DENV2 infected (P-value> 0.05) cells. (P-value<0.05 is significant) (P-values were calculated individually for each experimental group)
<table>
<thead>
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<th></th>
<th>Control</th>
<th>M1</th>
<th>M2</th>
</tr>
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<tbody>
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<td>(12 hours)</td>
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<tr>
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<tr>
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<tr>
<td>DENV2</td>
<td>70</td>
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<td>61.9</td>
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<tr>
<td><strong>Cell Viability (%)</strong></td>
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<tr>
<td>(24 hours)</td>
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<td>DENV2</td>
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<td>49.3</td>
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</table>

**Table 1.** Shows the percentage cell viability in no virus treated, HSV-1 treated and DENV2 treated macrophage populations 12 hours and 24 hours post-cytokine treatment
Table 2. Shows the percentage of SOCS1 and SOCS3 expression in no virus treated, HSV-1 treated and DENV2 treated macrophage populations 24 hours post-cytokine treatment

<table>
<thead>
<tr>
<th>SOCS (%)</th>
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<th>M2</th>
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<tr>
<td></td>
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<td>SOCS3</td>
<td>SOCS1</td>
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<td>177.1</td>
<td>60</td>
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<tr>
<td>DENV2</td>
<td>96.3</td>
<td>98.1</td>
<td>58.2</td>
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Figure 34. HSV-1 titers in infected macrophage subsets at 24 hours post infection with 0.1 MOI HSV-1. 3 or 3.5 fold lower virus yield was seen in culture fluids of M1 cells compared with either M0 or M2 cell cultures, respectively.
REFERENCES


