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**The Expression of Aryl Hydrocarbon Receptor in RAW 264.7 Macrophages in the
Presence of SOCS1 Peptide and SOCS3 Peptide Mimetic and Cells Infected with HSV-1**

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

By

MAHER SALEM ALWETHAYNANI

B.S., Umm Al Qura University, 2010

2016

Wright State University

WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

December 2, 2016

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Maher Salem Alwethaynani ENTITLED The Expression of Aryl Hydrocarbon Receptor in RAW 264.7 Macrophages in the Presence of SOCS1 Peptide and SOCS3 Peptide Mimetic and Cells Infected with HSV-1 BE ACCEPTED IN PARTIALFULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Alwethaynani, Maher Salem. M.S. Program of Microbiology and Immunology, Wright State University, 2016. The Expression of Aryl Hydrocarbon Receptor in RAW 264.7 Macrophages in the Presence of SOCS1 Peptide and SOCS3 Peptide Mimetic and Cells Infected with HSV-1

Macrophages play a crucial role for our immune system and protect our body from infection. Suppressor of cytokine signaling (SOCS) proteins negatively regulate cytokine receptor and TLRs. The aryl hydrocarbon receptor (AhR) also performs an important role in immunity. This study investigated the changes in expression of AhR in RAW 264.7 macrophage cells after the addition of SOCS1 and SOCS3 peptide mimetics and also examined AhR expression in RAW 264.7 macrophage cells before and after the addition of HSV-1. RAW 264.7 murine macrophage cell lines which are from male BALB/c mice were used in this study. The addition of the SOCS1 peptide mimetic treatment of uninfected RAW 264.7 macrophages caused a significant increase in AhR expression ($p < 0.001$) associated with production of the pro-inflammatory cytokines such as TNF- α . However, treatment of uninfected RAW 264.7 macrophages with SOCS3 peptide mimetic caused a significant decrease in AhR expression compared to uninfected control cells ($p < 0.01$) associated with production of IL-10. Following viral challenge, there was an overall decrease in AhR expression in all treated RAW 264.7 macrophages. During the course of the study, viabilities of RAW 264.7 macrophages with and without HSV-1 were assessed. Treatment of macrophages with SOCS3 increased cell viability compared to SOCS1 treatment while viability following both treatments was reduced in virus infected cells. These observations suggest that SOCS3 plays a critical role in controlling the effect of cytotoxic molecules. This study shows that SOCS1 peptide and SOCS3 peptidemimetic can impact AhR expression and cell survival of murine macrophages.

HYPOTHESIS

SOCS3 peptide mimetic induces anti-inflammatory cytokines such as IL-10 and less pro-inflammatory cytokine such as nitric oxide and TNF- α . SOCS1 peptide mimetic is known to increase the production of pro-inflammatory cytokines such as TNF- α . During virus infection, SOCS functions to inhibit immune response allowing virus invasion and replication. I predicted that the AhR expression cells in RAW 264.7 murine macrophage cells would be upregulated in the presence of SOCS1 peptide mimetic, while the AhR expression in RAW 264.7 murine macrophage cells would be downregulated in the presence of SOCS3 peptide mimetic. The AhR expression by RAW 264.7 murine macrophage cells will be decreased following infection with HSV-1 with or without SOCS1 and SOCS3.

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LIST OF ABBREVIATIONS

AhR = Aryl hydrocarbon receptor

Arnt = AhR nuclear translocator protein

ATCC = American Type Culture Collection

BSA = Bovine Serum Albumin

DMEM = Dulbecco's Modified Eagle's Medium

F-actin = Filamentous actin

gD = Glycoprotein D

HSV= Herpes Simplex

HVEM = Herpes Virus Entry Mediator

ICP0 = Infected Cell Protein Null

IFN- γ = Interferon gamma

IL = Interleukin

iNOS = Inducible Nitric Oxide Synthase

β -7 integrin = Integrin beta-7

JAK = Janus Kinase

KIR = Kinase Inhibitory Region

LPS = Lipopolysaccharide

MHC = Major Histocompatibility complex

MOI = Multiplicity of Infection

NF κ B = Nuclear factor κ B

PBS = Phosphate buffered saline

qROI = Qualified region of interest

SOCS = Suppressor of Cytokine Signaling

TNF- α = Tumor necrosis factor-alpha

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DEDICATION

I would like to dedicate my thesis project to my dad, my mom and my wife for their support and endless love.

INTRODUCTION

Macrophages and dendritic cells are formed upon differentiation of monocytes. They protect our body from infection and are phagocytic in nature. Any antigen that enters the body gets engulfed by these macrophages which in turn generate adaptive immune response.

Macrophages, mast cells and neutrophils are mediated as professional phagocytic cells (Murray and Wynn, 2012). Macrophages can be identified by surface markers like F4/80 which differentiates them from dendritic cells. All the tissues contain resident macrophages. During the time of embryonic development, macrophages develop with the tissue. Macrophages become functionally specialized based on the type of cytokines they encounter (Lavin et al., 2015).

Macrophages are activated by either classical or alternative pathway. Apart from engulfing microbes, they also play a role in wound healing and clearing cell debris (Mosser and Edwards, 2008).

The aryl hydrocarbon receptor (AhR) is activated in the presence of a ligand. It is present in the cytosol but is inactivated there with the help of proteins that are bound to it (Abel and Haarmann-Stemann, 2010). The three kinds of proteins binding AhR are chaperones, hepatitis B virus X associated protein-2 and p23. Ligand binding to AhR receptor leads to upregulation of the enzymes that are responsible for metabolizing the ligand. Cytochrome P-450 is one example of an enzyme that is activated by this signaling. A compound that has high affinity for AhR is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The binding of STAT1 and AhR occurs in response to LPS stimulations. Plasminogen activator inhibitor (Pai-2) is induced by LPS in RAW 264.7 macrophages (Nguyen et al., 2013). AhR and Pai-2 together regulate pro-inflammatory cytokine production in macrophages. AhR also mediates the differentiation of IL-17 Th cells and IL-17 treg cells (Veldhoen, 2010).

Suppressor of cytokine signaling (SOCS) proteins are negative regulators of cytokine receptors and Toll Like Receptors (TLR). These proteins have a Src homology 2 (SH-2) domain and a carboxy terminal region having amino acids that forms SOCS box. There are eight kinds of SOCS proteins present, namely SOCS1-SOCS7 and CIS. SH2 domain interacts with phosphotyrosine phosphorylated proteins and the interaction of SOCS box takes place with elongin BC complex. This interaction inhibits the degradation of SOCS proteins. There is also a kinase inhibiting region present in SOCS protein. SOCS1 binding to JAK inhibits its catalytic activity. SOCS3 binds to the cytokine receptor area, present adjacent to JAK binding site and thus it inhibits the binding of JAK. Only by ubiquitination can these SOCS proteins be degraded (Yoshimura, 2005).

Herpes Simplex Virus Type 1 (HSV-1) is a virus that has a double stranded DNA genome which is linear and is of approximate size of 152kbp (McGeoch *et al.*, 2006). The viral DNA is enclosed in a capsid which is icosahedral in shape and surrounded by a proteinaceous cluster called tegument. The tegument is also inside an envelope. This envelope has different multifunction glycoproteins which play the role of attachment of the viral cell with the host. This virus causes mouth sores and may cause genital sores as well (World Health Organisation [WHO], 2016). It can affect humans efficiently as it has several modes (Karasneh and Shukla, 2011). The virus first attaches itself to the host and then follows the anchoring of the viral cell to the host. HSV-1 inhibits immune response by preventing the activation of lymphocytes (Karasneh and Shukla, 2011).

LITERATURE REVIEW

Herpes Simplex Virus Type 1 (HSV-1)

HSV-1 or oral herpes is a highly contagious infection which mostly causes sores around the mouth (cold sores) but can also cause genital herpes (World Health Organisation [WHO], 2016). The virus possesses a large sized, linear, double-stranded DNA genome which is approximately 152kbp long (McGeoch *et al.*, 2006). This viral DNA genome is encased in an icosahedral shaped nucleocapsid which is surrounded by a tegument (a proteinaceous cluster forming a layer) which is also in turn encased in an envelope. This envelope is a polymorphic lipid bilayer which contains several copies of different multifunction glycoproteins which play various roles in attachment to and entry of host cells (Liu and Zhou, 2007). These glycoproteins are encoded in the genome of the virus by at least 74 different genes (McGeoch *et al.*, 2006).

HSV-1 which belongs to the alphaherpesvirus subfamily has excellent ability to infect human cells (Spear and Longnecker, 2003). This is due to several factors, one of which is its multiple entry modes (Karasneh and Shukla, 2011). The virus has the ability to infect host cells either by endocytosis or by direct fusion (fusion with the host cell's plasma membrane). This is brought about by the interaction of seven glycoproteins (gK, gD, gC, gH, gB, gM and gL) (Heldwein and Krummenacher, 2008) which interact with their cognate receptors on the cell surface in a series of steps (Karasneh and Shukla, 2011).

First step is the attachment of the virus to the host cell by binding of gB and/or gC to heparin sulphate proteoglycans on the host cell's surface (Spear, 2004). This is followed by interaction of gD with a gD receptor which enhances tight anchoring of the virus to the host cell's plasma membrane (Campadelli-Fiume, Cocchi, Menotti and Lopez, 2000; Shukla and Spear, 2001).

Finally, gD undergoes conformational changes during receptor binding which is suspected to signal gB and gH/gL thereby facilitating membrane fusion followed by the release of the viral tegument proteins and viral nucleocapsid into the host cell's cytoplasm (Akhtar and Shukla, 2009; Karasneh and Shukla, 2011). This is followed by uncoating of the nucleocapsid which allows the injection of viral DNA into the host cell's nucleus through nuclear pores for onward replication and capsid assembly (Liu and Zhou, 2007).

In human hosts, HSV-1 inhibits immune response by preventing the activation of cytotoxic T-lymphocytes (CTLs) which are major antiviral immune cells by secreting infected cell protein 47 (ICP-47) that binds specifically to transporters associated with antigen processing (TAP) (EMBI-EBI, 2016). This obstructs peptide-binding and translocation which subsequently prevents peptides from being loaded onto MHC class I molecules leading to retention of empty MHC I molecules in the endoplasmic reticulum and ultimately resulting in proteosomal degradation (EMBI-EBI, 2016). In human hosts, HSV-1 exhibits both latent and lytic modes (James, 2004). As a result of certain illnesses or stress, the latent mode can however be reactivated into the lytic cycle, causing cutaneous disease (Alsharif, 2015; James, 2004).

The host immune actions against HSV-1 are both complex and multifactorial involving both the innate and adaptive immune response (Chew et al., 2009) with macrophages playing anti-herpetic roles in the first stages of infection (Ellermann-Eriksen, 2005). The first wave of response is the production of cytokines (primarily type I interferons [IFN]) and tumour necrosis factor (TNF) (Ellermann-Eriksen, 2005). These have anti-viral activity and also stimulate the macrophages; this is followed by release of IL-12 and cytokines such as IFN- γ mainly by NK cells (Ellermann-Eriksen, 2005). The macrophages and other cells serve as a network for the control of the

replication of HSV-1 (Mantovani *et al.*, 2004). Macrophages also inactivate the virus and protect other cells from infection (Mantovani *et al.*, 2004).

Macrophages

Macrophages are a type of leukocytes (white blood cells) which are phagocytic in nature and protect our body against infecting agents. They engulf antigens which enter the body and generate immune responses. The precursor of macrophage is called monocyte. Monocytes then give rise to macrophages and dendritic cells. These, along with mast cells and neutrophils, are termed as professional phagocytic cells (Murray and Wynn, 2012). Macrophages can be differentiated from dendritic cells as they express surface markers like F4/80. They exist in nearly all tissues. During the time of embryonic development, macrophages develop with the tissue. The functional specialization of these cells depends on the type of cytokine they encounter (Lavin *et al.*, 2015).

Macrophage Functions

As mentioned earlier, macrophages exist in all tissues. In cases of inflammation, they migrate to the tissue as a response to inflammation. They also migrate in steady state. Macrophages originate from peripheral blood mononuclear cells (PBMCs). In the bone marrow, there is a common myeloid progenitor cell present which is responsible for the production of differentiated cells. The bone macrophages are called osteoclasts; microglial cells are present in brain; liver macrophages are termed as kupffer cells and histiocytes are the macrophages present in connective cells (Mosser and Edwards, 2008).

Functions of macrophages include defense from a range of microbes. An immune response is generated when antigens are engulfed which activates genes producing cytokines like IFN- γ and TNF α . Macrophages also perform anti-inflammatory function that play a significant

role in wound healing and clearing cell debris consequences from damaged and apoptotic cells (Mosser and Edwards, 2008). In addition, macrophages can recognize specific markers present on the cell surface by their receptors. Scavenger, complement and phosphatidyl serine receptors are present on the surface of macrophages for selection of only foreign cells or dying cells (Murray and Wynn, 2012). These receptors perform homeostatic functions independent of immune response (Mosser and Edwards, 2008).

Macrophage Activation

Macrophage activation happens because of many processes. One of them includes the endogenous danger signals. They trigger the activation of macrophages. They can respond to the environmental signals because of the plasticity they possess. Their physiology can also change based on the immune responses which can be either adaptive or innate. There are two pathways for macrophage activation. Classical pathway is the first pathway and it is called “M1” phenotype. Classical pathway includes macrophages that are activated during cell mediated immune responses (Mosser and Edwards, 2008; Mantovani, 2006).

M1 cells are formed by the activation of TLR in the presence of IFN- γ , TNF- α , Granulocyte Colony Stimulating Factor (GM-CSF), and microbial lipopolysaccharides (LPS) (Mosser and Edwards, 2008; Mantovani, 2006). High levels of reactive nitrogen species (RNS), reactive oxygen species (ROS), IL-12, IL-23 are produced by M1 cells which represent a proinflammatory role. M1 cells also produce low level of IL-10. M1 cells display a Th-1 like phenotype and also encourage inflammation, ECM destruction, and apoptosis (Mantovani, 2006). The combination of two signals IFN- γ and TNF- α is responsible for the production of macrophages that are high in microbicidal or tumoricidal capacity. To maintain these classically

activated macrophages, an anti-inflammatory immune response is required (Mosser and Edwards, 2008) (Figure 1).

The second pathway is alternative activation called “M2” phenotype. This gives rise to wound-healing macrophages. M2 cells also have anti-inflammatory functions. M2 cells are triggered by cytokines like IL-4, IL-10, and IL-13 or by glucocorticoid hormones. They express arginase-1, mannose receptor and IL-4 receptor- α . In compare to M1 cells, low levels of IL-12 and IL-23 are produced by M2 cells. Also, M2 cells produce high levels of IL-10. They are also involved in Th2 cell mediated response (Pesce et al., 2006; Mantovani, 2006) (Figure 1).

M1 and M2 cells can be differentiated from each other by understanding how arginine is metabolized by them. Arginine is catabolized into nitric oxide and citrulline by the enzyme nitric oxide synthase produced by M1 (Odegaard and Chawla, 2011). Bactericidal activity of nitric oxide helps in killing bacteria. Arginine is used to produce urea and polyamines which can support collagen synthesis. This is done by the enzyme arginase 1 that is produced by M2 cells (Odegaard and Chawla, 2011).

Alternatively activated macrophages also produce molecules like YM1 and YM2 which are chitin or chitin like molecules. These macrophages may act detrimentally to host cells. This can happen when their matrix-enhancing activity is disrupted. An example of this is the tissue fibrosis that occurs in chronic schistosomiasis. It happens because of the over activation of wound healing macrophages. Treatment with IL-4 specific antibodies caused reduction in accumulation of wound healing macrophages (Mosser and Edwards, 2008).

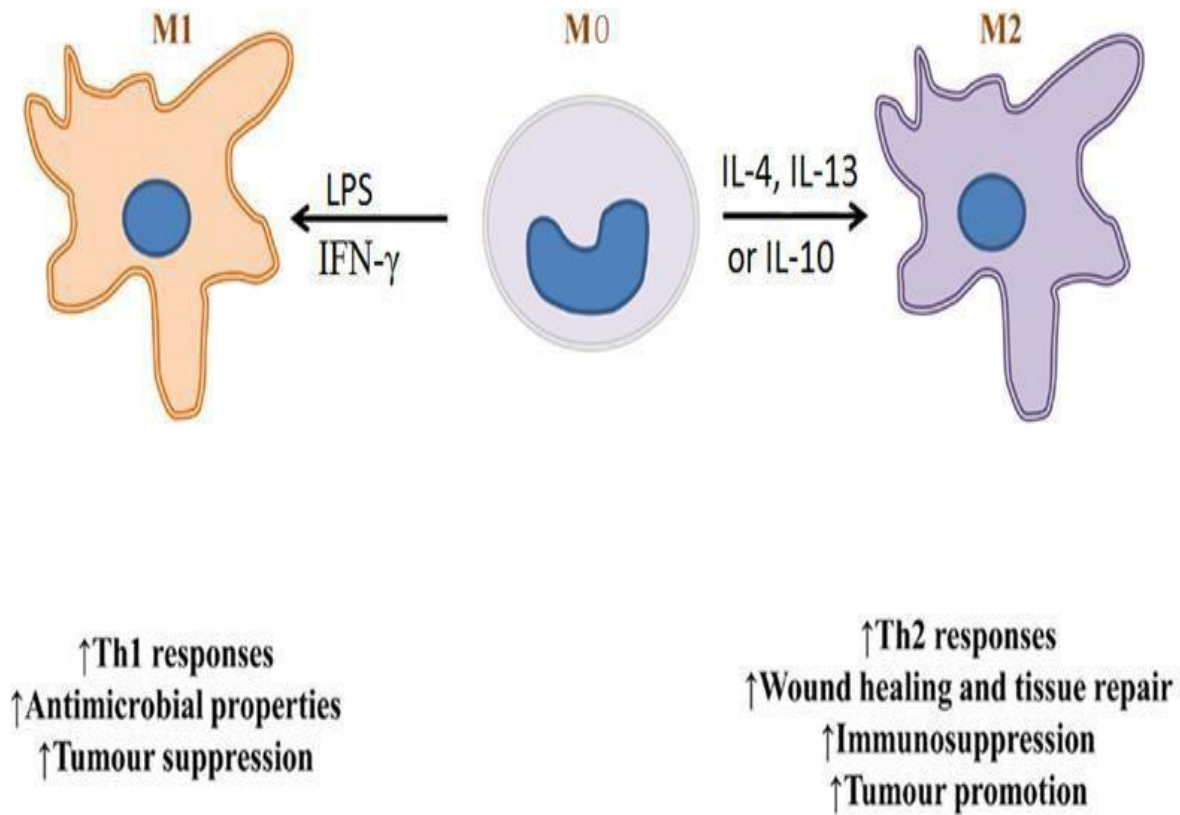


Figure 1. Polarization and activation of macrophages. LPS and IFN γ activate M1 macrophages to produce TNF α , IL-6, iNOS and ROS. IL-13 or IL-4 activates M2 macrophages to produce IL-10 (Modified from Mantovani, 2006).

The Aryl Hydrocarbon Receptor Signaling Pathway

AhR receptor is of Per-Arnt-Sim family which is also known as helix-loop-helix family. It is a transcription factor that gets activated by a ligand. This mediates the effects of polycyclic aromatic hydrocarbons. It is inactivated in the cytosol as protective proteins are bound to it to stabilize the receptor (Abel and Haarmann-Stemmann, 2010).

These protective proteins are as follows: -

1. Two chaperone proteins which perform the function of protecting cells from increasing in cell temperature (Feder and Hofmann, 1999).
2. Hepatitis B virus X associated protein 2 (XAP-2). The function of this protein is to prevent the degradation of AhR (Lees, Peet, and Whitelaw, 2003).
3. P23 protects the receptor from proteolysis (Nguyen et al., 2012).

Ligands that are responsible for the activation of AhR include compounds that are either dietary or pharmaceutical. This activation leads to the up-regulation of the enzymes responsible for the metabolizing these xenobiotics. An example of such enzyme is Cytochrome P450A1 (CYP450A1) (Nguyen et al., 2013). Ligands that are known to bind to this receptor and thus activating it are polycyclic aromatic hydrocarbons (PAH), and halogenated aromatic hydrocarbons (HAH). They are known to have high affinity with the AhR receptor. One of the compounds belonging to HAH family, TCDD, is known to have an extremely high affinity towards this receptor (Nguyen et al., 2013). When AhR comes in contact to its ligand, it gets transferred to the nucleus. In the nucleus, the AhR nuclear translocator (ARNT) leads to the detachment of protective proteins present in AhR. ARNT attaches itself to AhR leading to the formation of a functional transcription factor. This AhR- ARNT transcription factor binds to the

DNA at dioxin- responsive element (DRE). This binding leads to the regulation of expression of CYP450A1 enzyme (Meyer and Perdew, 1999). The deactivation of this transcription factor is done by the processes of phosphorylation/dephosphorylation resulting in the AhR existence degraded in the cytosol (Abel and Haarmann-Stemmann, 2010) (Figure 2).

Macrophages are affected by the activation of AhR, resulting in the dysregulation of vitamin D3 catabolism. Vitamin D3 in its active form regulates immune responses. Deficiency of this vitamin D3 can result in many disorders. The deficiency of vitamin D3 induced by BaP and the activation of AhR in macrophages may mediate some of the smoking effects (Matsunawa et al., 2009). BaP activates AhR which in turns stimulates the catabolism of vitamin D3 thus modulating its signaling. AhR also co-operates with a transcription factor known as cellular viral musculoaponeurotic fibrosarcoma oncogene homolog (c-maf) that controls integrin beta-7 (β -7 integrin) expression. β -7 integrin expression is also a molecular target of PAH (Monteiro et al., 2007).

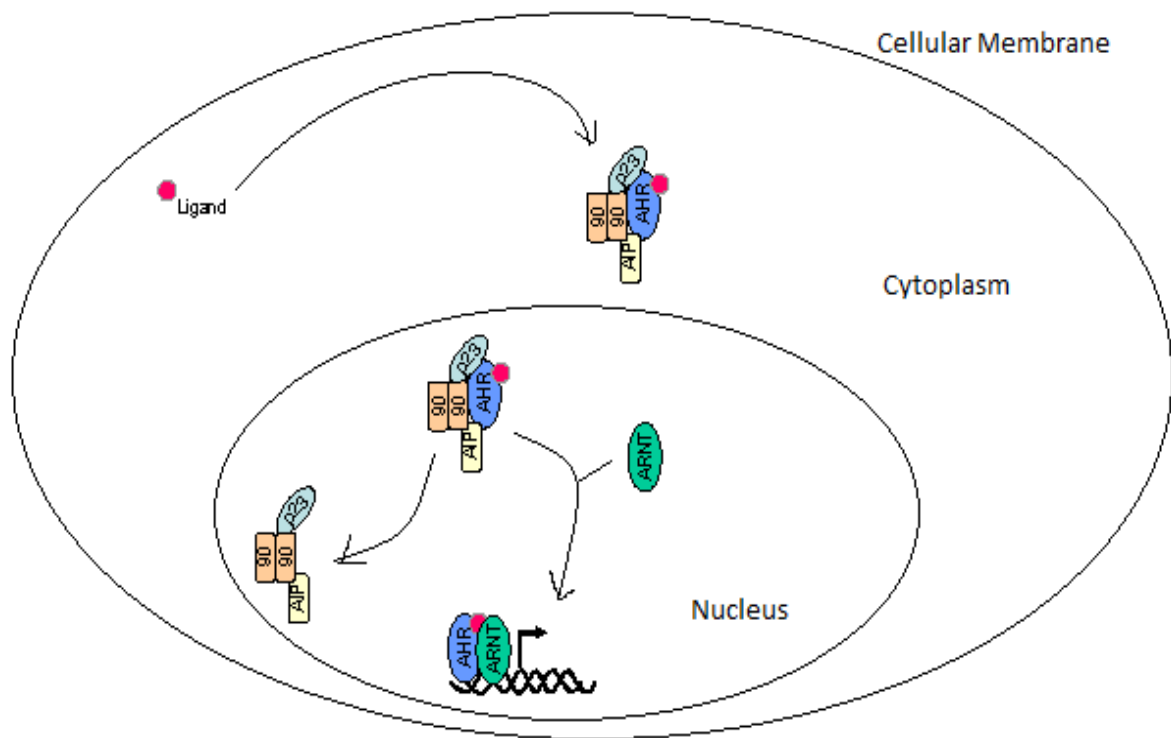


Figure 2. Aryl hydrocarbon receptor signaling pathway. AhR bind to its ligand then it gets transferred to the nucleus. AhR nuclear translocator (ARNT) leads to the detachment of protective proteins present in AhR. ARNT attaches itself to AhR leading to the formation of transcriptional activation of genes (Modified from Meyer and Perdew, 1999).

Suppressor of Cytokine Signaling

SOCS proteins negatively regulated cytokine receptor and TLR. They consist of eight kinds, namely SOCS1- SOCS7 and CIS. They contain a Src homology 2 (SH2) domain and a ~50 amino acid carboxy-terminal SOCS box. Interaction of SH2 domain takes place with the phosphotyrosine phosphorylated proteins while the SOCS box interacts with the elongin BC complex. This results in inhibiting the degradation of SOCS proteins (Yoshimura, 2005).

Cytokines such as IFN- γ or TLR ligands (by LPS) induce SOCS1 and SOCS3. Phosphorylation of JAK1 and JAK2 takes place when these cytokines bind to their receptors. This activates the receptor to which STAT binds resulting in phosphorylation of STAT. Post phosphorylation, STAT dimerizes and this complex enters the nucleus and binds to the genes responsible for production of SOCS1 and SOCS3, causing their up-regulation (Hu et. al., 2002; Alexander, 2002).

SOCS proteins also contain a kinase inhibitory region (KIR) that is responsible for eliminating the activity of JAK. KIR is present near the amino-terminal domain. The binding of SOCS1 to JAK prevents it from performing its catalytic activity. The cytokine receptor area adjacent to the site of JAK binding then gets bound by SOCS3. Degradation of both the SOCS complexes can then be done by ubiquitination, allowing SOCS proteins to control the JAK-STAT signaling (Yoshimura, 2005; Yoshimura and Yasukaw, 2012). SOCS3 is also responsible for regulation of STAT3 signaling. An increase in SOCS3 causes anti-inflammatory signals. In macrophage polarization, the signaling pathways of NF- κ B, phosphoinositide 3-kinases (PI3Ks), and extracellular signal-regulated kinase (ERK) are also regulated by SOCS1 and SOCS3. The cytokine-induced activation of SMAD3, STAT3, STAT6 and PIK3 are inhibited by SOCS3 (Wilson, 2014).

Materials and Methods

Cell Line

Cell line of RAW 264.7 murine macrophages was obtained from the American Type Culture Collection, Manassas, VA. Petri plates were obtained from BD Biosciences. The ten percent of fetal bovine serum was acquired from Fisher Scientific and the 1% penicillin-streptomycin from MP Biomedical, LLC. Our African green monkey epithelial cells were provided by Dr. Nancy Sawtell at Children's Hospital Medical Center in Cincinnati, OH.

We began by using the RAW 264.7 murine macrophage cell line. This cell line originated from the Abelson murine leukemia virus-induced tumor, which was procured from an adult male BALB/C mouse. The murine cell line was cultured on 100mm x 20mm petri plates. The medium used was Dulbecco's Modified Eagle's Medium (DMEM), obtained from BD Biosciences, along with 10% heat-inactivate fetal bovine serum, and 1% penicillin-streptomycin antibiotic. The cells were grown in humidified 5% incubator set at 37°C. The cells were split two to three times per week. African green monkey epithelial cells, also known as Vero 76 cells, (CCL-81, ATCC) were used to proliferate HSV-1 (Syn 17+) and to calculate their titers. The cells were then infected with a 0.1 multiplicity of infection (MOI).

Polarization Induction

Once the RAW 264.7 cells reached 70% confluency, they were treated with 100ng/ml lipopolysaccharide (LPS), to induce an M1 phenotype. Cells were polarized for a period of twenty-four hours. The cells were collected using a cell scraper after the twenty-four-hour period for the purpose of cell viability and flow cytometry. LPS from E. coli 0111:B4 was obtained from Chondrex Inc, (Redmond, VA).

Cell Viability

The RAW murine macrophages were grown to a confluency of approximately 70%. Cells were then stimulated with the LPS (100ng/ml), SOCS1 peptide (35µm/ml), and SOCS3 peptide (35µm/ml) with a (0.1 MOI) of virus or without a virus. Untreated-cells were the controls. Cells were incubated for a period of twenty-four hours. The cells that were grown using 24-well plates during the twenty-four-hour incubation period were collected using a cell scraper. They were centrifuged at 1500 rpm for 5 mins, at 4°C, after that the medium was removed. One ml of DMEM medium was added to the suspension of pellet. The cells were then stained with trypan blue at a ratio of 1:2. This equation was used to detect cell viability:

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

Immunofluorescent Staining

RAW 264.7 murine macrophages were grown in 8 wells removable silicone cultivation chambers (purchased from Ibidi) to approximately 50% confluency is reached. Cells were stimulated with LPS, SOCS1 peptide (35 µM/ml), and SOCS3 peptide (35 µM/ml) with a (0.1 MOI) of virus or without a virus. Untreated-cells were used as a control. RAW 264.7 macrophages were incubated 24 hours. Following the incubation period, the culture medium was immediately aspirated. Bovine Serum Albumin (1% BSA) was suspended in phosphate buffered saline. 1% BSA was used to wash the cells (3-5 minutes for 2 times). Then, four percent (4%) paraformaldehyde was used to fix the cells for 15 minutes at room temperature. The cells were washed with 1% BSA in PBS for three times (five minutes each). By using 0.25% Triton X-100 diluted in PBS, cells were permeabilized and incubated for 10 minutes at room temperature. The cells were washed with 1% BSA in PBS for three times (five minutes each). Cells were incubated for 2 hours at room temperature with a blocking buffer (5% Goat serum, 3% BSA, and

0.05% tween) to limit non-specific binding. The cells were washed with 1% BSA in PBS for three times (5 mins each). The concentrations of primary antibody and dilutions were applied with a blocking buffer as shown in (Table 1). In blocking buffer, cells were incubated in diluted primary antibodies at a temperature of 4C overnight. Then, the cells were washed with 1% BSA in PBS for three times (five minutes each). In the dark, cells were incubated in Texas Red Phalloidin X and the secondary antibody as shown at (Table 1) for 2 hours at room temperature. The cells were washed with 1% BSA in PBS for three times (5 mins each). Cells were applied to one drop of Vectashield hardset mounting medium (H-1400), Vector Laboratories). Then cells were visualized an Olympus Epi-fluorescence microscope with a 'spot' digital camera.

Table 1. Summary of fluorescent label and antibodies used in immunofluorescence experiment.

ANTIBODY / STAIN	Dilution	COMPANY	Action
Texas Red-Phalloidin X	1:40	Life Technologies	F-actin stain
Anti-Aryl hydrocarbon Receptor antibody	1:200	abcam	Primary antibody
Goat Anti-Mouse IgG H&L (DyLight® 488)	1:500	abcam	Secondary antibody

Flow Cytometric Analysis

Approximately 1.5-2 million cells from each treatment were placed in 1.5mL microcentrifuge tubes. The cells were washed with 1% BSA in PBS for three times (5 minutes each). Cells were spun at 153 RCF for five minutes at 4°C. Cells were then fixed with four percent of paraformaldehyde for twenty minutes at room temperature. The cells were washed with 1% BSA in PBS for three times (5 minutes each). Cells were permeabilized using 0.1% saponin for fifteen minutes at room temperature. Next, cells were blocked with a blocking buffer (3% BSA and 0.1% saponin) for thirty minutes. The cells were washed with 1% BSA and 0.1% saponin in PBS for three times (5 minutes each). Cells were stained with anti-mouse AhR primary antibody (RPT9), in 100µL of 3% BSA for (30 mins) at 4°C (Perdew et al., 1995). The cells were washed with 1% BSA and 0.1% saponin in PBS for three times (5 minutes each). Cells were suspended with 500µL of 10% FBS and 0.1% sodium azide in ice cold PBS. Then cells were analyzed using flow cytometry on an Accuri C6 Flow Cytometer. The FCS Express program was used to analyze the results from flow cytometry. In FCS Express program, isotype control was subtracted from the sample to generate the % of positive stained cells.

SOCS Experiments

Twenty-four hours before any treatment, murine macrophage cells were grown in triplicate, using 24-well plates. The original culture medium was replaced by fresh DMEM which contained 10% FBS and which was treated with LPS to obtain the M1 phenotype. We added the SOCS1 peptide and the SOCS3 peptide either with or without the HSV-1, after which the cells were incubated for twenty-four hours at 37°C. After the incubation period, cells were collected using a cell scraper and were then centrifuged at 1500rpm for five minutes. The pellet was then

re-suspended in 1 ml of 10% DMEM medium and the cells were stained with trypan blue to determine the cell viability.

Statistical Analysis

Experiments were repeated a minimum of three successive times. Data were collected from representative experiments in order to measure cell viability and flow cytometric analysis.

Using Sigma Plot 13.0 software, one-way ANOVA was applied to analyze the differences between the experimental testing groups. P values ≤ 0.05 were defined as a statistically significant. Data were depicted as the mean \pm the standard error of the mean.

RESULTS

RAW 264.7 Macrophage, treated with LPS, virus-infected macrophages and uninfected macrophages displayed morphological changes when compared to untreated macrophages

RAW 264.7 Macrophages were activated with LPS for twenty-four hours to stimulate the M1 phenotype. LPS-treated macrophages without HSV-1 appeared enlarged and flattened with intracellular vacuoles, while control cells appeared rounded at twenty-four hours. Following viral challenge, control cells and LPS-treated macrophages exhibited rounded shapes at twenty-four hours (Figures 3, 4, 5 and 6).

Cell Viabilities of RAW 264.7 Macrophage Untreated and Treated with LPS, SOCS1 and SOCS3 Peptide Mimetic after 24 Hours with and without HSV-1 Infection:

RAW 264.7 Macrophages were treated with LPS, SOCS1 peptide and SOCS3 peptide mimetic for twenty-four hours with and without HSV-1 (MOI 0.1). Trypan blue assay was performed to detect the number of viable cells after 24 hours of treatment with LPS, SOCS1 and SOCS3 with and without HSV-1. Treated RAW 264.7 Macrophage cells were compared to the cell viabilities of the untreated M0 macrophages.

RAW 264.7 Macrophages following treatment with LPS showed significant decrease (~50%, P-value <0.001) in cell viability compared to M0 control cells (~85%) after 24 hours. Macrophages treated with SOCS1 and SOCS3 Peptide mimetic displayed slight decrease (79%, 82%, respectively) in cell viability compared to M0 control after 24 hours. M1 macrophages (~50%) treated with LPS in comparison with macrophages treated with SOCS1 Peptide and SOCS3 Peptide mimetic (79%, 82%, respectively) exhibited a significant decreased (with p value <0.001) in cell viability 24 hours post-treatment (Figure A7).

Following viral challenge, viabilities of RAW 264.7 macrophages following treatment with LPS or SOCS1 or SOCS3 peptide mimetic with HSV-1 were assessed. At 24 hours following treatment, M1 macrophages (~40%, $p < 0.001$) exhibited a reduction in cell viability of ~10% whereas both macrophages treated with SOCS1 Peptide and SOCS3 Peptide mimetic showed minor decreases in viability (77%, 79%, respectively) compared to M0 control cells. There was no significant difference in cell viability between cells treated with of SOCS1 and SOCS3 compared to M0 control cells (~83%). Both groups of cells treated with SOCS1 and SOCS3 (77%, 79%, respectively) showed significant increases (with p value < 0.001) in cell viability compared to macrophages treated with LPS (Figure B 7).

SOCS1-treated Macrophages Showed High Expression Levels of AhR, while Both SOCS3-treated and Control Macrophages Expressed Lower Levels of AhR in Comparison to SOCS1-treated Cells

AhR expression by un-treated and treated macrophages following 24 hours of LPS, SOCS1 and SOCS3 peptide mimetic treatment in the presence and absence of HSV-1 infection was evaluated. Immunofluorescence staining and flow cytometry were performed after 24 hours of treatment. Immunofluorescent images suggested that SOCS1 macrophages appeared to express more AhR expression, while SOCS3 macrophages appeared to express less of AhR expression compared to control cells. LPS macrophages appeared to express more of AhR expression compared to SOCS3 and control cells (Figures 3, 4). Flow cytometric analysis of SOCS1 and LPS macrophages exhibited statistically significant increases level of AhR expression (45%, 42%, respectively, p value < 0.001) compared to M0 (28%). By comparison, SOCS3 macrophages showed decreased level of AhR expression (19%, p value < 0.05) compared

to M0. On the other hand, SOCS3 macrophages exhibited statistically significant decreased levels of AhR expression (p value <0.001) compared to SOCS1 and LPS (Figures 8 and 10).

Virus Challenge Up Regulates AhR Expression in SOCS1-treated Macrophages, and Down Regulates AhR Expression in SOCS3-treated Macrophages

In immunofluorescent images, infected cells with virus suggested that SOCS1 macrophages appeared to express more AhR expression, while SOCS3 macrophages and control cells appeared to express less of AhR expression compared to SOCS1 (Figures 5 and 6). Infected control cells with virus showed a decrease in AhR expression compared to un-infected control cells, while AhR expression was decreased in SOCS1 and SOCS3 following virus infection compared to uninfected cells (Figures 3, 4, 5, 6). Flow cytometric analysis of infected cells that treated with SOCS1 suggested that virus leads to up-regulation of AhR expression (Figures 9 and 11). Following virus challenge, SOCS1-treated macrophages exhibited decreased levels of AhR expression (35%) compared to uninfected SOCS1-treated macrophages (45%) (Figures 8, 9, 10, 11 and Table 2). AhR expression appeared slightly changed in SOCS3 macrophages that infected with virus when compared to un-infected SOCS3 macrophages (Figures 8, 9, 10, 11 and Table 2). Both samples expressed statistically significant increased levels of AhR in SOCS1-treated macrophages compared to SOCS3-treated macrophages. Virus-infected SOCS3-treated macrophages showed slightly a decreased level of AhR expression (15%, p value <0.001) while uninfected SOCS3-treated macrophages cells expressed (19%, p value <0.001). Virus- infected LPS-treated cells showed strongly decreased expression levels of AhR (23%, p value <0.01) when compared to uninfected LPS-treated cells (42%, p value <0.001). Similarly, virus-infected control cells expressed a high decreased in expression levels of AhR (9%, p value <0.001) when compared to uninfected control cells (28%, p value <0.001) (Figures 8, 9, 10, 11 and Table 2).

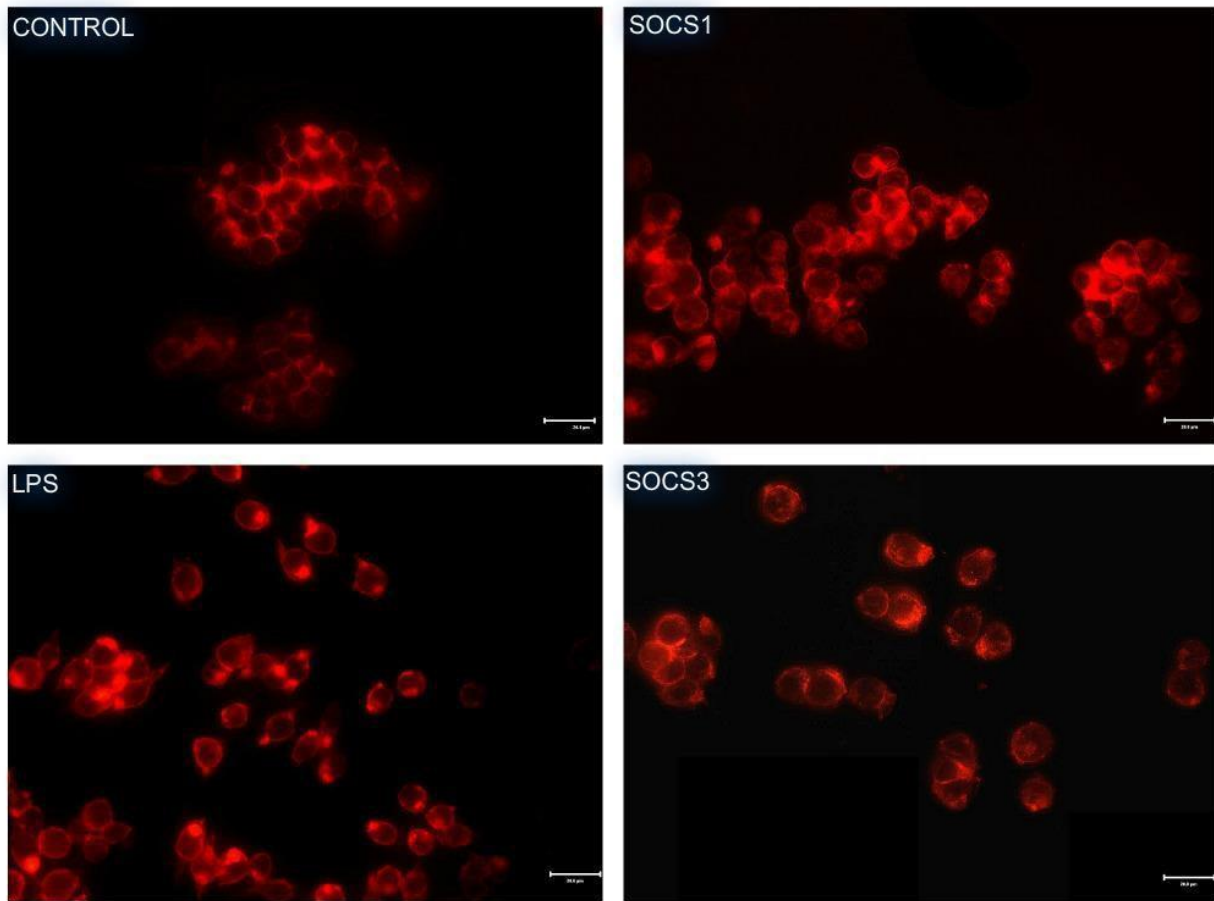


Figure 3. Un-treated RAW 264.7 macrophages and macrophages treated with LPS, SOCS 1, or SOCS 3 peptide mimetic after 24 hours and stained with Texas-Red Phalloidin X. (Scale bar=20 μm).

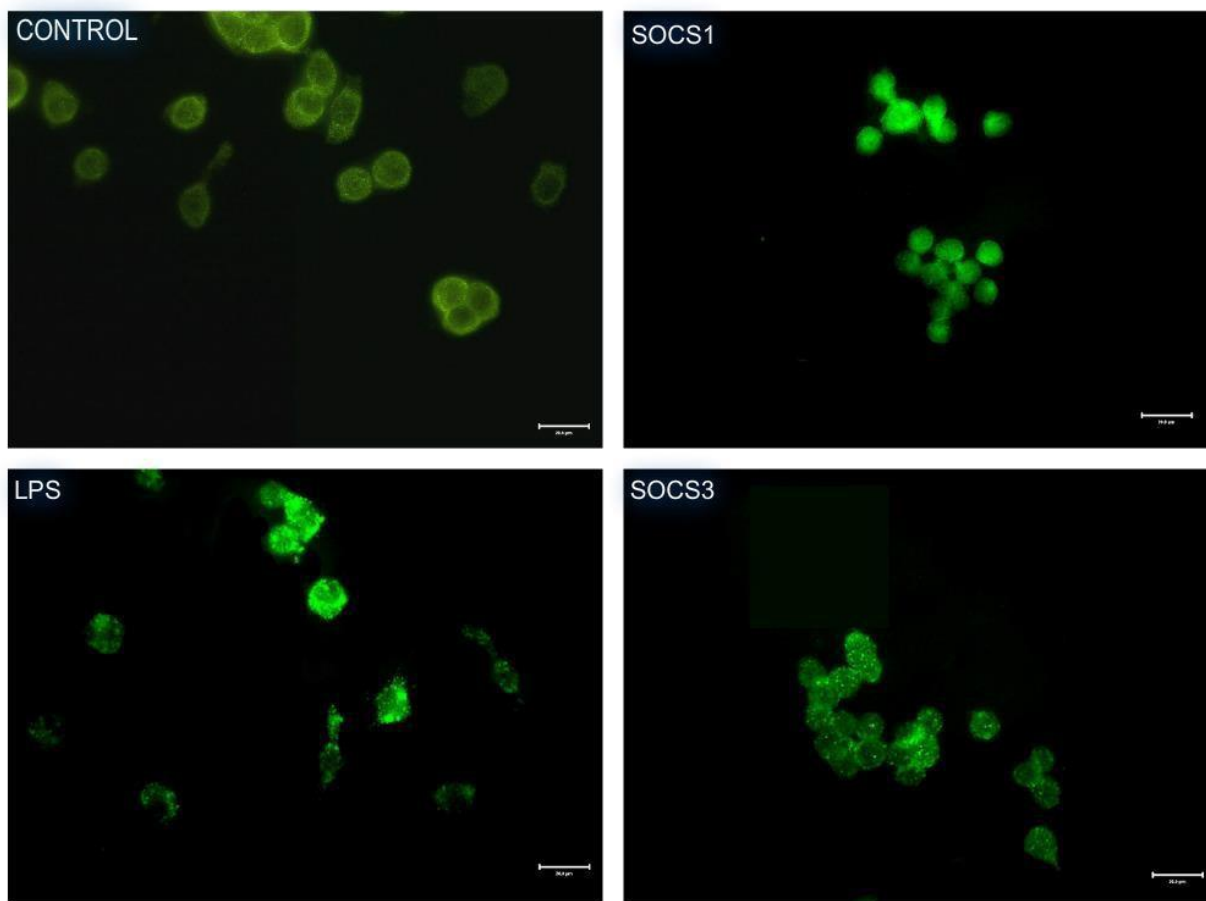


Figure 4. Immunofluorescence images for AhR expression in un-treated RAW 264.7 macrophage and macrophages treated with LPS, SOCS 1, and SOCS 3 peptide mimetic after 24 hours and stained with anti-AhR antibody. (Scale bar =20 μm).

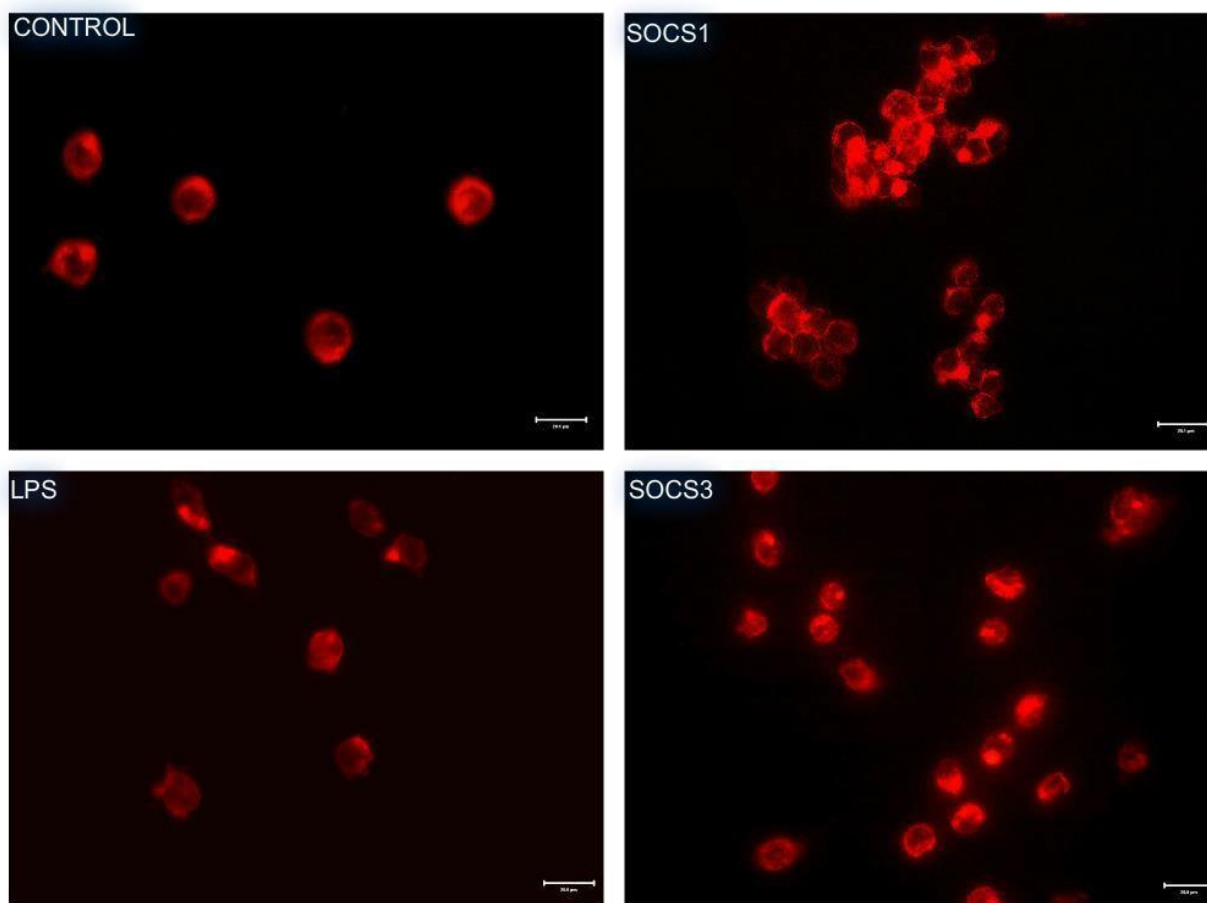


Figure 5. HSV-1 infected un-treated RAW 264.7 macrophages and HSV-1 infected macrophages treated with LPS, SOCS 1, or SOCS 3 peptide mimetic after 24 hours and stained with Texas-Red PhalloidinX. (Scale bar =20 μ m).

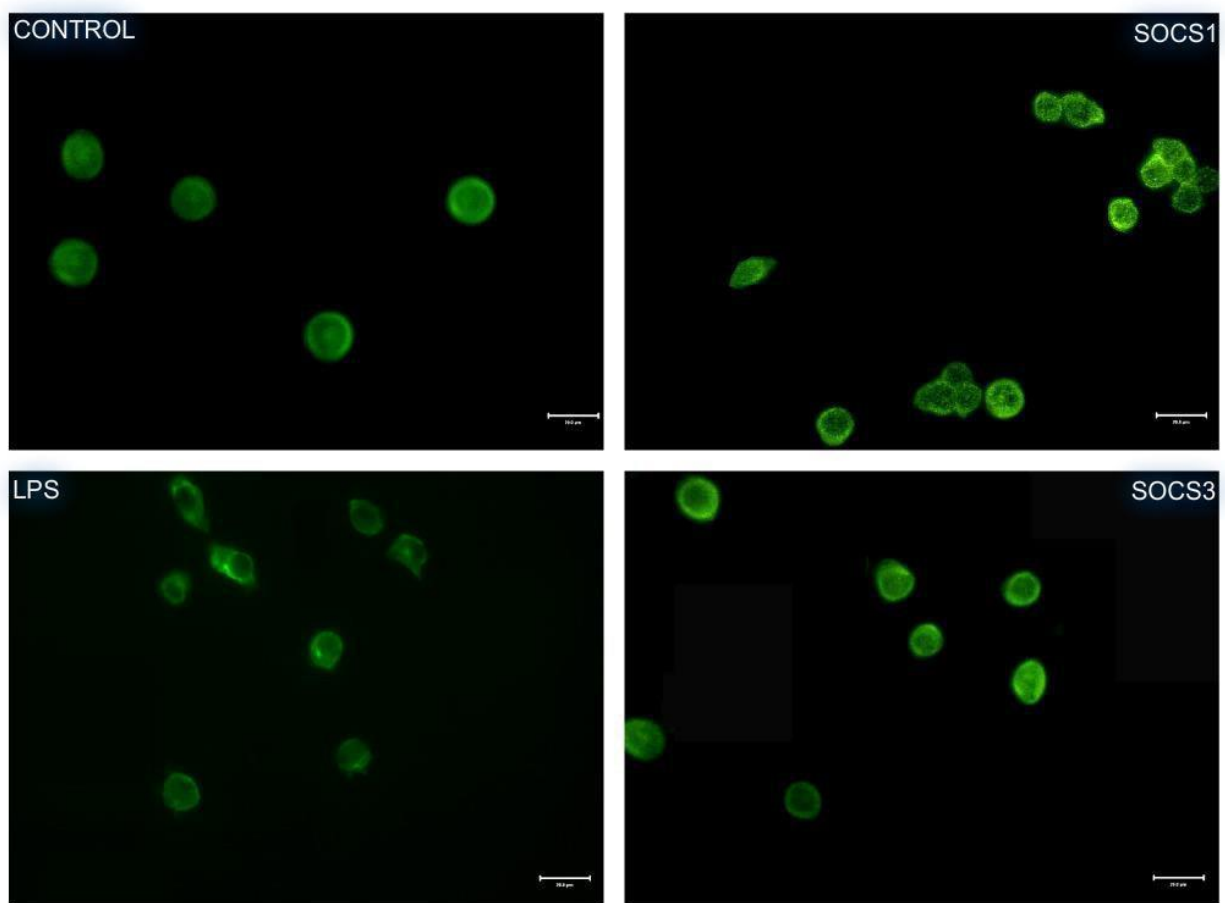


Figure 6. Immunofluorescence images for AhR expression in HSV-1 infected un-treated RAW 264.7 macrophage and HSV-1 infected macrophages treated with LPS, SOCS 1, or SOCS 3 peptide mimetic after 24 hours and stained with anti-AhR antibody. (Scale bar =20 μm).

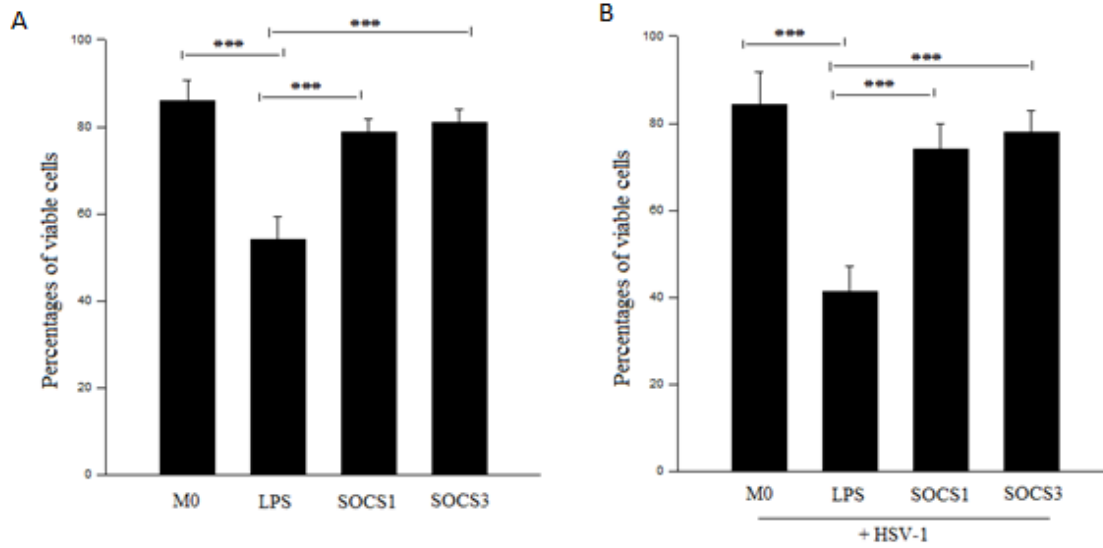


Figure 7. Cell viability of un-treated-macrophages (M0), Macrophages treated with LPS, macrophages treated with SOCS 1, and macrophages treated with SOCS 3 peptide mimetic after 24 hours. (A) Shows un-infected cells. (B) Shows infected cells. Each value represents mean \pm standard error (SE) of three separate experiments. ***; $p \leq 0.001$.

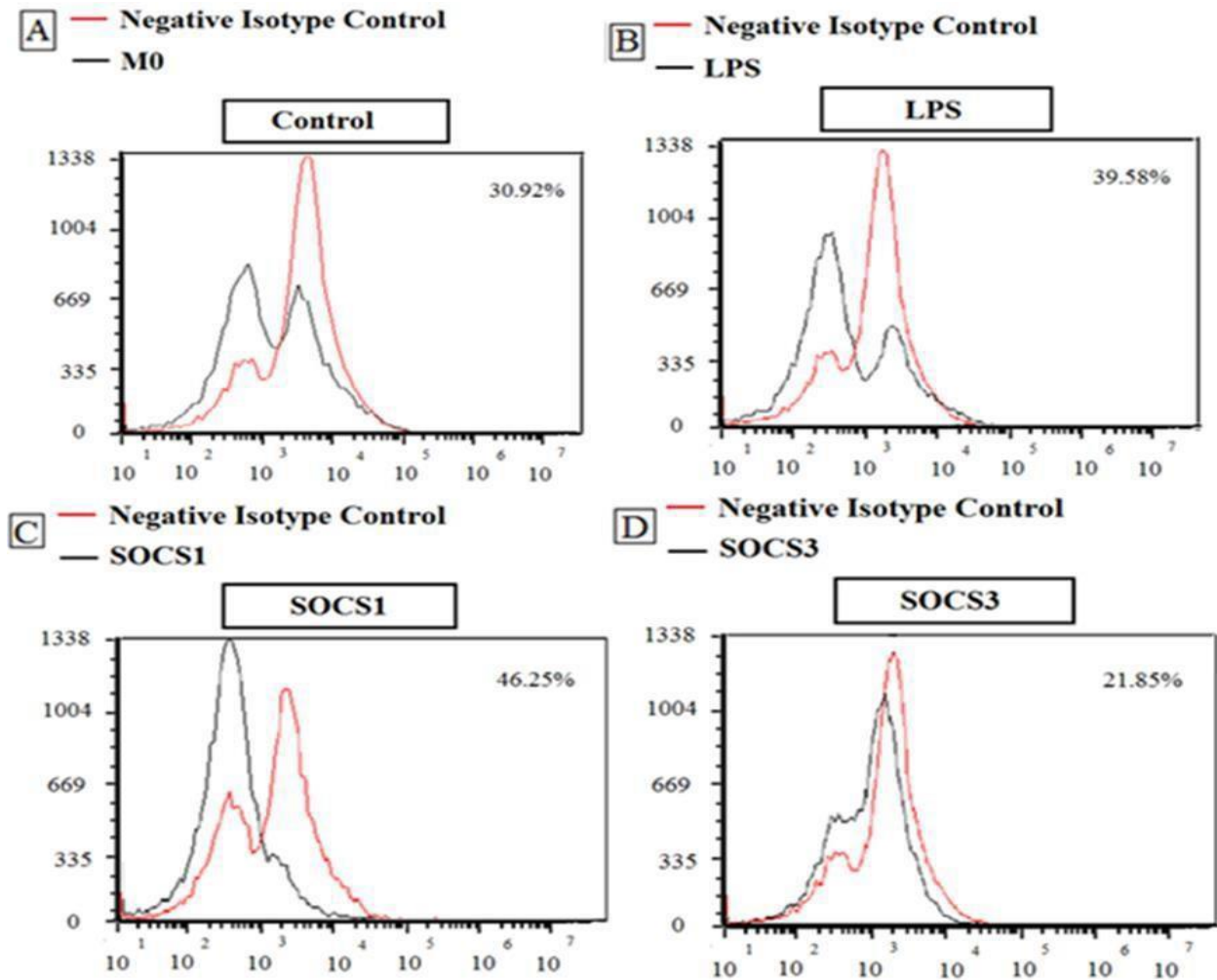


Figure 8. Flow cytometry analysis of AhR expression levels in un-treated RAW 264.7 macrophages after 24 hours. A, B, C, and D histograms show the percentage of cells positive for AhR expression in un-treated macrophages (M0), macrophages treated with LPS, SOCS 1, or SOCS 3 peptide mimetic after 24 hours and stained with anti-AhR antibody. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed.

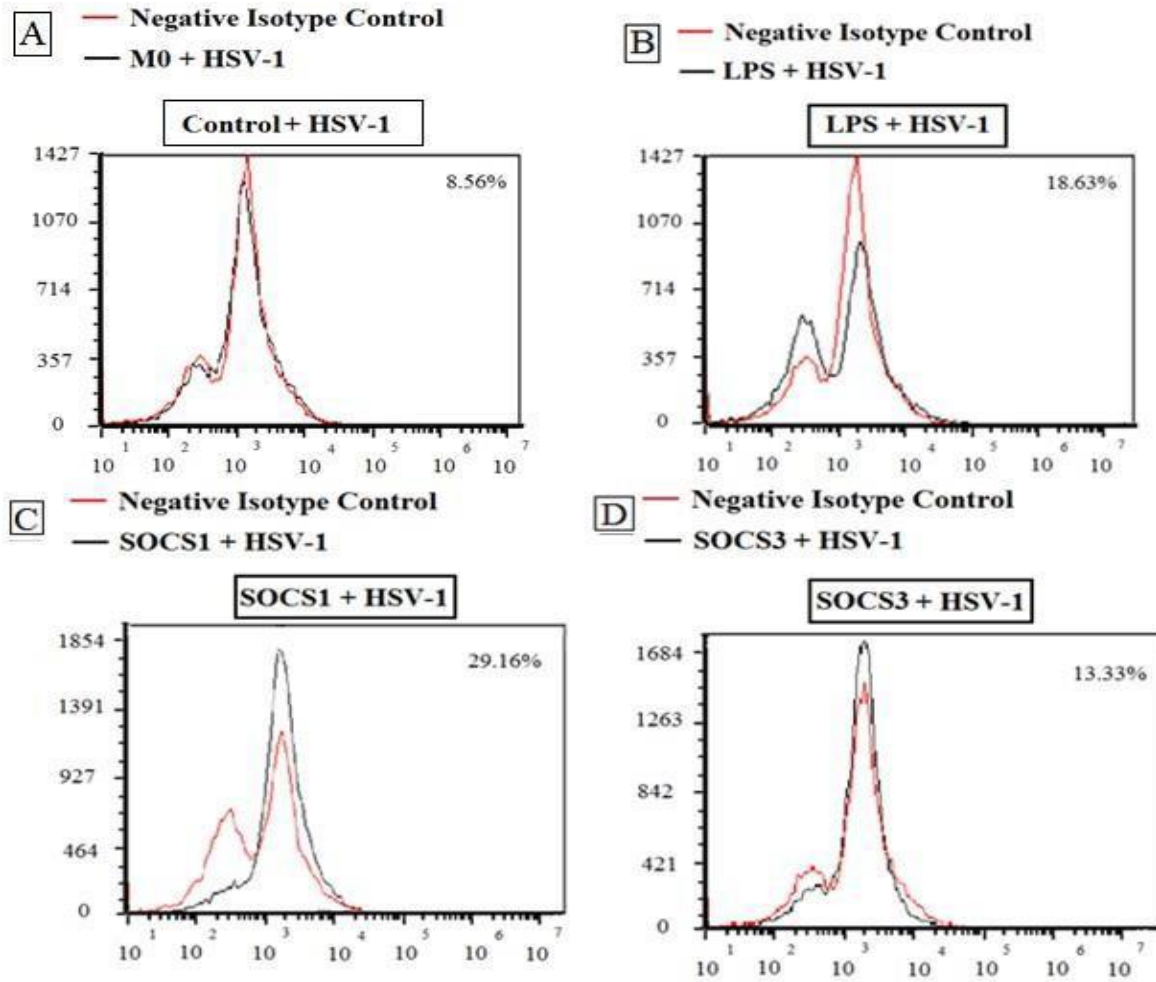


Figure 9. Flow cytometry analysis of AhR expression levels in un-treated RAW 264.7 macrophages after 24 hours. A, B, C, and D histograms show the percentage of cells positive for AhR expression in un-treated macrophages (M0), macrophages treated with LPS, SOCS 1, or SOCS 3 peptide mimetic after 24 hours with HSV-1 infection and stained with anti-AhR antibody. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed.

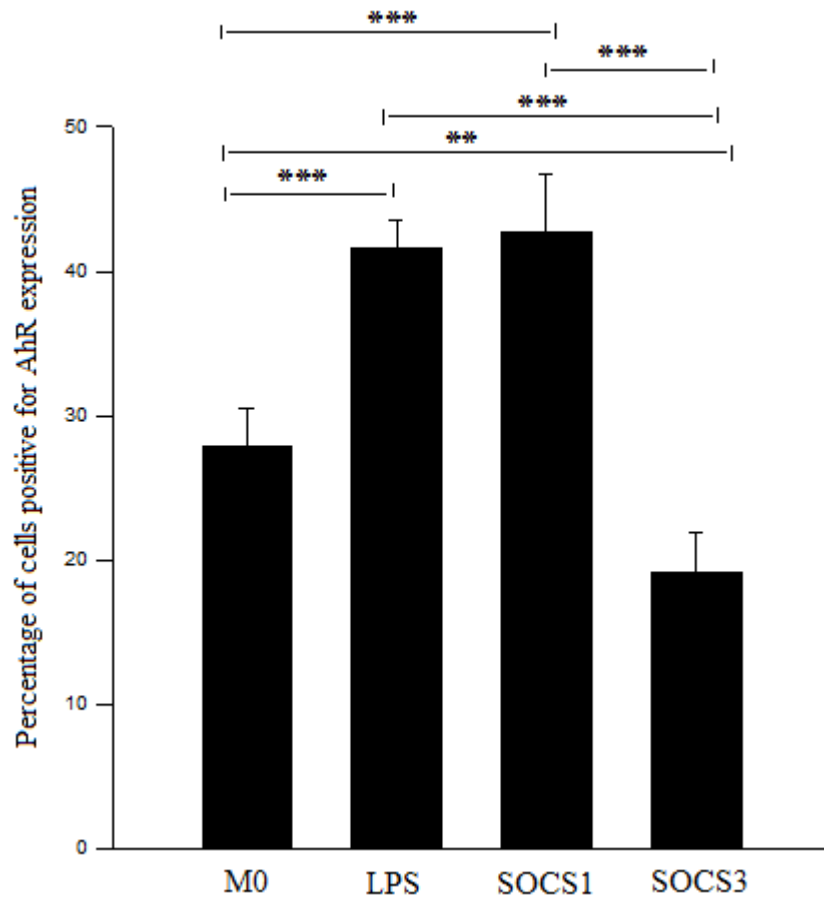


Figure 10. Un-treated RAW 264.7 macrophages (M0), macrophages treated with LPS, SOCS 1, or SOCS 3 Peptide Mimetic after 24 hours and stained with anti-AhR antibody. Each value characterizes mean \pm standard error (SE) of three separate experiments. ***, $p \leq 0.001$ **, $p < 0.01$.

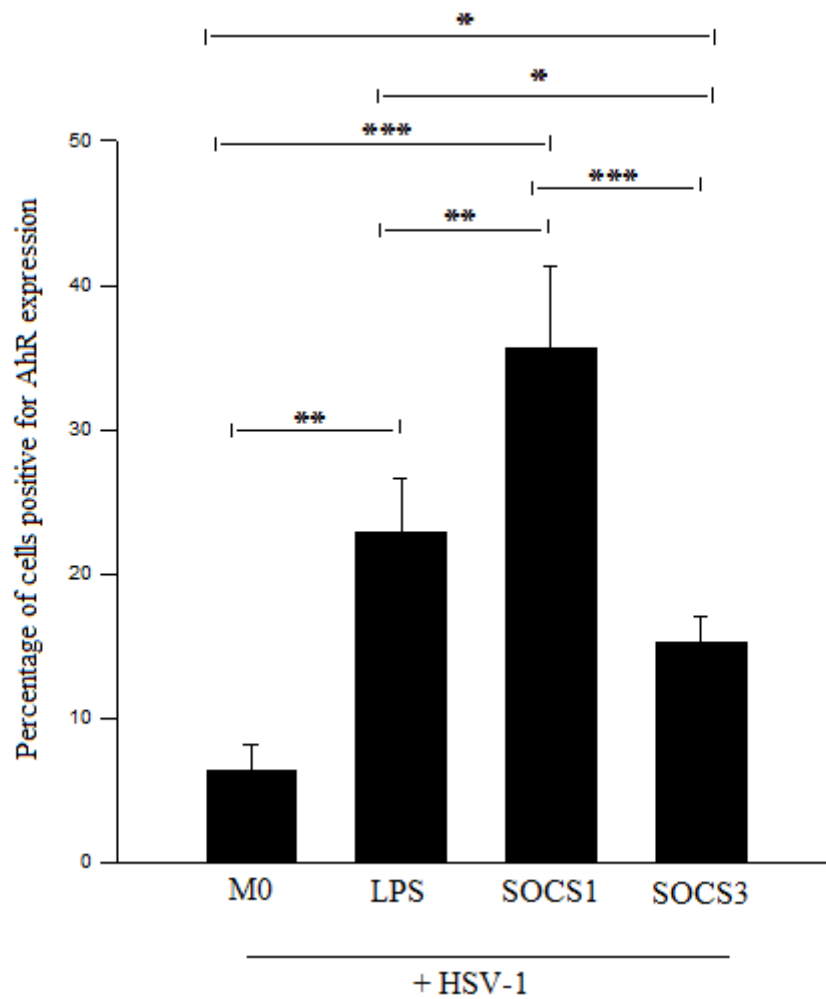


Figure 11. Un-treated infected RAW 264.7 macrophages (M0), infected macrophages treated with LPS, SOCS 1, or SOCS 3 peptide mimetic after 24 hours and stained with anti-AhR antibody. Each value characterizes mean \pm standard error (SE) of three separate experiments.

***, $p \leq 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Table 2. Summary of number of un-polarized and polarized macrophages pre and post- HSV-1 infection that stained positive for AhR expression

Percentage of cells positive for AhR expression				
	Uninfected cells		Infected cells	
TREATMENT	Mean	SEM	Mean	SEM
CONTROL	27.78	1.57	6.37	1.05
LPS	41.56	1.15	22.78	2.18
SOCS1	43.72	2.30	35.65	3.27
SOCS3	19.08	1.59	15.21	1.02

DISCUSSION

In this study, RAW 264.7 macrophages treated with LPS were flattened, extended, with irregular shape and contained visible intracellular vacuoles. These cells were strongly adherent in F-actin stains compared to uninfected control cells, while control cells appeared rounded and elongated. Following challenge with HSV-1, all RAW 264.7 macrophages including LPS-treated cells were rounded. This morphology of macrophages after exposure to HSV-1 made it difficult to distinguish different treatments. Morphological changes were seen possibly due to changes of the actin cytoskeleton that happen during the normal virus life cycle (Bigley, 2014; Reichard, 2012).

Viabilities of RAW 264.7 macrophages following treatment with LPS or SOCS1 or SOCS3 peptide mimetic with and without HSV-1 were assessed. Macrophages exposed to LPS showed a significant decrease in the cell viability with and without HSV-1 compared to the control cells (Figure 5). LPS-treated cells are known to produce high levels of reactive nitrogen species (RNS), reactive oxygen species (ROS), and TNF- α functioning as pro-inflammatory molecules. These cytotoxic molecules are implicated in decreased the cell viability of M1 macrophages (Schachtele et al., 2010).

SOCS molecules control JAK/STAT signaling pathway mediating cytokine production (Frey et. al., 2009). During viral infection, these proteins play an important role in controlling intracellular immune responses. SOCS3-treated macrophages displayed an increase in cell viability with and without HSV-1 infection compared to SOCS1-treated macrophages (Figure 5). Alsharif (2015) suggested that treatment of macrophages with SOCS3 peptide mimetic led to a decrease in the production of inflammatory cytokines (TNF- α and IL-6) and an increase in anti-inflammatory IL-10. In this study, increases in viability of SOCS3-treated macrophages support

the suggestion that SOCS3 plays a critical role in controlling the effect of cytotoxic molecules (Schachtele et al., 2010).

AhR is also known to regulate the differentiation of inflammatory CD4⁺ Th IL-17 cells and T reg cells (Veldhoen, 2010). AhR might also interact with factors like interferon regulatory factor-4 (IRF-4) which regulates the formation of subsets of T cells, including the production of IL-17 cells (Veldhoen, 2010). Deficiency of IRF-4 producing cells can hinder the differentiation of Th17 cells (Brüstle et al., 2007). In the LPS response of macrophages, IRF-4 is responsible for negative regulation of TLR-4 signaling and in subsequent production of the pro-inflammatory cytokines such as TNF- α and IL-12 (Honma et al., 2005). AhR and IRF-4 interaction may be responsible for different roles performed by AhR. AhR is also involved in signaling in NF κ B. The AhR suppresses the LPS induced activation of IL-6 by interacting with STAT1 on IL-6 promoter that is known to inhibit the transcriptional activation of NF κ B (Nguyen et al., 2013).

In this study using flow cytometric analysis, LPS and SOCS1 peptide mimetic treatments of uninfected RAW 264.7 macrophages caused a significant increase in AhR expression ($p < 0.001$) (Fig 10) associated with production of the pro-inflammatory cytokines such as TNF- α . AhR expression is induced by LPS and TLR ligands in murine macrophages (Kimura et al., 2009). Reichard (2012) found that SOCS1 expression predominated in the pro-inflammatory macrophages.

SOCS3 peptide mimetic-treated of uninfected macrophages induced significant decrease in AhR expression compared to uninfected control cells ($p < 0.01$) (Fig 10). SOCS3 protein has been shown to mediate IL-10 production and inhibition of nitric oxide and TNF- α production (Qasimi et al., 2006). Alsharif (2015) suggested that SOCS3 protects macrophages from the pro-inflammatory cytokines and cell toxicity. The SOCS3 peptide mimetic exerts an anti-

inflammatory response that protects against lytic effect of LPS and IFN- γ . Another explanation for anti-inflammatory effect of SOCS3 may be its use of SOCS3 signaling which lead to production of anti-inflammatory IL-10 (Qin et al., 2012).

Following viral challenge, AhR expression decreased in all treated macrophages (Figure 11). Cell viabilities were reduced in virus infected cells (Fig 5). This reduction in overall of AhR expression and cell viability was likely due to the crucial role of macrophages against viral infection (Reichard, 2012). This cell death may due to the pro-inflammatory products such as IFN- γ /TNF- α (Wang et al., 2011). However, inhibiting the JAK-STAT signaling by SOCS will inhibit cytokine production and inhibit immune system against infection (Cooney, 2002). Nowoslawski et al. (2010) found that viral proteins were able to hijack SOCS functions and made SOCS the targets of virus. Therefore, SOCS inhibits immune response, allowing virus invasion and replication (Nowoslawski et al., 2010).

LPS activates STAT1 in macrophages (Kimura et al., 2009). AhR and STAT1 binding takes place in response to the stimulation given by LPS. Thus, AhR plays an important role in the JAK-STAT signaling due to the binding between AhR and STAT1. It is probable that certain factors that are responsible for Ahr-STAT1 binding are produced only by LPS. LPS is also known as the inducer of plasminogen-activator inhibitor (Pai-2) in RAW 264.7 macrophages (Nguyen et al., 2013). AhR co-operates with Pai-2 and regulates pro-inflammatory cytokine production in macrophages. This mechanism involves in NF κ B. Pai-2 may be expressed in response to LPS and is a factor required for the binding of AhR and STAT1. (Nguyen et al., 2013).

SOCS3-treated macrophages displayed an increase in cell viability in infected cells and induced a high reduction in AhR expression (Figures 5 and 11). This finding supports the

important role of SOCS3 during viral replication. Yokota et al. (2004) found that SOCS3 induction would have a dramatic impact on the immune system helping HSV-1 replication. SOCS3 inhibits antiviral immune responses (Nowoslawski et al., 2010). HSV-1 infection activates the induction of SOCS3 thus inhibiting the IFN production system. IFN- β is well known to activate JAK-STAT pathway through IRF-3 and NF- κ B (Yokota et al., 2004). SOCS3 may suppress the JAK-STAT pathway by inhibiting pro-inflammatory production including IFN- β (Yokota et al., 2004). SOCS3 promotes Th2 development by inhibiting IL-12- mediated STAT4 activation in T cells also, it inhibits IL-6 signaling in macrophages (Yokota et al., 2004). The reduction of AhR expression post-infection may result from the upregulation in SOCS3 due to the infection as found in this study (Figures 5 and Fig 11).

FUTURE STUDIES

SOCS proteins inhibit the JAK-STAT signaling allowing, inhibition of cytokine production and including the response of the immune system infection (Cooney, 2002). Viral proteins hijack SOCS functions and make them the targets of virus resulting inhibition of immune responses allowing virus invasion and replication (Nowoslawski et al., 2010). It would be beneficial to study the morphological changes and the AhR expression in macrophage cells after the addition of SOCS1 peptide and SOCS3 peptide mimics during the early stages of HSV-1 infection. These effects can be evaluated using RAW 264.7 macrophages treated with LPS or SOCS1 peptide or SOCS3 peptide mimetic or control cells with or without HSV-1 (0.1 MOI) for 4, 6, and 12 hours to monitor the actin cytoskeleton during the early stage of infection. The cell morphology could be evaluated via immunofluorescent microscope and AhR expression via flow cytometry. Based on the results of the present study SOCS1 peptide mimetic will be associated with production of increased inflammatory cytokines such as TNF- α and increased expression of AhR and treatments with SOCS3 peptide will be associated with production of anti-inflammatory IL-10 and decreased expression of AhR. I would expect that the actin cytoskeleton in SOCS1 macrophages would be more elongation than SOCS3 macrophages infected cells. Cytokine production could be measured using enzyme-linked immunosorbent assay (ELISA) or Luminex Multiplex Immunoassays of culture supernatant fluids to verify that there is a reduction in pro-inflammatory cytokines in SOCS3-treated macrophages as the population is shifted to the anti-inflammatory state.

Cell viability studies should include measurements of apoptosis such as immunostaining for annexin V. AhR plays an important role in the apoptosis pathway. My study of cell viability with trypan blue displayed an increase in cell viability in SOCS3-treated macrophages with and

without HSV-1 while it showed a decrease in AhR expression post-infection. This decrease should be examined through annexin V assay to determine if there is any link between low AhR expression and higher cell survival in SOCS3-treated macrophages. This way will better define the specifics of the role the AhR plays in a macrophage's apoptotic pathway.

Stimulation of AhR using an exogenous AhR ligand such as TCDD or natural AhR ligand would be beneficial to see how AhR expression is affected. Exogenous AhR ligand may change the expression levels of AhR. AhR would bind to its exogenous ligand such as TCDD resulting to activate the AhR expression. In order to compare these conditions with this study, macrophages should be exposed to TCDD or natural AhR and measure the change of AhR expression level before and after the addition of the stimulation. I would expect that TCDD would bind the AhR and upregulate the AhR expression.

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APPENDIX

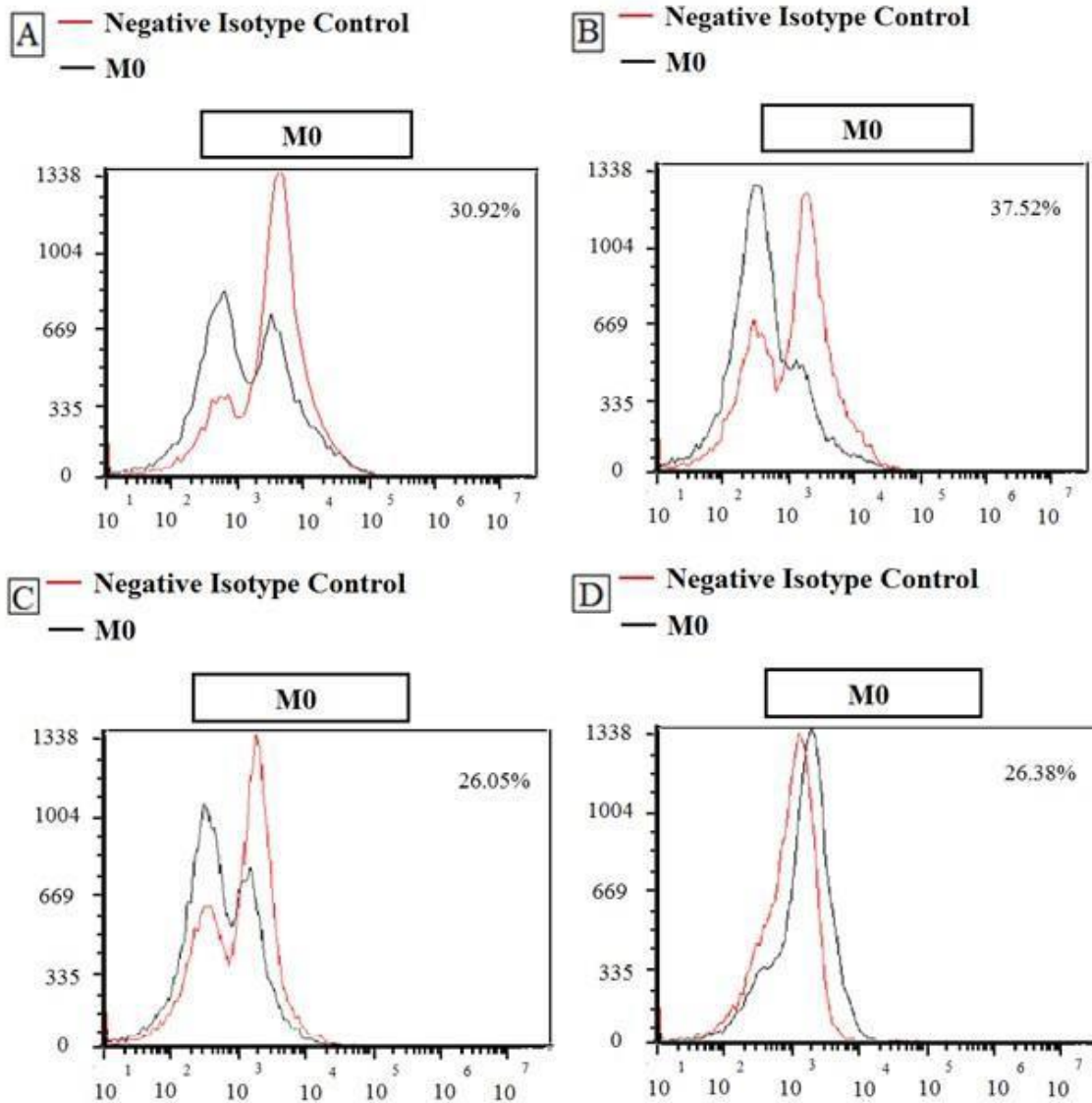


Figure 12. Flow cytometry analysis of AhR expression levels in un-treated RAW 264.7 control macrophages. A, B, C, and D histograms show the percentage of cells positive for AhR expression in control macrophages after 24 hours. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.

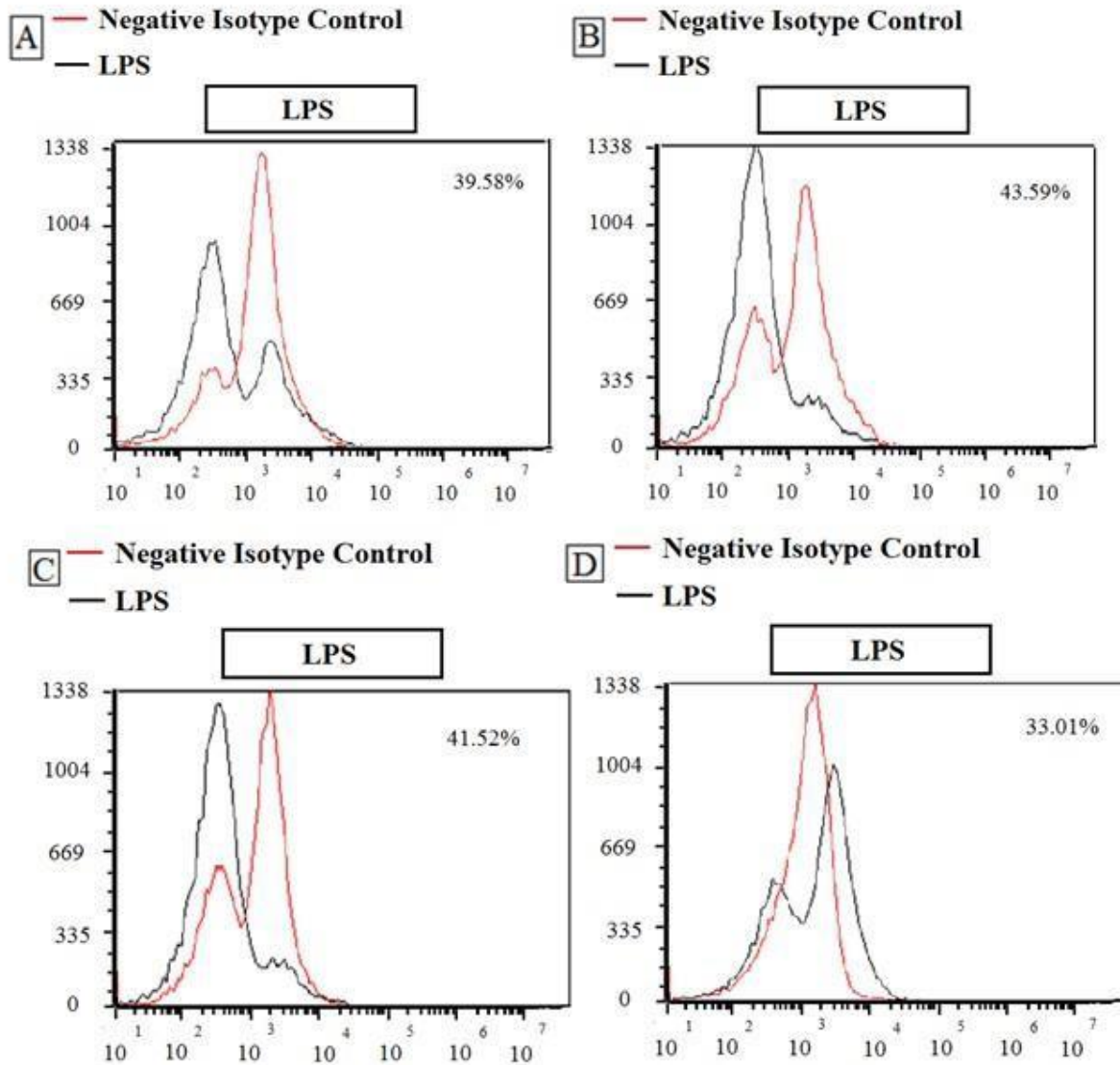


Figure 13. Flow cytometry analysis of AhR expression levels in RAW 264.7 macrophages treated with LPS. A, B, C, and D histograms show the percentage of cells positive for AhR expression in macrophages treated with LPS after 24 hours. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.

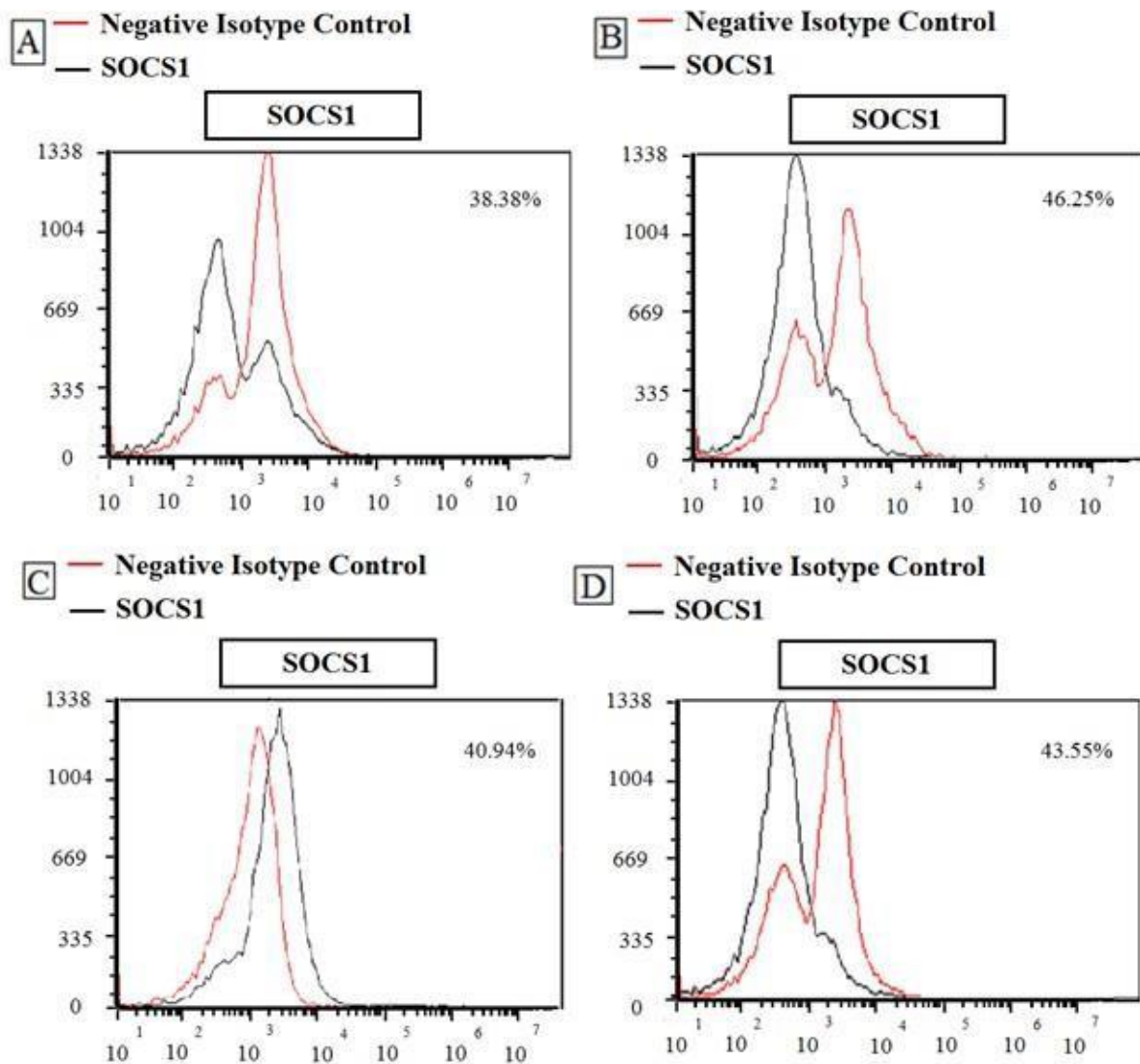


Figure 14. Flow cytometry analysis of AhR expression levels in RAW 264.7 macrophages treated with SOCS1. A, B, C, and D histograms show the percentage of cells positive for AhR expression in macrophages treated with SOCS1 after 24 hours. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.

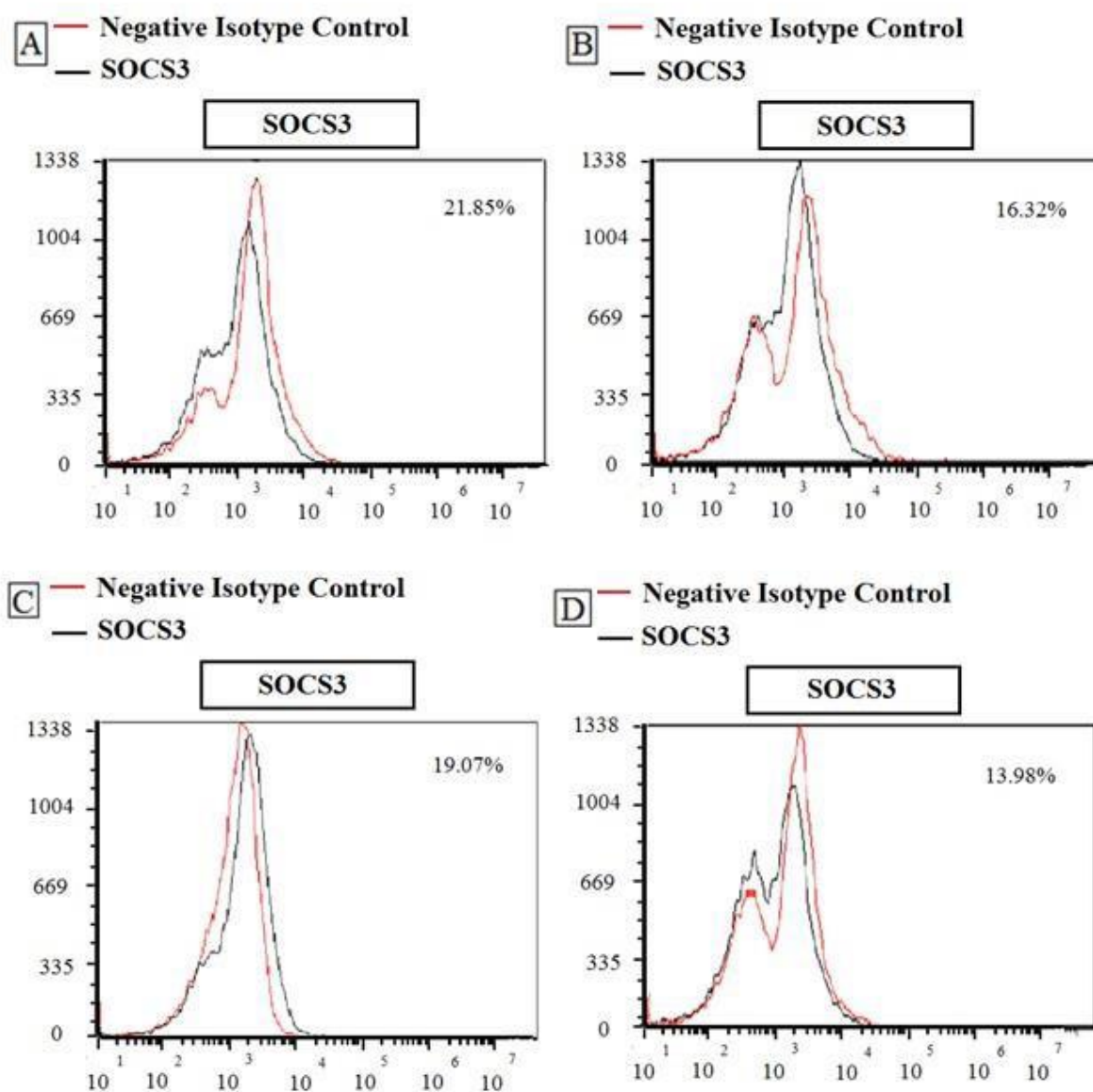


Figure 15. Flow cytometry analysis of AhR expression levels in RAW 264.7 macrophages treated with SOCS3. A, B, C, and D histograms show the percentage of cells positive for AhR expression in macrophages treated with SOCS3 after 24 hours. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.

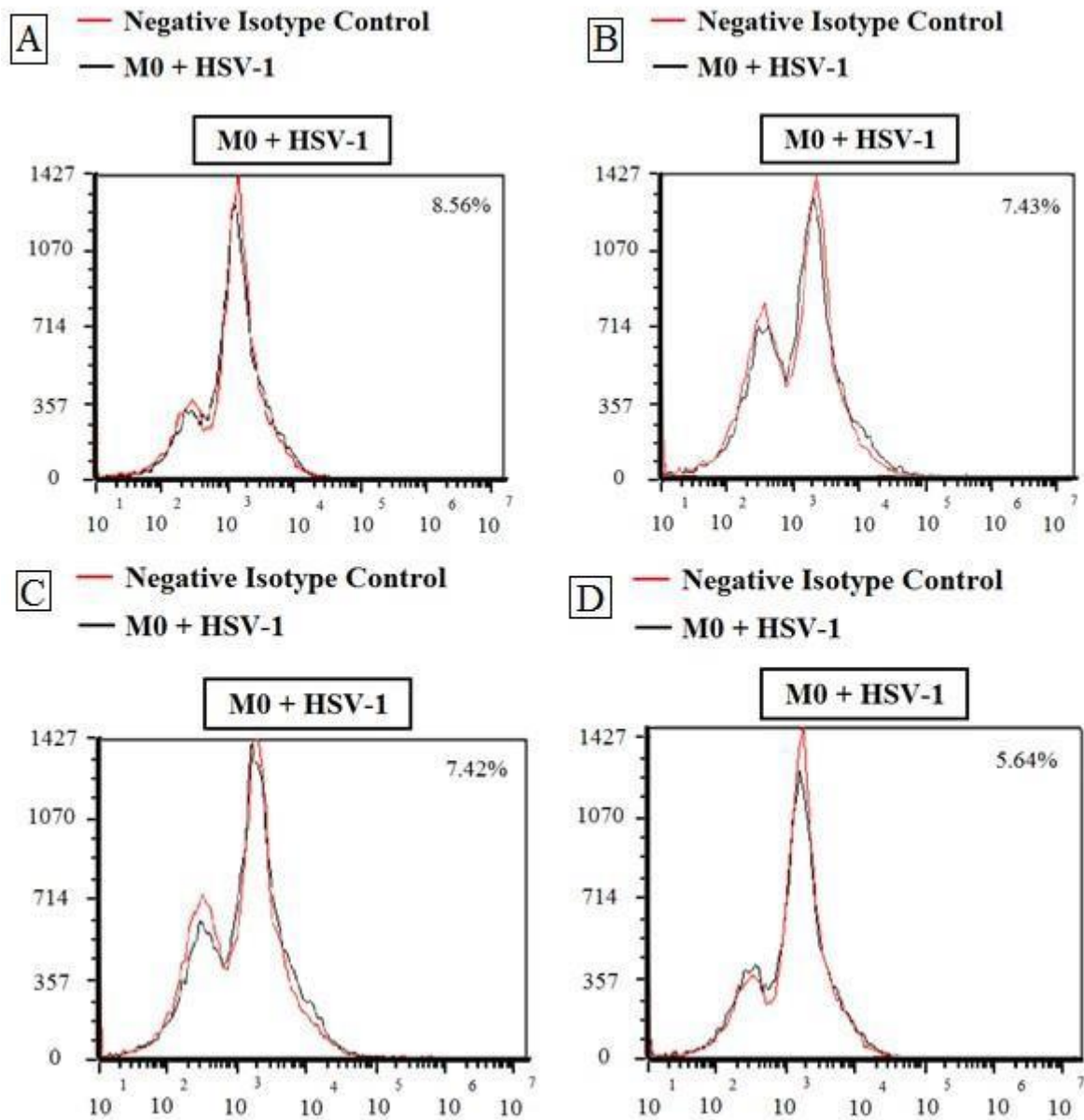


Figure 16. Flow cytometry analysis of AhR expression levels in un-treated RAW 264.7 control macrophages. A, B, C, and D histograms show the percentage of cells positive for AhR expression in control macrophages after 24 hours with HSV-1 infection. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.

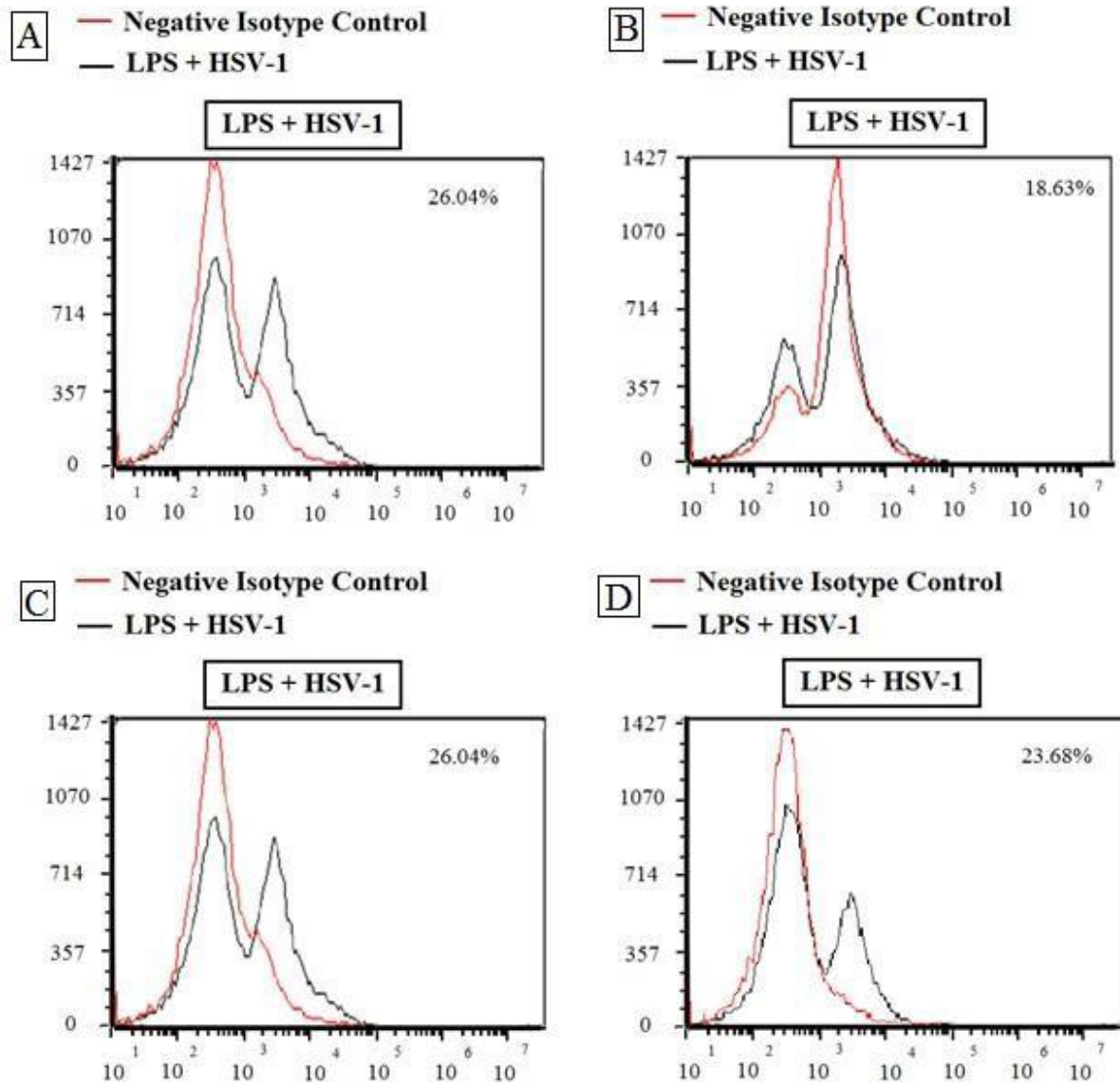


Figure 17. Flow cytometry analysis of AhR expression levels in RAW 264.7 macrophages treated with LPS. A, B, C, and D histograms show the percentage of cells positive for AhR expression in macrophages treated with LPS after 24 hours with HSV-1 infection. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.

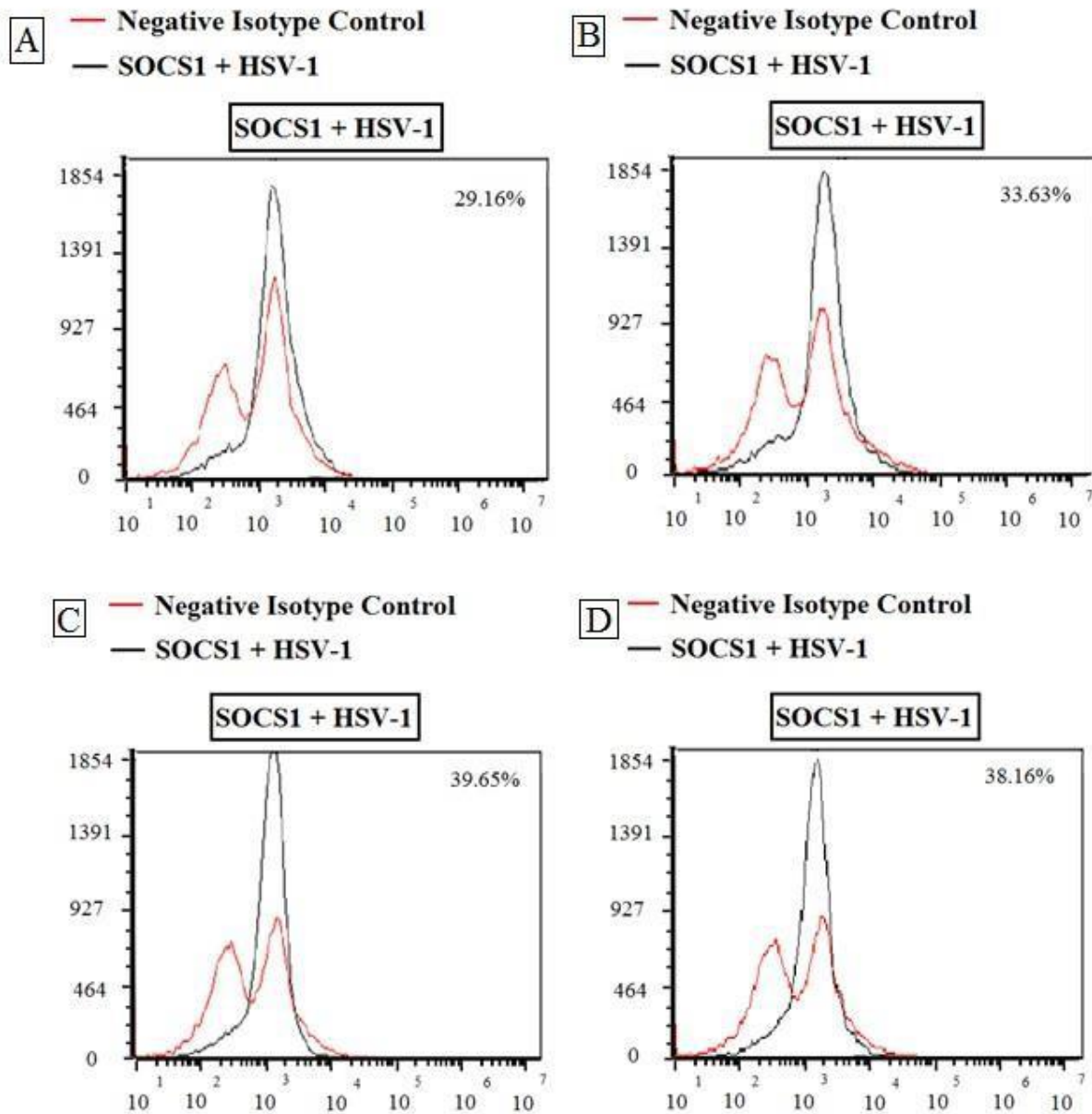


Figure 18. Flow cytometry analysis of AhR expression levels in RAW 264.7 macrophages treated with SOCS1. A, B, C, and D histograms show the percentage of cells positive for AhR expression in macrophages treated with SOCS1 after 24 hours with HSV-1 infection. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.

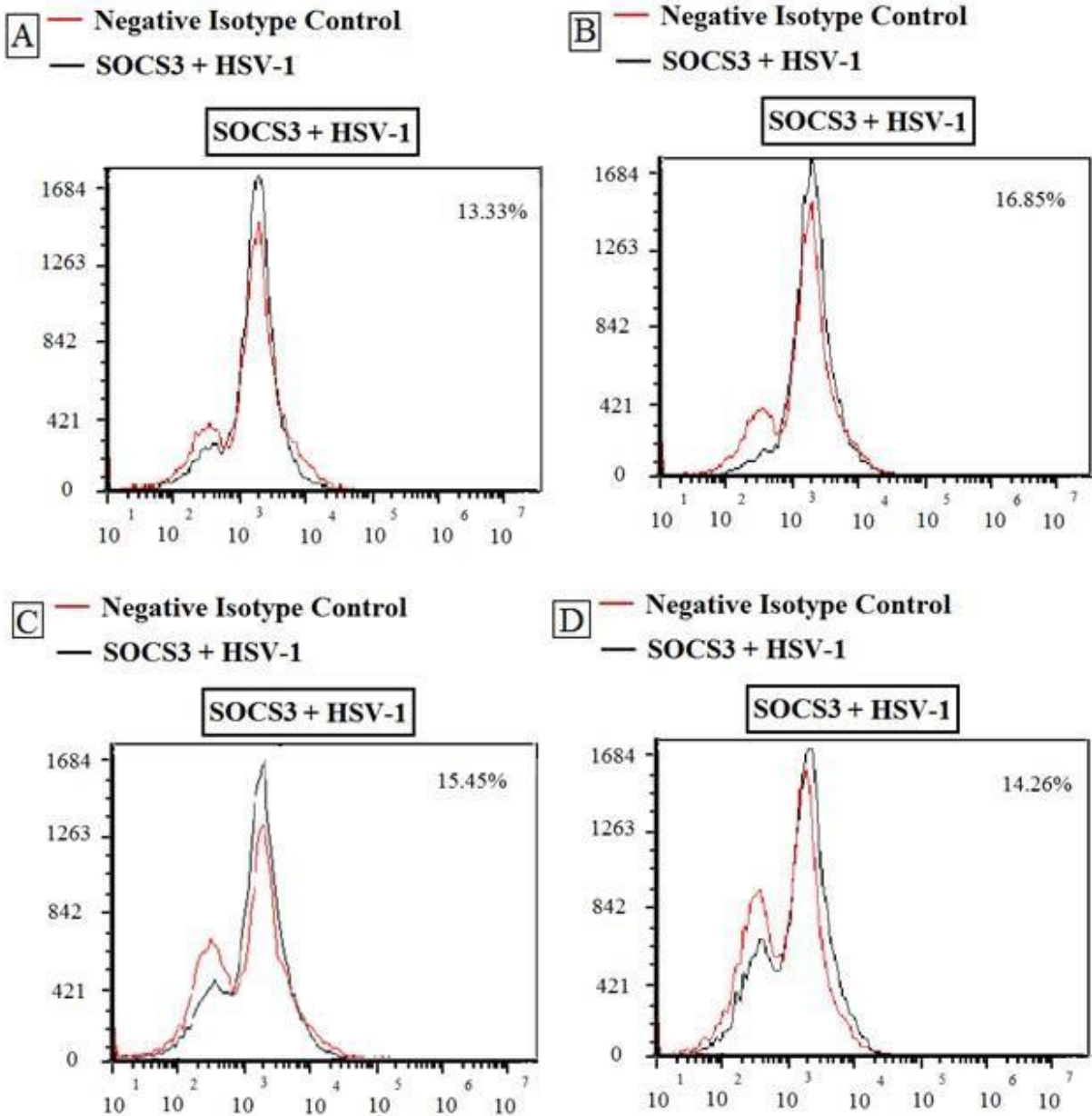


Figure 19. Flow cytometry analysis of AhR expression levels in RAW 264.7 macrophages treated with SOCS3. A, B, C, and D histograms show the percentage of cells positive for AhR expression in macrophages treated with SOCS3 after 24 hours with HSV-1 infection. Red: negative isotype control; black: anti-mouse AhR primary antibody, conjugated with PE. Three independent experiments were performed with four representative histograms.