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The Impact of HSV-1 Infection on Cell Viability, Morphology, and CD Markers Expression by Unpolarized and Cytokine-Polarized J774A.1 Mouse Macrophages

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The Impact of HSV-1 Infection on Cell Viability, Morphology, and CD Markers Expression by

Unpolarized and Cytokine-Polarized J774A.1 Mouse Macrophages

A thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science.

By

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B.S., Umm Al-Qura University, 2009

2015

Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE STUDIES

MAY 27, 2015

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Sarah Saad Alsharif ENTITLED The Impact of HSV-1 Infection on Cell Viability, Morphology, and CD Markers Expression by Unpolarized and Cytokine-Polarized J774A.1 Mouse Macrophages BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

AlSharif, Sarah Saad. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2015. The Impact of HSV-1 Infection on Cell Viability, Morphology, and CD Markers Expression by Unpolarized and Cytokine-Polarized J774A.1 Mouse Macrophages

Macrophages play an important role in the immune system, particularly in neutralizing pathogens via phagocytosis and the production of multiple cytokines and chemokines that control infection after exposure to specific stimuli. Macrophages exhibit two different phenotypes, M1 and M2. This study evaluated the role of Suppressors of cytokine signaling (SOCS)1 and SOCS3 on Herpes Simplex Virus (HSV-1) infection of polarized macrophages, cell viability, cell morphology, and the expression of cell surface CD markers. I hypothesized that J774A.1 murine macrophages cells in the naïve state (M0), and M1 and M2 phenotypes would display differences in CD markers CD80,CD163, and CD200R, cell morphology, and viability following HSV-1infection and that the SOCS3 peptide mimetic would reduce the cytotoxicity associated with the M1 phenotype or HSV-1 infection.

Unpolarized J774A.1 macrophages (M0) or polarized M1 and M2 phenotypes displayed differences in CD surface proteins at 18, 24, and 48 hours following HSV-1 infection and there was ~22% decrease in viability in M1 macrophages regardless of whether the macrophages were uninfected or infected with HSV-1. M2 macrophages demonstrated ~10% reductions in viability compared with infected M0 macrophage. Differences in cell morphology were noticed in uninfected polarized and unpolarized cells. M1 macrophages displayed irregular shapes and intracellular vacuoles were more pronounced at 24 and 48 hours compared to 18 hours. M0 appeared rounded or elongated, and M2 macrophages induced by IL-4, IL-10, or IL-13 showed

elongation with the majority of cells appearing rounded. HSV-1-infected M0, M1 or M2 macrophages all became rounded and aggregated following HSV-1 infection.

We found CD80 expression (by flow cytometry) was slightly deceased on uninfected M1 macrophages and M2 macrophages compared to uninfected M0 macrophages and infected M1 macrophages and IL-10-induced M2 macrophages also exhibited a minor increase in CD80. A reduction of CD163 expression occurred on M1 macrophages uninfected or infected with HSV-1. The level of CD163 on infected M2 macrophages was increased compared to uninfected M2 macrophage cells with the level being higher on infected IL-10-induced M2 macrophages. These observations were not statistically significant; however, the level of CD200R significantly increased on infected M2 macrophages polarized by IL-13 compared to CD200R expression in virus-infected M0 macrophages.

Treatment with either SOCS3 peptide mimetic or pJAK2 increased the viability of polarized J774A.1 M1 macrophages. Production of pro-inflammatory cytokines TNF-α and IL-6 were reduced in polarized M1 macrophage treated with SOCS3 or pJAK2 peptide mimetic compared with polarized M1 untreated cells. Also, the SOCS3 peptide mimetic increased cell viability in M1 and HSV-1-infected M1 RAW264.7 and J774A.1 macrophages, but this increase was only significant in the RAW264.7 cells. This result suggests that SOCS3 mimetic exerts an anti-inflammatory effect by diminishing the lytic effect of M1 polarization. M1 cells treated with SOCS3 behave similar to the M2 phenotype and produce the anti-inflammatory cytokine IL-10. This study highlights the events that lead to M1 versus M2 polarization and the knowledge gained regarding SOCS3's ability to reduce inflammation may make it a potential therapeutic target for inflammatory diseases.

SPECIFIC AIMS and HYPOTHESIS

A previous study by Reichard (2012) showed that J774A.1 murine macrophages exhibited variability in cell morphology, viability, and surface proteins expression 24 hours after exposure to IL-4 or IFN-γ and LPS and /or HSV-1 infection. Based on these previous observations, in the present study we evaluate the impact of HSV-1 on macrophages of different polarization phenotypes and the impact of SOCS1 and SOCS3 during HSV-1 infection. Aim 1 is to examine the effects of HSV-1 infection and macrophage polarization on cell viability, morphology, and surface expression of CD80, CD163, and CD200R. We hypothesize that J774A.1 murine macrophages cells in the naïve state (M0), and M1 and M2 phenotypes will display differences in cell morphology, viability, and surface proteins at 18, 24, 48 hours following HSV-1infection.

Aim 2 is to quantitate cell viability and measure cytokine secretion for HSV-1-infected M1 macrophages supplemented with SOCS1, SOCS3, or phosphorylated-JAK2. We hypothesize that a SOCS3 peptide mimetic will reduce the cytotoxicity associated with the M1 phenotype or HSV-1 infection. Macrophages treated with either SOCS3 or SOCS1 inhibitor will produce more anti-inflammatory cytokine (IL-10) and less pro-inflammatory cytokines (IL-6 and TNF-α) than untreated M1 macrophages. We further anticipate that macrophages treated with SOCS1 will increase the production of TNF-α.

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LIST OF ABBREVIATIONS

AD= Alzheimer's disease

ATCC= American Type Culture Collection

BSA= Bovine Serum Albumin

CD= Clusters of Differentiation

CD200R= Clusters of Differentiation 200 Receptor

CP-SCS3= Cell-Penetrating Form of SOCS3

DMEM= Dulbecco's Modified Eagle's Medium

ERK= Extracellular Signal-Regulated Kinase

HSV= Herpes Simplex

HVEMs= Herpes Virus Entry Mediators

ICP0= Infected Cell Protein Null

IFNγ= Interferon Gamma

IL= Interleukin

iNOS= Inducible Nitric Oxide Synthase

JAK= Janus Kinase

KIR= Kinase Inhibitory Region

LPS= Lipopolysaccharide

MHC class-I= Major Histocompatibility Complex I

MOI= Multiplicity of Infection

NFκB= Nuclear Factor kappa-light-chain-enhancer of Activated B Cells

NK= Natural Killer Cells

OaS1a= 2′-5′-Oligoadenylate Synthetase

PBS = Phosphate Buffered Saline

PI3Ks= Phosphoinositide 3-Kinases

ROS= Reactive Oxygen Species

sCD163= soluble CD163

SH2= Src Homology 2 Domain

SOCS= Suppressors of Cytokine Signaling

STAT= Signal Transducer and Activator of Transcription

TGF-β= Transforming Growth Factor Beta

TH1= T Helper Cells 1

TH2= T Helper Cells 2

TLR= Toll-Like Receptor

TNFα= Tumor Necrosis Factor Alpha

ACKNOWLEDGEMENT

I would like to thank my thesis advisor, Dr. Nancy Bigely, for her insightful comments and her valuable suggestions that greatly helped toward the successful completion of this thesis. I appreciate having had this wonderful opportunity to work with her. I would also like to acknowledge Dr. Babra Hull, the program director, who has indispensable support throughout my master's degree. I would also like to thank Dr. F. Javier Alvarez-Leefmans for serving as a member on my thesis committee and for his timing and guidance. I am thankful to my mother, sisters, and brothers for their unwavering support and love throughout my life. I am grateful to my friends, especially May Albalawi, who has always encouraged me and shared this journey. I am gratitude for all those who gave me help in the completion of this study

Thank you, Lord, for always being there for me.

DEDICATION

This thesis is dedicated to my Parents, Sisters, Friends, and Colleagues who have a strong belief and trust in me. I hope that this achievement will complete the dream that my mother had for me all those many years ago when she chose to give me the best education she could.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a member of the *Herpesviridae* family. It has a double-stranded DNA genome packaged into an icosahedral protein capsid (Steven and Spear, 1997). HSV-1 is a highly significant virus in terms of its clinical relevance, infecting 70-80% of the world's adult population (Dakvist *et. al.*, 1995). During the primary infection of HSV-1, patients suffer from cold sores and fever blisters. Following the primary infection, the symptoms tend to disappear as HSV-1 becomes latent in neurons of the dorsal root ganglia. Reactivation of HSV-1 from latency leads to a relapse in symptoms (Sawtell, 1998; Spruance *et. al.*, 1977). The immune system, both the innate and adaptive arms, responds effectively to HSV-1 infection through activation of macrophages, natural killer (NK) cells, and T cells that release a variety of effector molecules. These molecules interfere with viral replication and help to control the infection in host cells (Mogensen *et. al.*, 1989).

Macrophages are derived from monocytes and play an important role in the immune system, particularly in neutralizing pathogens via phagocytosis and the production of multiple cytokines and chemokines that control infection and promote tissue healing (Mills, 2012). Macrophages exhibit two different phenotypes after exposure to specific stimuli. These phenotypes are referred to as M1 and M2 macrophages (Figure 1). Macrophages are able to convert between the M1 and M2 phenotypes continuously in order to provide optimal macrophage functioning and homeostasis during an inflammatory event (Stout *et. al*., 2005, Davis *et. al*., 2013). M1 cells are induced by the combination of lipopolysaccharide (LPS) and the pro-inflammatory cytokine interferon-γ (IFN-γ). These M1 cells produce high levels of pro-inflammatory effector molecules such as interleukins (IL), including IL-1β, IL-6, IL-12, and IL-23, tumor necrosis factor

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(TNF), and inducible nitric oxide synthase (iNOS), which are vital in mediating cytotoxic functions and contributing to host immune defenses (Figure 1). Over the course of these pro-inflammatory events, it is likely that even healthy tissue surrounding the infected area may be damaged (Martinez and Gordon, 2014; Ding *et. al.*, 1988). To help reduce the likelihood of potential damage, stimulation with IL-4, IL-10, or IL-13 promotes macrophage polarization to the M2 phenotype. The M2 phenotype has an anti-inflammatory effect by generating IL-10 and transforming growth factor beta (TGF-β) (Mantovani *et. al*., 2004). These mediators participate in wound healing, induce angiogenesis, and suppress immune responses (Novak and Koh, 2013) (Figure 1).

Macrophage Polarization and their Properties/Functions
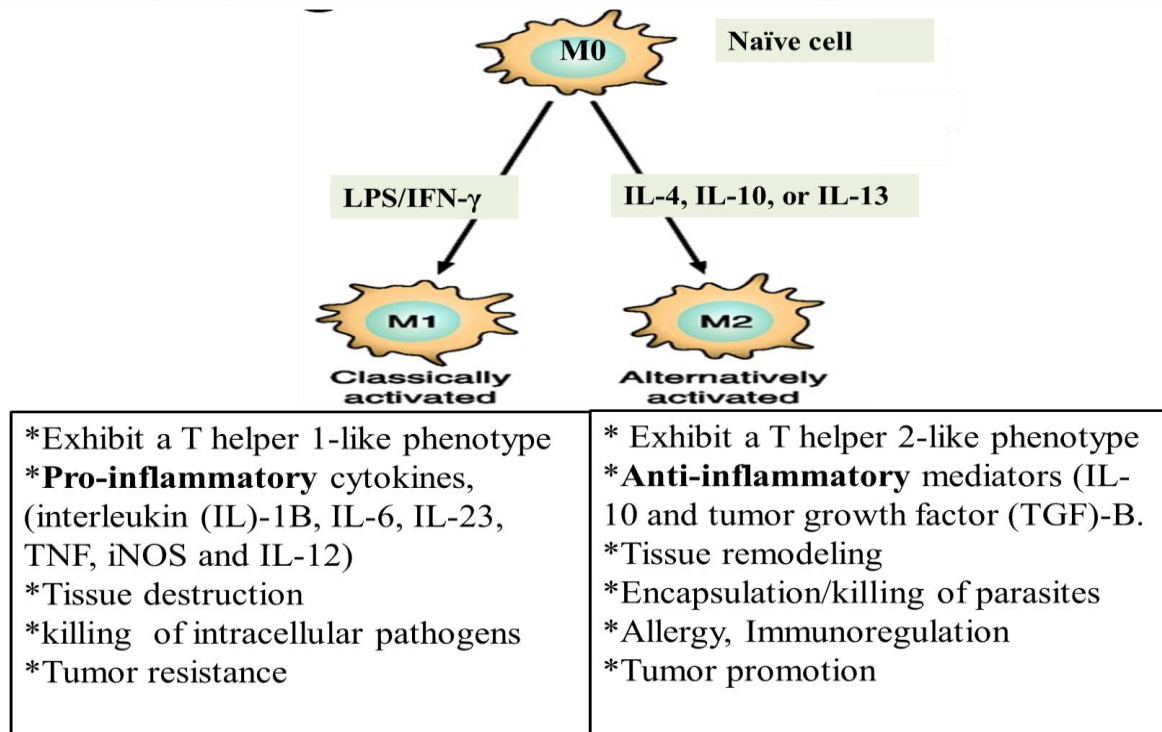
Figure 1. Macrophage phenotypes have separate functions. M1 and M2 are converted from a naïve state (M0) upon specific stimuli. M1 cells are induced by a variety of different stimuli, including the combination of LPS and the pro-inflammatory cytokine IFN-γ. M1 macrophages are pro-

inflammatory and fight infection. M2 macrophages can be activated by IL-4, IL-10, or IL-13. M2 macrophages are anti-inflammatory and repair tissue damage. (Adapted from Nagy *et al.*, 2012)

M1 and M2 macrophages can be distinguished by the cluster of differentiation (CD) markers on their cell surface. Specifically, CD80, CD200R, and CD163, can differentiate between M1 and M2 activated macrophages (Mantovani et. al., 2004). Based on a study done in human monocyte-derived macrophages, Ambarus et al. (2012) found that CD80 expression levels were specific to M1 macrophages; however, CD163 and CD200R were up-regulated in M2 macrophages induced by IL-10 and IL-4 or IL-13, respectively. These alterations in CD levels are driven by the cells' need to respond to the infection. CD80 acts as a co-stimulatory signal on the surface of antigen presenting cells, activated B cells and monocytes. CD80 participates in T cell initiation and maintenance (Linsley *et. al*., 1991). The CD200 receptor (CD200R) is responsible for inhibiting inflammatory macrophage activation and is expressed on a number of immune cells including myeloid cells, monocyte/macrophages, dendritic cells, and on certain T and B cells (Hoek *et. al*., 2000). Both CD163 and CD200R are involved in anti-inflammatory actions that serve to control the pro-inflammatory responses that may cause damage. They accomplish this by decreasing the production of pro-inflammatory cytokines (Jenmalm *et. al*., 2006).

An additional layer of control over cytokine production comes in the form of the suppressor of cytokine signaling (SOCS) proteins. SOCS proteins serve to negatively regulate cytokine receptor signaling and toll-like receptors (TLR). For example, SOCS1 and SOCS3 are potent inhibitors of the JAK/STAT pathway that ultimately activates the gene expression of pro-inflammatory molecules (Yoshimura et al., 2005). Therefore, the activity of SOCS proteins is indirectly involved in the activation of macrophages to the M1 or M2 state, controlling which cytokines are being produced (Wilson, 2014).

LITERATURE REVIEW AND PRELIMINARY DATA

HSV-1

Herpes simplex virus type 1 (HSV-1) is a common virus that infects 70-80% of the adult population (Dakvist *et. al.,* 1995). In humans, the primary symptoms of active infections are sores or lesions on mucosal surfaces or the skin. The virus establishes lifelong latency in neurons of the dorsal root ganglia, during latency the infected individual does not display symptoms. During stress or illness, latent HSV-1 infections can reactivate into an active infection and initiate cellular damage and lysis, thereby causing the individual to develop symptoms again (Sawtell, 1998; Spruance *et. al.,* 1977).

HSV-1 is a member of the *Herpesviridae* family. It is a large DNA virus, consisting of a linear, double-stranded DNA genome packaged into an icosahedral protein capsid and wrapped in a lipid bilayer envelope (Steven and Spear, 1997). HSV infection utilizes different receptors, depending on the cell type. HSV-1's envelope has several surface glycoproteins that are required for viral entry into host cells. The glycoproteins gB, gC, gD, gH and gL are primarily responsible for attachment to cellular receptors and fusion of membranes (Pertel *et. al*., 2001). The interaction between gB/gC surface proteoglycans and heparan-sulphate moieties on the host cell facilitates attachment of virus particles. gB interacts and binds with the paired immunoglobulin-like type 2 receptor-alpha (PILR-alpha). Similarly, gD binds to herpes virus entry mediators (HVEMs) (Campadelli-Fiume *et. al*., 2000). These HVEMs include nectin-1, nectin-2 or 3-O-sulphotransferase-modified heparan sulphate. Binding of both gB and gD to their host cell counterparts initiates viral fusion with the membrane. Fusion events are further mediated by gB and the gH–gL heterodimer, which ultimately results in the release of the viral nucleocapsid into

the cytoplasm of the host cell (Figure 2). The nucleocapsid is then transported to the nuclear membrane by utilizing the cell's microtubule network. The viral DNA then enters the nucleus. Once the viral DNA has breached the host cell nucleus, transcription of viral genes commences and the process of creating new virus particles begins (Sodeik *et. al.,*1997; Campadelli-Fiume *et. al.*, 2000).
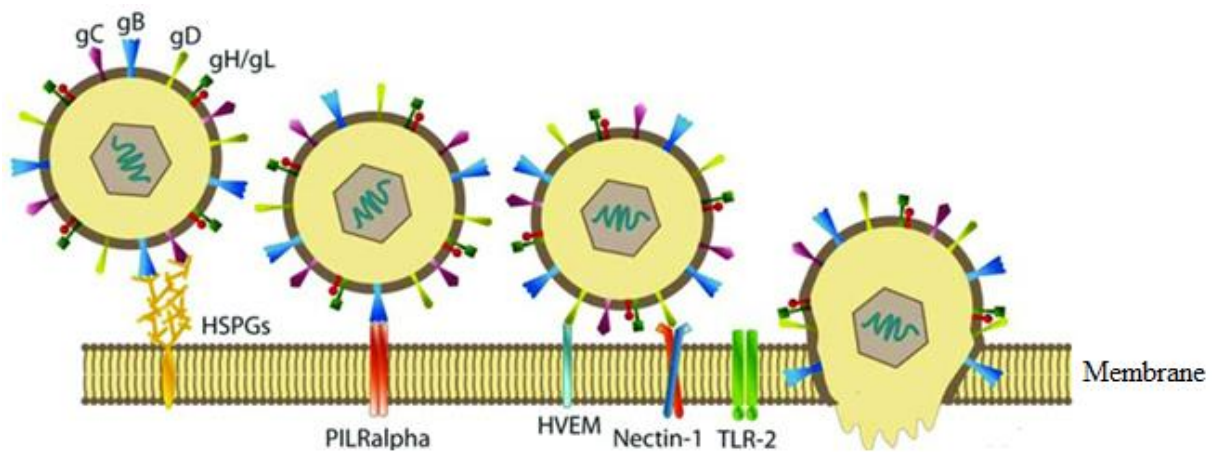
Figure 2: HSV-1 attachment and entry into a host cell membrane. gB/gC bind heparan-sulphate moieties on the host cell facilitates attachment of virus particles. gB interacts and binds with the PILR-alpha. gD binds HVEMs including nectin and initiates viral fusion with the membrane. Fusion events are further mediated by gB and the gH–gL heterodimer, which ultimately results in the release of the viral nucleocapsid into the cytoplasm of the host cell (Reproduced from Manservigi et. al., 2010).

Ligand binding to HVEM activates nuclear factor kappa B (NF-κB) and stimulates T cells. However, binding of HSV-1's glycoprotein gD inhibits these events and is one example of how HSV-1 alters normal immune strategies (La *et. al.*, 2002). It is in this way that HSV produces many proteins that contribute to immune evasion. Infected-cell protein null (ICP0) is a viral protein

secreted during the early stage of infection and is responsible for viral replication and reactivation of latent herpes virus. ICP0 is associated with a decrease in IFN-γ production and is believed to stimulate the degradation of cellular proteins through the proteasome (Lin *et. al*., 2004; Boutell and Everett, 2003). Additionally, ICP47 binds to the transporter associated with antigen processing 1/2 (TAP1/2) and blocks HSV peptide presentation by the major histocompatibility complex I (MHC class I) (Bauer and Tampe, 2002).

Macrophages

Macrophages are specialized cells of the innate immune system that are derived from monocytes found in the circulatory system. Macrophages are capable of killing extracellular pathogens that enter the blood, and help to clear tissue debris by phagocytosis. These cells also play a role in repairing damaged tissue (Mills, 2012). To combat intracellular pathogens, macrophages activate T and B cells through the production of multiple cytokines (Small *et. al*., 1994). When T lymphocytes are stimulated by a microbe, they release cytokines that encourage their differentiation into T helper 1 (Th1) cells and T helper 2 (Th2) cells. These helper cells mediate the functions of effector cells, which is crucial for host defense (Mosmann *et. al*., 1986).

M1 vs. M2 Macrophage Polarization

M1 and M2 macrophages phenotypes are converted from a naïve state upon specific stimuli. Under special conditions (i.e., during infection), Th1 cells, NK cells, and macrophages produce IFN-γ that induces the classically activated M1-macrophage phenotype (Dale *et. al*. 2008). The addition of IFN-γ and LPS to the J774A.1 macrophage cell line induces the M1 state that appears as irregularly flattened cells with visible intracellular vacuoles (Reichard, 2012). M1 cells secrete large amounts of pro-inflammatory cytokines such as IL-1B, IL-6, IL-23, TNF, iNOS, and

IL-12 and are bactericidal. In addition, they release chemokines and display increased levels of MHC molecules responsible for presenting antigens to the rest of the immune system (Martinez and Gordon, 2014; Ding *et. al.*, 1988). The M1 macrophage response is necessary to control viral replication; however, the inflammation that ensues can cause substantial host tissue damage (Ding *et. al.*, 1988).

In contrast with the M1 state, the alternatively activated M2 macrophage exhibits anti-inflammatory functions, tissue damage repair, and induces angiogenesis (Novak and Koh, 2013). M2 has more than three subclasses based on gene expression profiles, M2a, M2b, and M2c. M2 is activated by IL-4 produced from Th2 cells, macrophages themselves, eosinophils, and basophils (Martinez and Gordon, 2014). Additionally, IL-13, IL-1R, and IL-10 from Th2 and leukocytes induce M2a, M2b, and M2c subclasses, respectively. IL-10 and TGF-β, two anti-inflammatory mediators, are produced by M2 macrophages (Mantovani *et. al*., 2004) (Table 1). IL-4 knockout mice display susceptibility to parasites such as, *Cryptococcus neoformans*, demonstrating the significance of M2 macrophages in host protection (Martinez *et. al*., 2009).

During inflammation, M1 and M2 macrophages display plasticity by being able to transition from one phenotype to the other continuously in order to achieve optimal macrophage function and homeostasis (Stout *et. al*., 2005, Davis *et. al*., 2013). An imbalance between M1 and M2 phenotypes is related to neurodegenerative diseases including Parkinson's disease (Tang and Le, 2015).

Table 1: Phenotypes of M1 and M2 macrophage subsets and their functions. M1 and M2 polarized macrophages show different opposing activities, origin, and molecular basis (Adapted from Hao et. al., 2012 and Duluc et. al., 2007).

Macrophage polarization				
		M2 program		
	M1 program	**M2a**	**M2b**	**M2c**
Stimulation	IFN-γ and LPS	IL4/IL13	LTR/IL-1R	IL-10
Expression	CD86, CD80, MHC II ↑, and iNOS	CD163, MHC II,	CD86 MHC II	CD163
Cytokines Produced by macrophage sub-sets	TNF- α IL-1-β IL-6, IL-12 IL-23	IL-10, TGF- β, and IL-1ra	IL-1, IL-6, IL-10, and TNF- α	IL-10 and TGF- β

Macrophage Events during HSV Infection and Cytokine Involvement

The innate immune system responds to HSV infection within a few hours through activation of macrophages and NK cells. Antiviral activity appears within the first 8 hours *in vitro* and 12 hours *in vivo* (Mogensen, *et. al.*, 1989). HSV-1 infection induces macrophages to release galectins 1 and 3 in the extracellular space. Galectins block viral entry to the target cell by binding to HSV-1's membrane glycoproteins (Miettinen *et. al.*, 2012).

Macrophages detect the presence of HSV-1 through pattern recognition receptors (PRRs) on their surface that bind pathogen-associated molecular patterns (PAMPs) expressed on HSV-1, ultimately leading to macrophage stimulation (Medzhitov and Janeway, 2002). While IFN is present prior to HSV-1 infection, or through autocrine signaling of IFN-α/β on macrophages (Mogensen and Virelizier, 1987), macrophages infected with HSV produce high levels of IFN-α/β and TNF cytokines. TNF-α signaling activates NF-κB, and its translocation from the cytosol into the nucleus. Once in the nucleus, NF-κB turns on gene expression of numerous inflammatory cytokines. IFN-α/β and TNF-α mediators activate macrophages to release reactive oxygen species (ROS) and reduce the spread of HSV to other cells by inactivating extracellular viruses (Paludan *et. al.*, 1998) (Figure 3).
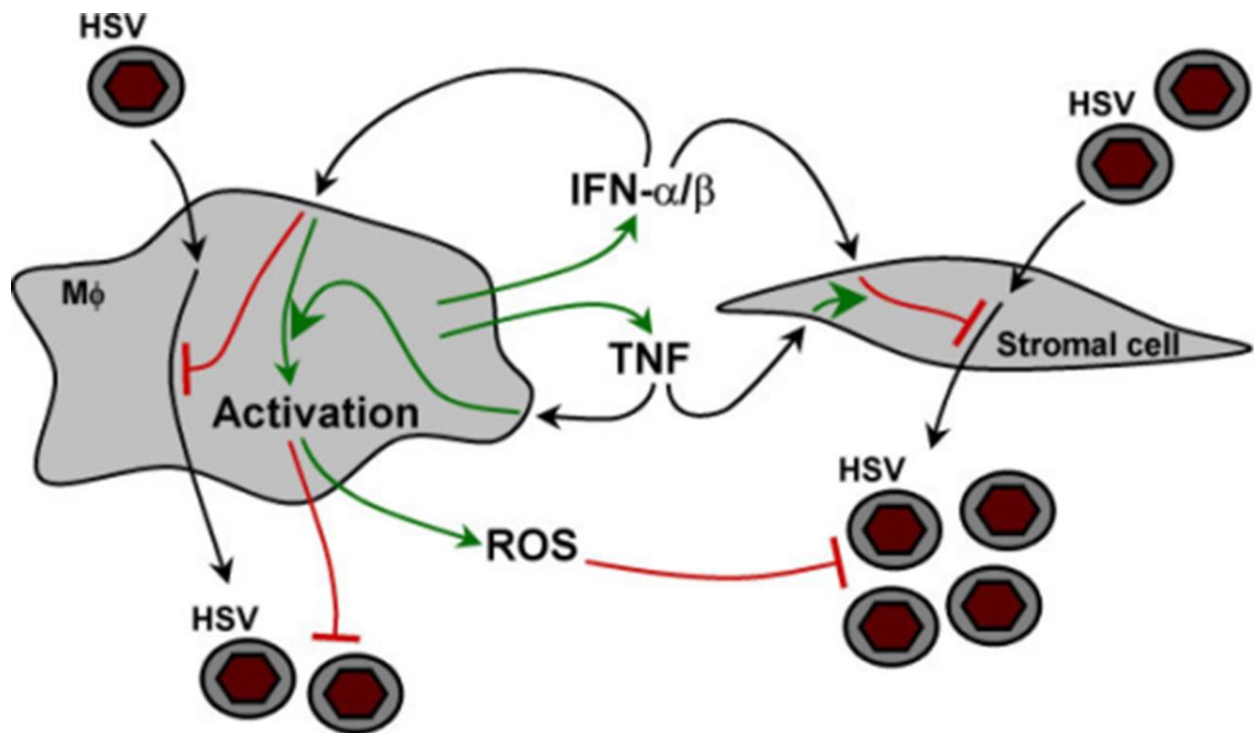
Figure 3: Initial macrophage responses to HSV-1 infection result in the generation (shown in green) of IFN-α/β and TNF cytokines and ROS that serve to prevent (shown in red) the spread of the virus to other cells (Reproduced from Ellermann-Eriksen, 2005).

A 2004 study by Al-Khatib *et. al.* delivered INF-β into the eyes of mice via an adenovirus vector prior to infection of the eye with HSV-1. They found that the mice demonstrated reductions in viral titer, viral gene expression, IFN-γ levels, and CD8+ T lymphocyte and NK cell infiltration. These effects were attributed to IFN-α /β's ability to prevent viral protein translation by inhibiting the eukaryotic initiation factor (eIF-2α) and inducing 2′-5′-oligoadenylate synthetase (OaS1a). OaS1a binds to the endoribonuclease RNase L that cleaves single-stranded mRNA and rRNA and blocks protein synthesis.

IFN and TNF also induce the generation of iNOS, which creates nitric oxide and inhibits viral replication. Conversely, IL-4 inhibits iNOS; however, TNF and IFN-γ are able to prevent IL-4 from inhibiting iNOS formation (Figure 4).
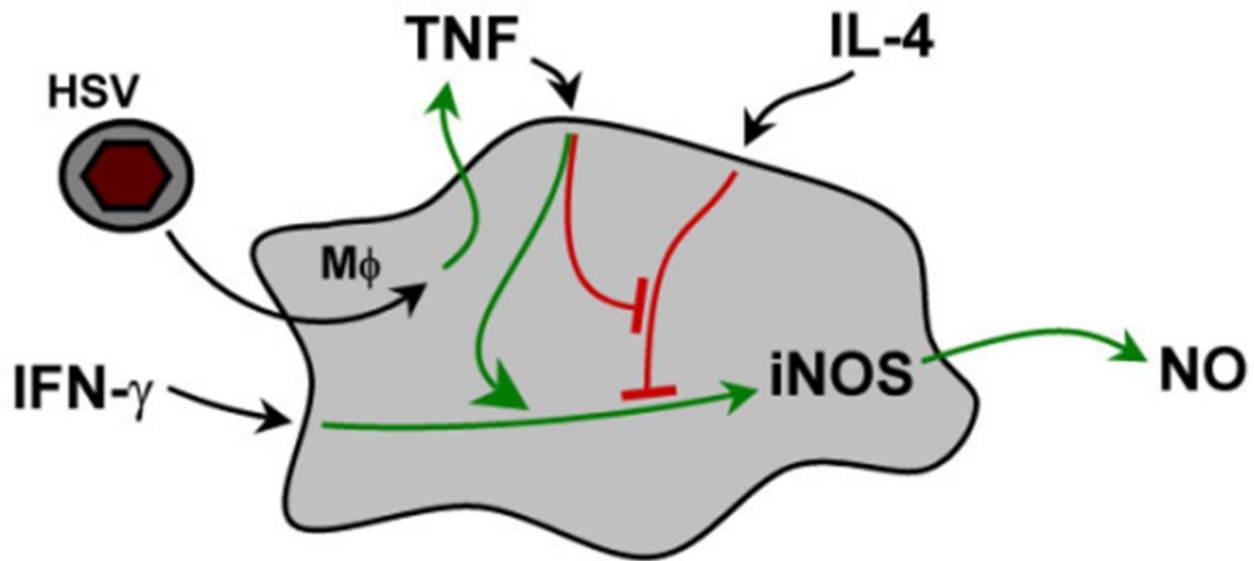
Figure 4: The role of cytokines in the regulation of macrophage iNOS production in response to HSV-1 infection. IFN--γ and TNF stimulate the production of iNOS, which creates nitric oxide in order to prevent viral replication generation (shown in green). iNOS generation is inhibited by IL-4. TNF and IFN-γ are able to overcome the effect of IL-4 (shown in red) (Reproduced from Ellermann-Eriksen, 2005).

INF-γ is also produced in response to HSV-1 infection. It circulates in the blood stream and plays a role in both innate and adaptive immunity. It activates different types of cells through specific receptors and helps to protect the cells from HSV-1 and other viral infections. INF-γ activates Janus kinase (JAK), thereby mediating signal transducer and activator of transcription (STAT)1 activation, converting M0 macrophages to M1 and inducing the secretion of pro-inflammatory cytokines (Zhou *et. al*., 2014) (Figure 5).
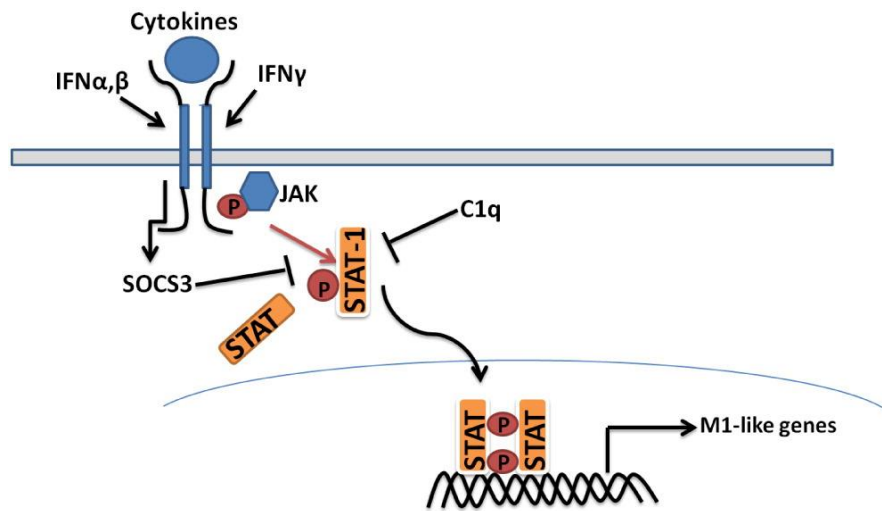
Figure 5: Interferon-mediated M1 polarization through the JAK/STAT pathway. INF-α, β, or γ bind to their receptor on the macrophage surface; this leads to the activation of the receptor and phosphorylation of JAK. JAK phosphorylates STAT-1 and phosphorylated STAT-1 dimerizes and the STAT complex translocates to the nucleus. STAT then activates the expression of genes involved in converting M0 macrophages to M1 and inducing the secretion of pro-inflammatory cytokines. SOCS3 inhibits STAT-1 activation, ultimately blocking the conversion of M0 to M1 macrophage phenotypes (Reproduced from Zhou et. al., 2014).

Cluster of Differentiation (CD) markers

In addition to displaying separate phenotypes, M1 and M2 have different functions, gene expression, and cell surface markers (Mantovani *et. al*., 2004). M1 and M2 macrophages can be distinguished by using different cluster of differentiation (CD) markers antibodies, CD80, CD163, and CD200R. The anti-inflammatory M2 cells display scavenger, mannose, and galactose-type receptors (Peiser and Gordon, 2001). CD80 is specific to human M1 polarized macrophages and CD200R is a specific marker to polarized human M2 (Jaguin *et. al*., 2013).

CD80, or B7-1, is a co-stimulatory signal that is involved in initiation and maintenance of T cells. It is expressed on the surface of antigen presenting cells, activated B cells, and monocytes. CD80/CD86 can bind to their cognate receptor, CD28 or cytotoxic T-lymphocyte-associated protein 4 (CTLA4), resulting in initiation or inhibition of naive T-cell activation (Linsley *et. al*., 1991; Walunas *et. al*., 1996, respectively). A previous study performed in J774A.1 cells showed that CD86 expression was up regulated in uninfected M1 polarized macrophages compared to uninfected M2 polarized cells. In contrast, CD86 expression reduced in M1, M2, and control macrophages infected with HSV-1 (Reichard, 2012).

CD163 is a glycoprotein of the scavenger receptor cysteine-rich (SRCR) superfamily type B (Law *et. al*., 1993; Kristiansen *et. al*., 2001). It is a marker for anti-inflammatory activities in monocyte/macrophage cell populations. CD163 is the endocytic receptor binding hemoglobin complexed with the plasma protein haptoglobin: it is produced during the early response to host injury. CD163 on the surface of the macrophage binds to either hemoglobin or heme when present at high levels. CD163 expression is enhanced by stimulation of monocyte/macrophage cells with IL-6, IL-10, and glucocorticoids and is not affected by IL-4 or IL-13 (Koning *et. al*., 2010), whereas LPS, INF-γ, and TNF-α reduce its expression (Buechler *et. al.*, 2000; Högger, *et. al.*,

1998). CD163 was classified as a marker of M2C cells. It was recently found that CD163 also exists as a soluble form in plasma (sCD163): this form is induced by LPS and other stimuli (Sulahian *et. al.*, 2001). The majority of sCD163 is expressed in patients with myelo-monocytic leukemia and infection (Møller *et. al*., 2002). In this study, CD163 was selected to distinguish among macrophage populations and was used as a marker for the M2 phenotype.

The CD200 receptor (CD200R) is a membrane glycoprotein that acts as an immune-inhibitory receptor thereby preventing inflammatory macrophage activation (Hoek *et. al.*, 2000). CD200R is displayed predominantly on myeloid cells, monocyte/macrophages, dendritic cells, and on a subgroup of activated T and B cells. CD200R binds CD200, which is a type 1 membrane glycoprotein of the Ig superfamily and is expressed in several cell types (Rijkers *et. al*., 2008). CD200R on M2 cells, specifically M2a, induces an anti-inflammatory response that limits potentially damaging pro-inflammatory responses, in part by reducing the production of pro-inflammatory cytokines (Jenmalm *et. al*., 2006).

INF-γ can stimulate expression of CD200R in murine macrophages, unlike human macrophages where it is unable to do so. When human cells were stimulated with IL-4 for 3 days *in vitro*, their expression of CD200R expression increased (Koning *et. al.*, 2010). In this study, we investigate the effect of HSV-1 infection on CD200R expression on murine macrophages.

Suppressor of Cytokine Signaling Proteins

Suppressor of cytokine signaling (SOCS) proteins negatively regulate cytokine receptor signaling and TLR. There are eight types of SOCS proteins currently identified: CIS and SOCS1 - SOCS7. All SOCS proteins consist of a ~50 amino acid carboxy-terminal SOCS box and an Src homology 2 (SH2) domain. The SH2 domain of SOCS proteins interact with phosphotyrosine

phosphorylated proteins. The SOCS box domain interacts with the elongin BC complex, which inhibits the SOCS proteins degradation (Yoshimura, 2005).

SOCS1 and SOCS3 can be induced by cytokines (such as IFN-γ) or TLR ligands (through LPS). When one of these cytokines binds to its receptor, JAK1 and JAK2 are phosphorylated and the receptor becomes activated. STAT binds to the activated receptor and is activated by phosphorylation. Upon dissociation from the receptor, phosphorylated STAT dimerizes and the STAT complex translocates to the nucleus. STAT then binds to the genes for SOCS1 and SOCS3, as well as other cytokine-induced genes leading to expression (Hu *et. al*., 2002; Alexander, 2002) (Figure 6A).

SOCS1 and SOCS3 inhibit JAK activity

SOCS1 and SOCS3 proteins also have a kinase inhibitory region (KIR) near the amino-terminal domain (Figure 6B) that eliminates JAK activity. SOCS1 binds directly to JAK, preventing its catalytic activity. Conversely, SOCS3 binds to areas of cytokine receptors adjacent to JAK's binding location, inhibiting JAK activity through the KIR. Both the SOCS1 and SOCS3 complexes can then be targeted for degradation by ubiquitination recruited through the SOCS box. Therefore, SOCS molecules tightly control the duration and intensity of cytokine actions by JAK-STAT signaling (Yoshimura, 2005; Yoshimura and Yasukaw, 2012). Furthermore, SOCS3 regulates STAT3 signaling and therefore controls whether STAT3 leads to pro-inflammatory or anti-inflammatory signals. Ultimately, increases in SOCS3 lead to anti-inflammatory signals.
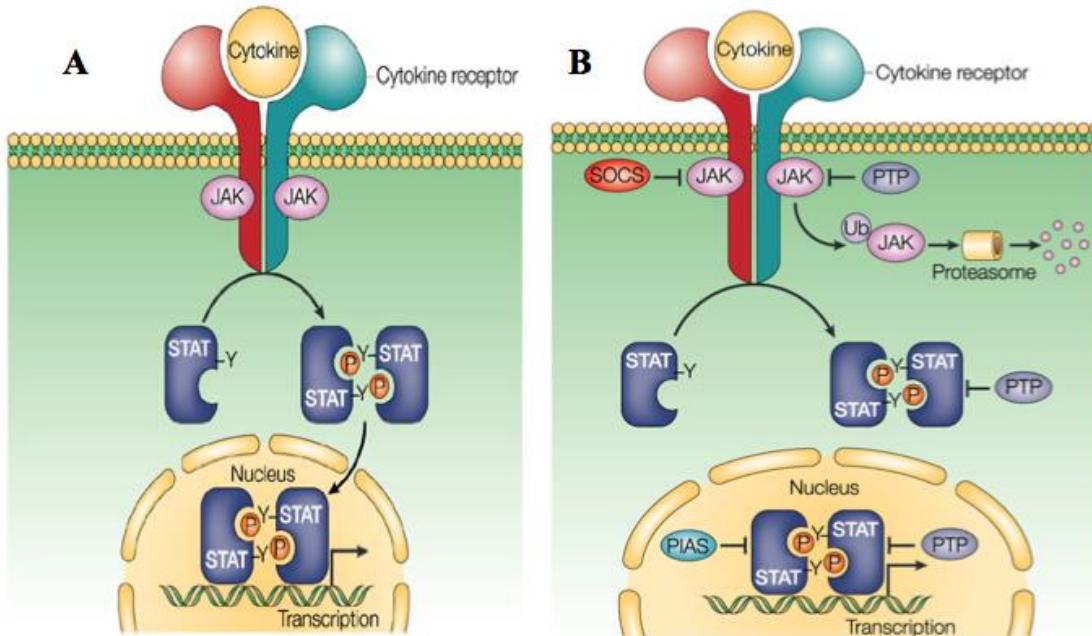
Figure 6: Activation of JAK–STAT signaling through cytokines binding its receptor. A) Cytokines induce JAK activity. B) SOCS proteins inhibit JAK activity (Reproduced from Alexander, 2002; Shuai and Liu, 2003)

SOCS1 and SOCS3 also play roles in macrophage polarization by regulating NF-κB, phosphoinositide 3-kinases (PI3Ks), and extracellular signal-regulated kinase (ERK) signaling pathways. Specifically, SOCS3 inhibits cytokine-induced activation of SMAD3, STAT3, STAT6, and PI3K, thereby inhibiting macrophage polarization to the M2 state. Conversely, SOCS1 inhibits STAT1 and NF-kB activity induced by cytokines, preventing M1 polarization (Wilson, 2014).

MATERIALS AND METHODS

HSV-1 and Cell Lines

HSV-1 (syn17$^+$) (courtesy of Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH) was titrated in Vero cells (CCL-81). J774A.1 and RAW 264.7 are murine macrophage-like cells. J774A.1 is a reticulum cell sarcoma, an adherent macrophage cell line derived from an adult female BALB/cN mouse. RAW 264.7 is Abelson murine leukemia virus-induced tumor derived from an adult male BALB/C mice. All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). J774A.1 cells were maintained in a medium consisting of Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented with 10% Fetal Bovine Serum (FBS; Fisher Scientific) and 1% antibiotic antimycotic solution (10000 U/mL penicillin, 10000 μg/mL streptomycin, and 25 μg/ml Ampholericin B) PSG (1% penicillin-streptomycin and 0.1% gentamycin, each at 10000U/mL) in a humidified 5% CO_2 incubator at 37ºC. J774A.1 cells were cultured in 100 mm x 20 mm cell culture treated Petri dishes (BD Biosciences). Cultures were split 1:5 every other day.

Polarization Induction

When J774A.1 macrophages reached approximately 50-70% confluency (by observation), culture medium was replenished with IFN-γ and LPS supplemented media. A final concentration of 20 ng/ml IFN-γ (Peprotech)/ 100 ng/mL LPS (Chondrex) was used to induce the M1 phenotype. The M2 phenotype was induced with 20 ng/ml of either IL-4 or IL-13, or IL-10 at 10 ng/ml (Peprotech). Cultures were allowed to polarize for 18, 24, or 48 hours. Cells were collected from the culture dishes using a cell scraper to analyze cell viability, morphological changes, and CD markers by flow cytometry and immunofluorescence.

Cell Viability

J774A.1 macrophages were grown to approximately 70% confluency (by observation), at which time either IFN-γ/LPS (M1) or IL-4, IL-10, or IL-13 (M2) was added with or without infection at a multiplicity of infection (MOI) of 0.1 HSV-1 per cell. Untreated cells were used as a control. After 18, 24, and 48 hours, cells were collected from the 24-well plates using a cell scraper. The supernatant was aspirated after centrifugation (1500 rpm for 5 minutes, at 4°C). The cell pellet was suspended in 1 mL of 10% DMEM medium. The cells were stained with trypan blue (Fisher Scientific) to determine cell viability using a haemocytometer using the following equation.

% Cell Viability = [Total Viable cells (Unstained) / Total cells (Viable +Dead)] X 100.

Immunofluorescence Staining

J774A.1 macrophages were plated and grown on sterile glass coverslips (Fisher Scientific) at 3×10^5 cells per well of a 6-well plate. Once the coverslips reached approximately 50% confluency, the cells were stimulated with IFN-γ/LPS or IL-4, IL-10, or IL-13, with or without HSV-1 (MOI of 0.1). Following incubation for 18, 24, or 48 hours, culture media was aspirated and cells were washed with 1% bovine serum albumin (BSA) suspended in phosphate buffered saline (PBS). Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were then rinsed three times with 1% BSA in PBS for three minutes each. Cells were blocked for non-specific binding with 3% BSA in PBS and incubated for one hour at room temperature. Cells were then rinsed three times with 1% BSA in PBS for three minutes each. Diluted primary antibodies (Table 2) in the blocking buffer (3% BSA in PBS) were applied directly

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onto the cells and incubated overnight at 4°C. Cells were then rinsed three times with 1% BSA in PBS for three minutes each. Stained cells on coverslip were mounted with drop of Vectashield hard-set mounting medium (H-1400, Vector Laboratories) onto microscope slides and visualized on an Olympus Epi-fluorescence microscop equipped with a "spot" digital camera.

Table 2: Summary of Primary Antibody Concentrations used to show CD makers expression on the un-polarized and polarized macrophages for Immunofluorescent staining.

Antibody	Concentration/Dilution	Company
Texas Red-Phalloidin X	(3μg/million cells)	Life Technologies
Anti-CD80 antibody conjugated to Pacific blue	(2μg/million cells)	Biolegend
Anti-CD163 antibody conjugated to FITC	(2μg/million cells)	Antibodies-Online Inc.
Anti-CD200R antibody conjugated to Alexa Fluor 488	(2μg/million cells)	Antibodies-Online Inc

Flow Cytometric Analysis

J774A.1 macrophages were plated on 150 mm x 15 mm petri dishes (BD Biosciences) and incubated until 50% confluent. The following day, cells were treated with either IFN-γ/LPS, IL-4, IL-10, or IL-13 with or without HSV-1 for 24 hours. Cells were collected using a cell scraper and viability was calculated with a hemocytometer using the trypan blue exclusion test. Cells were washed three times with 1% BSA in PBS. Cells were blocked for non-specific binding with 3% BSA in PBS and incubated for one hour at room temperature. Cells were then rinsed three times with 1% BSA in PBS. The cells were incubated (light-protected) with the primary antibodies (Table 3) and isotype control antibodies for respective antibodies diluted in the blocking buffer (3% BSA in PBS) for 1 hour at 4°C and washed three times with 1% BSA in PBS. The cell pellet was then re-suspended with ice cold PBS with 0.5% sodium azide buffer and analyzed by the Accuri C6 flow cytometer.

Table 3: Summary of Primary Antibody Concentrations used to show CD makers expression on the un-polarized and polarized macrophages for flow cytometry.

Antibody	Concentration/Dilution	Company
Anti-mouse CD80 conjugated to PE/Cy5	(2µg/million cells)	Biolegend
Anti-CD163 conjugated to FITC	(2µg/million cells)	Antibodies-Online Inc.
Anti-CD200R conjugated to FITC	(2µg/million cells)	Biolegend

SOCS Experiments

Twenty-four hours prior to treatment, J774A.1 macrophages were grown, in triplicate, in 24-well plates at a density of 5×10^4 cells per well. The culture medium was then replaced by fresh DMEM containing 10% FBS and treated with 20 ng/ml IFN-γ and LPS (100 ng/mL) to convert the cells to the M1 phenotype. SOCS1 peptide (35μM/ml), SOCS3 peptide (35μM/ml) or PJAK2 (SOCS1 inhibitor, 35μM/ml) was added to the cells, with or without HSV-1 (MOI of 0.1). The cells were incubated for 24 hours.

In a separate experiment, SOCS3 peptide (35μM/ml) or tissue culture medium was added to the cell culture for 30 minutes. The cells were either infected with HSV-1 (MOI of 0.1) for 24 hours or induced to an M1 state using 20 ng/ml IFN-γ and LPS (100 ng/mL), or both.

After incubation cells were collected from the 24-well plates using a cell scraper and centrifuged at 1500 rpm for 5 minutes. The supernatants were collected and stored at −80°C for subsequent IL-1β, IL-6, TNF-α, and IL-10 measurements. The cell pellet was re-suspended in 1 mL of 10% DMEM medium. The cells were stained with trypan blue to determine cell viability.

Cytokine Measurements

The frozen supernatants were evaluated for IL-1β, IL-6, TNF-α or IL-10 concentrations using Luminex Multiplex Immunoassays (R&D Systems) in accordance with manufacturer's instructions.

Statistical Analysis

       All experiments were carried out at least three times. Flow cytometry and cell viability data shown were from representative experiments. The statistical significance of differences between testing and control experimental groups was analyzed by one way ANOVA using Sigma Plot 12.0 software. P values equal to or less than 0.05 were considered to be statistically significant, and data are represented as the mean ± standard error of the mean.

RESULTS

Uninfected and Infected with HSV-1 Control and Cytokines Polarized Macrophages

Polarization of J774 Macrophage Cells Line Alter Morphology

Macrophages were treated for 18, 24, or 48 hours with IFN-γ and LPS to induce the M1 phenotype or with IL-4, IL-10, or IL-13 to induce the M2 phenotype. Morphological examination showed that the polarized macrophages exhibited different morphologies with and without HSV-1 (MOI 0.1).

Uninfected M1 macrophages appeared as flattened, extended irregular shapes, contained visible intracellular vacuoles, and attached to the plates (adherent), whereas M0 and M2 cells displayed a rounded cell shape at 18 (Figure 7), 24 (Figure 8), and 48 hours (Figure 9), with some M2 macrophages appearing elongated. At 24 and 48 hours, M1 macrophages demonstrated the same morphological changes described above for 18 hours, although the irregular shape and intracellular vacuoles were more pronounced (Figure B in 8 & 9).

HSV-1 Infection of Polarized Macrophages Further Alters Morphology

M0, M1 and M2 macrophages became rounded following infection with HSV-1 (MOI 0.1) for 18, 24, and 48 hours (Figure 10, 11, &12 from A-E). Infected M1 macrophages showed different morphological changes compared with uninfected M1 macrophages. Uninfected M1 cells were flattened, displayed extended irregular shapes, and had intracellular vacuoles (Figure B in 7, 8, & 9); however, HSV-1 infected M1 macrophages were rounded (Figure B in 10, 11, &12).

Cell Viabilities of Unpolarized of Polarized Macrophages after 18, 24, and 48 Hours With and Without HSV-1 Infection

J774A.1 macrophages were treated with IFN-γ/LPS (M1) or IL-4, IL-10, or IL-13 (M2) with or without HSV-1 (MOI 0.1) for 18, 24, and 48 hours. The cells were stained with trypan blue to determine the number of live versus dead cells. Cell viabilities for M1 and M2 macrophages were compared with those of untreated M0 cells as the control.

Polarization of J774A.1 macrophages for 18 hours leads to a statistically significant decrease in cell viability in M1 (25% p <0.001) and M2 macrophages induced by IL-4 (13%, p <0.05), but the decrease was not significant for M2 subgroups induced by IL-10 (~11%) or IL-13(3%) or compared to M0 control cells (95%)(Figure 13A). At 24 hours following polarization, M1 macrophages (18%, p <0.001) displayed a reduction in cell viability whereas both IL-4 and IL-10-induced M2 macrophages also showed significant, although minor, decreases in viability (~13%, p <0.05) compared to M0 control cells. There was no significant difference in cell viability between IL-13-induced M2 macrophages (3%) and M0 control cells (~97%) (Figure 14A). Cell viability for M1 macrophages at 48 hours post-polarization was similar to that observed after 24 hours (~21%, p <0.001). M2 macrophages, regardless of method of induction, demonstrated minor, not statistically significant reductions in viability compared with M0 macrophage (Figure 15 A). The cell viability was significantly lower in uninfected M1 macrophages (77%) compared to uninfected M2 macrophages polarized by IL-4 (8%, p< 0.05), IL-10 (11%, p=0.001), and IL-13 (9%, p<0.01) (Figure 15 A).

At 18 hours following polarization and HSV-1 infection, M1 infected macrophages (21%, p <0.001) exhibited a significant decrease in cell viability compared to infected M0 control cells (85%); M2 infected macrophage induced by IL-4, IL-10, and IL-13 did not display a significant

reduction (Figure 13 B). Compared to infected M0 cells (~88%), a significant decrease in cell viability was seen in M1 infected macrophages cells (~21%, $p < 0.05$) (Figure 14 B). HSV-infected M2 macrophages activated by IL-4, or IL-13(~2%) did not show significant differences in cell viability compared to HSV-infected M0 control cells. HSV-infected M2 macrophages polarized by IL-10 (10%) exhibited a reduction in cell viability, although this reduction was not significant (Figure 14 B). The cell viability was significantly lower in infected M1 macrophages compared to M2 infected macrophages induced by IL-4 or IL-13 (19%, $p = 0.05$ or ~21%, $p < 0.001$, respectively) at 24 hours (Figure 14 B). Compared to the viability of infected M0 cells (80%) at 48 hours, decreases in cell viability were observed in infected M1 macrophages (18%, $p < 0.005$) and infected M2 macrophages activated by IL-4 (18%, $p < 0.001$), IL-10 (14, $p < 0.005$), and IL-13 (20%, $p < 0.001$) (Figure 15B).

CD Marker Expression of polarized and control macrophages before and after HSV-1 infection

CD marker expression by un-polarized and polarized macrophages following 18, 24, and 48 hours of cytokine treatment in the presence and absence of HSV-1 infection was evaluated. Immunofluorescence staining with flow cytometry was performed at 18, 24, or 48 hours. CD80 expression on uninfected M1 macrophages was the highest at 18 and 24 hours, whereas it decreased at 48 hours. There were no apparent differences in the CD80 expression between these macrophages at different points in time (Supplemental figure 1). Infected M1 macrophages displayed an increase in CD80 expression at 24 and 48 hours (Supplemental figure 2).

M2 macrophages induced by IL-10 or IL-13 showed higher expression of CD163 at 24 (M2/IL-10) and 18 hours (M2/IL-13). CD163 expression was down-regulated among macrophages (Supplemental figure 3). Infected M2 macrophages polarized by IL-10 or IL-4

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showed an up-regulation of CD163 expression at 24 and 48 hours. CD163 expression was down-regulated among macrophages (Supplemental figure 4).

Uninfected M2 macrophages polarized by IL-4 showed an up-regulation of CD200R expression at 18 and 24 hours. No difference in CD200R expression were observed among macrophages (Supplemental figure 5). CD200R expression was highly up-regulated in infected M2 macrophages induced by IL-13 at 24 hours, and slightly high in infected M2 macrophages induced by IL-4 and IL-13 at 24 hours (Supplemental figure 6).

CD80: Flow cytometry was used to evaluate CD marker expression for control and polarized macrophages following 24 hours of cytokine treatment with or without HSV-1 infection. CD80 expression was insignificantly reduced on uninfected M1 macrophages (49%), M2 macrophages activated by IL-4 (45%), IL-10 (55%), and IL-13 (51%) compared to uninfected M0 macrophages (63%) (Figure 16 A&B). Following HSV-1 infection for 24 hours, M1 macrophages and M2 macrophages stimulated by IL-10 exhibited a minor increase in CD80 expression (56% and 57%, respectively) compared to infected M0 macrophages (53%) while M2 macrophages stimulated by IL-4 and IL-13 displayed slightly decreased levels of CD80 (51%) (Figures 17 A & B). In comparison to uninfected CD80$^+$ M1 macrophages (49% of the population) and CD80$^+$ M2 macrophages stimulated by IL-4 (45% of the population) or IL-10 (55% of the population), CD80 expression was slightly up-regulated in infected M1 macrophages (56% of the population) and M2 macrophages stimulated by IL-4 or IL-10 (51% and 57% of the population, respectively) (Figure 18). There was no change in CD80 expression in M2 macrophages stimulated by IL-13 before and after HSV-1 infection (Figure 18). The CD80 expression results were not statistically significant.

CD 163: The level of CD 163 expression was similar in both uninfected M0 and M2 macrophages induced by IL-10 (31% of the population) (Figures 19 A and B). The slight decrease in CD 163 expression on uninfected M1 and M2 macrophages induced by IL-4 or IL-13 was not statistically significant (Figures 19 A and B). Infected M2 macrophages induced by IL-10 (34% of the population) showed a minor increase in CD163 expression compared to infected M0 control (29% of the population). CD163-expressing infected M1 macrophages (21% of the population) and infected M2 macrophages induced by IL-4 or IL-13 (~26% of the population for both) were slightly decreased compared to CD163-expressing infected M0 control macrophages (29% of the population) (Figures 20 A and B). Uninfected and HSV-infected M0, M1 and M2 macrophages, regardless of manner of induction, exhibted slightly variable levels of expression of CD163, with CD163+ macrophages represented 20-30% of the population in all conditions (Figure 21). There were no statistically significant differences among any of the macrophage phenotypes regardless of infectious state.

CD200R: CD200R was expressed on 28% of the population of uninfected M0 macrophages (Figures 22 A & B). CD200R expression was similar in uninfected M2 macrophages induced by IL-4 (25% of the population) or IL-10 (26% of the population). Uninfected M1 macrophages showed reduced CD200R expression (19% of the population) and only 20% of the population of M2 macrophages induced by IL-13 were CD200R$^+$ (Figures 22 A & B).

The CD200R expression slightly increased in infected M1 macrophages (21% of the population), and infected M2 macrophages induced by IL-4 (24% of the population), IL-10 (26% of the population), and IL-13 (27% of the population; $p < 0.05$) compared to infected M0 macrophages (Figures 23 A & B). There was an insignificant decrease in the number of CD200R$^+$ HSV-1 infected M0 macrophages (17% of the population) compared to uninfected M0

macrophages (28% of the population) (Figures 24). However, there was no significant change in CD200R expression in M1 and M2 macrophages stimulated by IL-4 and IL-10 pre- and post-HSV-1 infection (Figure 24). The number of CD200R+ in infected M2 macrophages stimulated by IL-13 (27% of the population) was greater than uninfected M2 macrophages stimulated by IL-13 (20% of the population) (Figure 24): these results were not statistically significant. These data are summarized in (Table 4).

Effects Of SOCS1, SOCS3 Peptide Mimetics, and pJAK2 (SOCS1 Inhibitor) on Cell Viabilities of Polarized M1 J774A.1 Macrophages and RAW 264.7 macrophages

Cell viabilities of polarized M1 and HSV-1-infected M1 J774A.1 macrophages were determined following treatment with SOCS1 and SOCS3 peptide mimetics and pJAK2 for 24 hours. The SOCS3 peptide mimetic and pJAK2 resulted in increased viability of polarized M1 macrophages compared with SOCS1 treated cells (p<0.001). There was no difference between infected and uninfected groups (Figure 25). J774A.1 M1 cells produce high levels of TNF-α and IL-6 compared to M1 cells treated with either SOCS3 peptide mimetics or pJAK2 (Table 5). M1 cells treated with SOCS3 behave similar to the M2 phenotype and produce the anti-inflammatory cytokine IL-10 (Table 5). The polarized M1 macrophage viability decreased in response to the SOCS1 peptide mimetic (p<0,001), with no significant difference observed between virus and non-virus infected cells (Figure 25).

The effect of the SOCS3 peptide mimetic was tested to examine its ability to modify the cytotoxic effects of M1 polarization or HSV-1 infection. RAW 264.7 macrophages were treated with SOCS3 and then were either infected with HSV-1, polarized by M1, or both. When compared

with the untreated controls (~65%), the SOCS3 peptide mimetic increased cell viability in the M1 (~78%) and HSV-1 + M1 group (~83%) (p< 0.05) (Figure 26). However, SOCS3 did not significantly affect HSV-1 only treated cells (Figure 26). Pro- inflammatory cytokines, IL-6 and TNF-α were increased in M1 cells compared to M1 treated with SOCS3 and M2 macrophages induced by IL-4, IL-10, or IL-13. However, IL-1β treatment did not show a change between M1, M1 treated with SOCS3, and M2 macrophages. A decrease in IL-10 production was seen in M1 cells, while M1 cells treated with SOCS3 and IL-4 or IL-13 polarized M2 macrophages increased the production of IL-10. IL-10 polarized M2 macrophages show high levels of IL-10 because IL-10 is both an inducer and an effector molecule in these cells (Figure 27). It appeared that the SOCS3 peptide mimetic protected RAW 264.7 macrophages from the lytic effects of M1 polarization and SOCS3 causes M1 macrophages to behave similar to the M2 phenotype. The same experiments were performed in the J774 cell line. The SOCS3 peptide mimetic showed no significant effect on HSV-1 infected, M1 polarized, or HSV-1 + M1-treated J774 cells; however, the SOCS3 peptide mimetic increased cell viability in HSV-1-infected M1 J774A.1 macrophages (Figure 28). This result suggests that SOC3 mimetic exerts an anti-inflammatory effect by diminishing the lytic effect of M1 polarization.

Figure 7: Changes in Cellular Morphology of Polarized and Un-polarized J774A.1 Macrophages 18 hours pre- HSV-1 infection. A-E show unpolarized and cytokine-stimulated (polarized) macrophages that were fixed and stained with phalloidin (for filamentous actin arrangement), and then evaluated by immunofluorescence microscopy. A shows unpolarized control (M0), B, M1 macrophages polarized by LPS and IFN-γ, and C- F shows M2 macrophages polarized by IL-4, IL-10, and IL-13 respectively. M1 macrophages were elongated, and begin to form intracellular

vacuoles in the cytoplasm. M2 macrophages induced by IL-4, IL-10, and IL-13 were rounded and displayed no changes in morphology as compared to control macrophages. (Scale bar =50μm).

Figure 8: Changes in Cellular Morphology of Polarized and Un-polarized J774A.1 Macrophages 24 hours pre- HSV-1 infection. A-E show unpolarized and cytokine-stimulated (polarized) macrophages that were fixed and stained with phalloidin (for filamentous actin arrangement), and then evaluated by immunofluorescence microscopy. A shows unpolarized control (M0), B, M1

macrophages polarized by LPS and IFN-γ, and C- F shows M2 macrophages polarized by IL-4, IL-10, and IL-13 respectively. M1 macrophages displayed irregular shapes and intracellular vacuoles were more pronounced than 18 hours. M0 appeared rounded or elongated, and M2 macrophages induced by IL-4 and IL-13 show elongation with the majority of cells appearing rounded. M2 macrophages induced by IL-10 were rounded. (Scale bar =50μm).
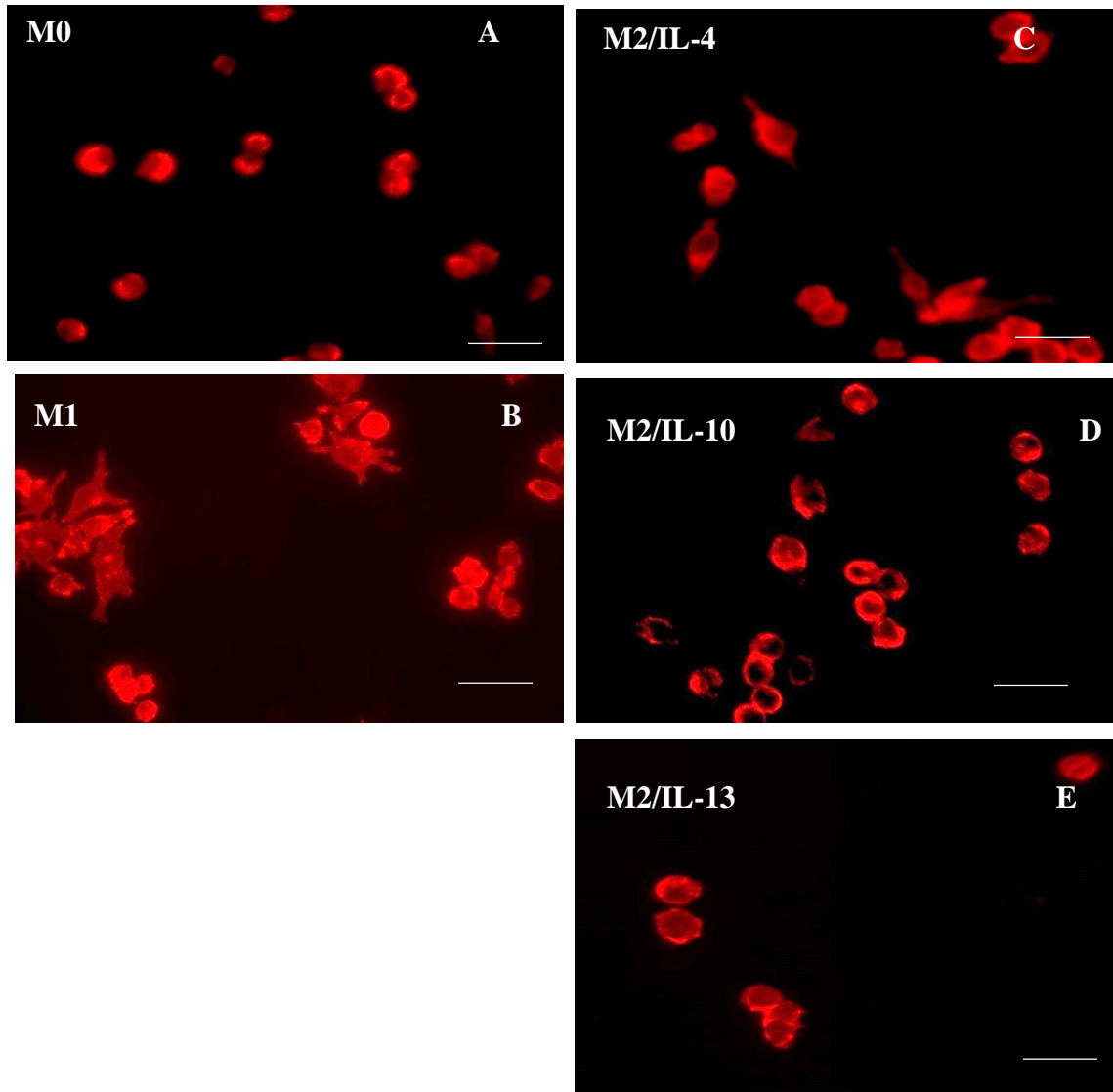
Figure 9: Changes in Cellular Morphology of Polarized and Un-polarized J774A.1 Macrophage 48 hours pre- HSV-1 infection. A-E showed un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained with phalloidin (for actin arrangement), and then evaluated by immunofluorescence microscopy. A showed un-polarized control (M0), B, polarized M1 macrophages by LPS and IFN-γ, and C- F showed polarized M2 macrophages by IL-4, IL-10, and IL-13 respectively. M1 macrophages exhibited the same morphological changes described at 18

and 24 hours. M2 macrophages induced by IL-4 were elongated and rounded compared to M2 macrophages induced by IL-10 or IL-13 and M0 macrophages. (Scale bar =50μm).
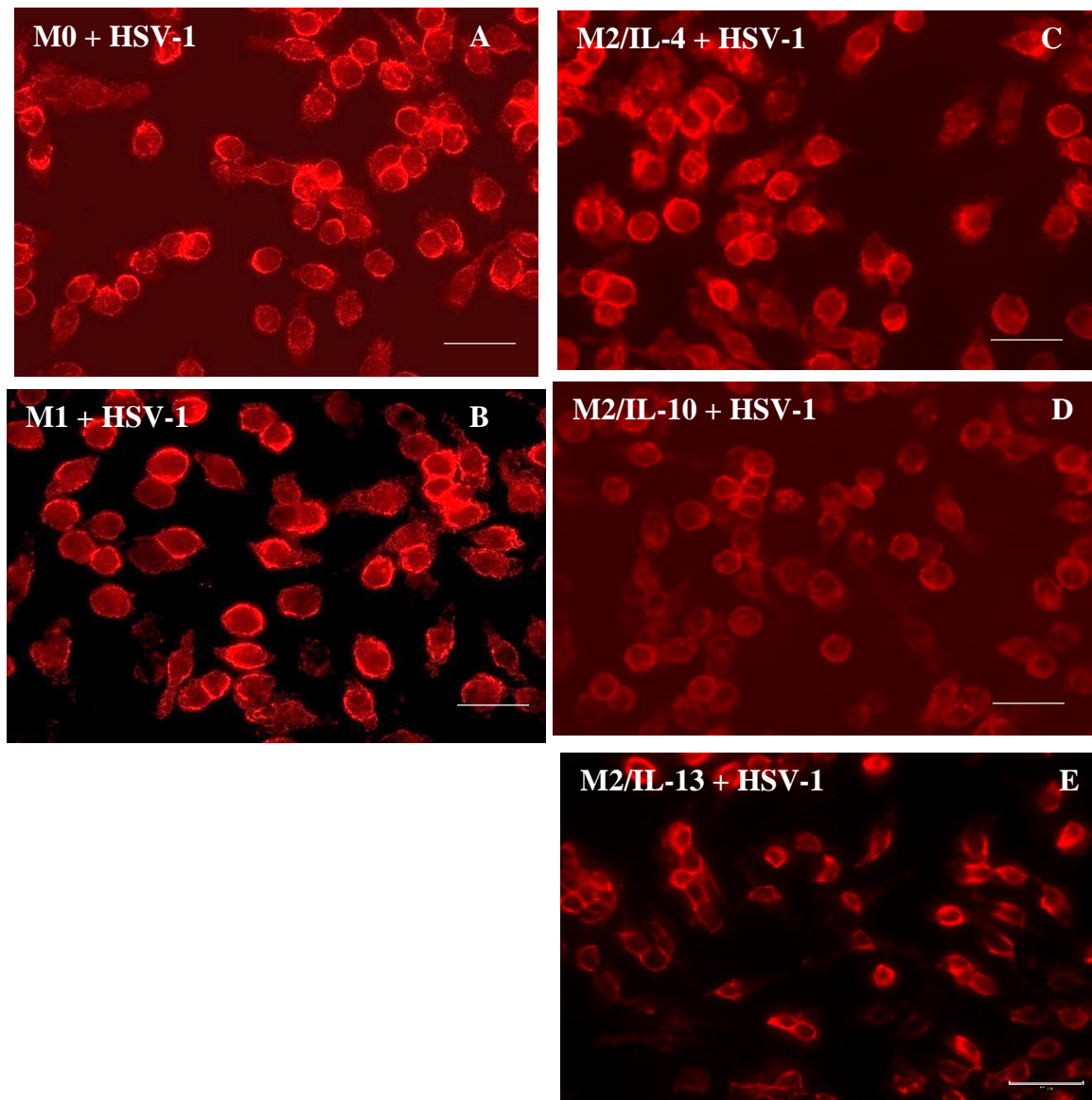
Figure 10: Changes in Cellular Morphology of Polarized and Un-polarized J774A.1 Macrophage 18 hours post- HSV-1 infection. A-E showed HSV-1 infected un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained with phalloidin (for actin arrangement), and then evaluated by immunofluorescence microscopy. A showed infected un-polarized control (M0), B, infected polarized M1 macrophages by LPS and IFN-γ, and C- F

showed infected polarized M2 macrophages by IL-4, IL-10, and IL-13 respectively. All infected

macrophage phenotypes were rounded and aggregated.   (Scale bar =50μm).
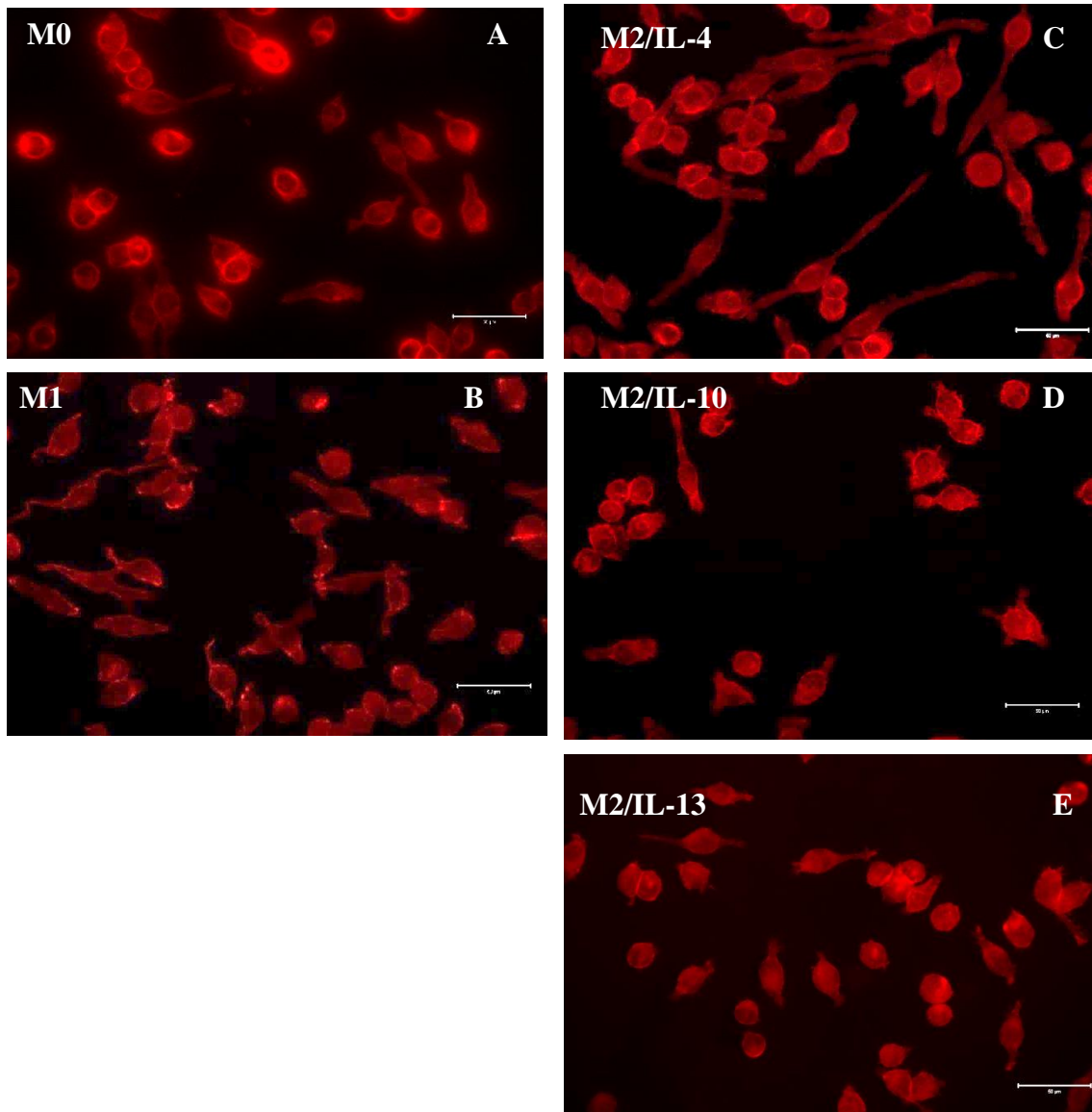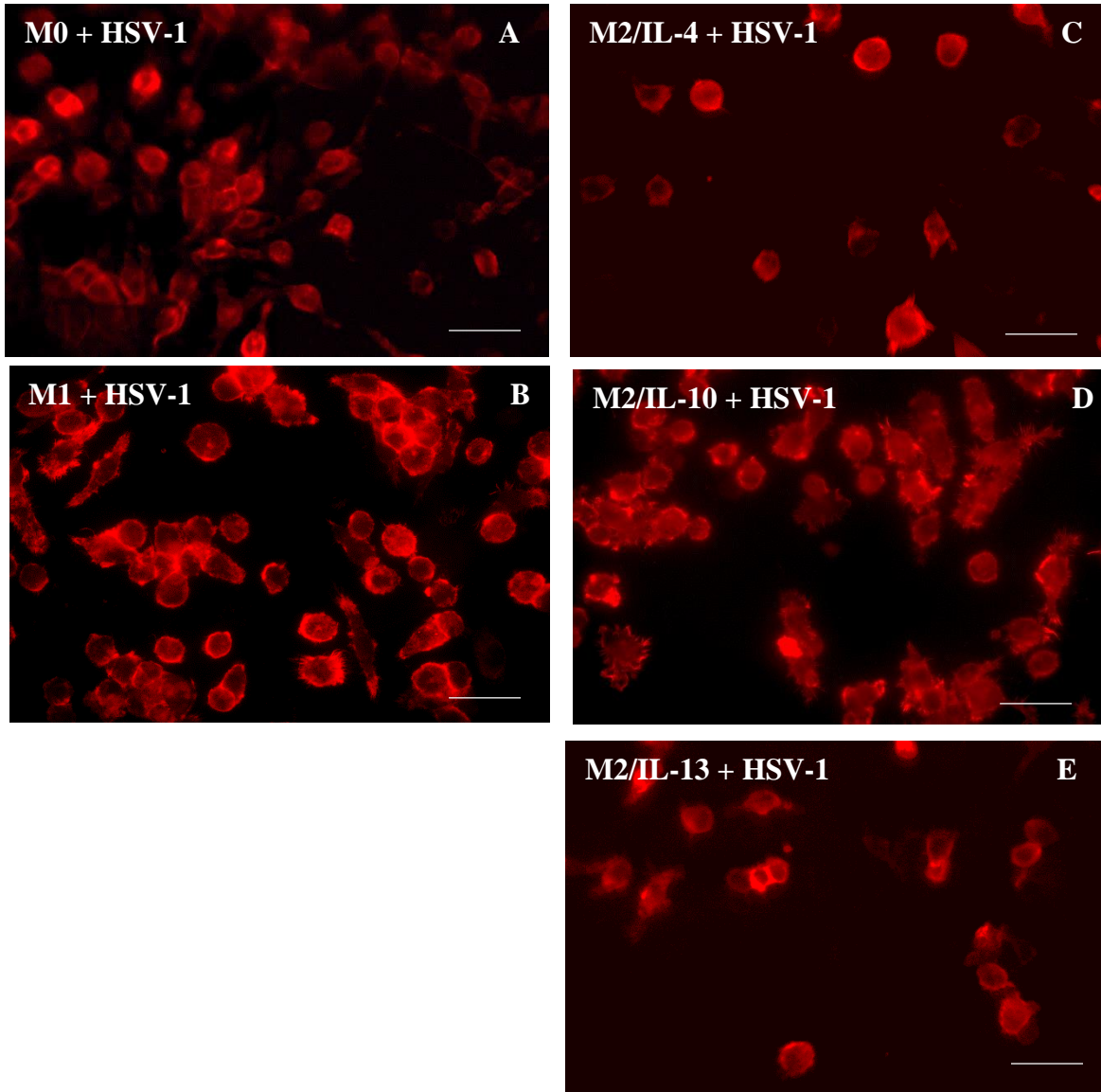
Figure 11: Changes in Cellular Morphology of Polarized and Un-polarized J774A.1 Macrophage 24 hours post- HSV-1 infection. A-E showed HSV-1 infected un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained with phalloidin (for actin arrangement), and then evaluated by immunofluorescence microscopy. A showed infected un-polarized control (M0), B, infected polarized M1 macrophages by LPS and IFN-γ, and C- F showed infected polarized M2 macrophages by IL-4, IL-10, and IL-13 respectively. Infected macrophages were rounded and aggregated. (Scale bar =50μm).
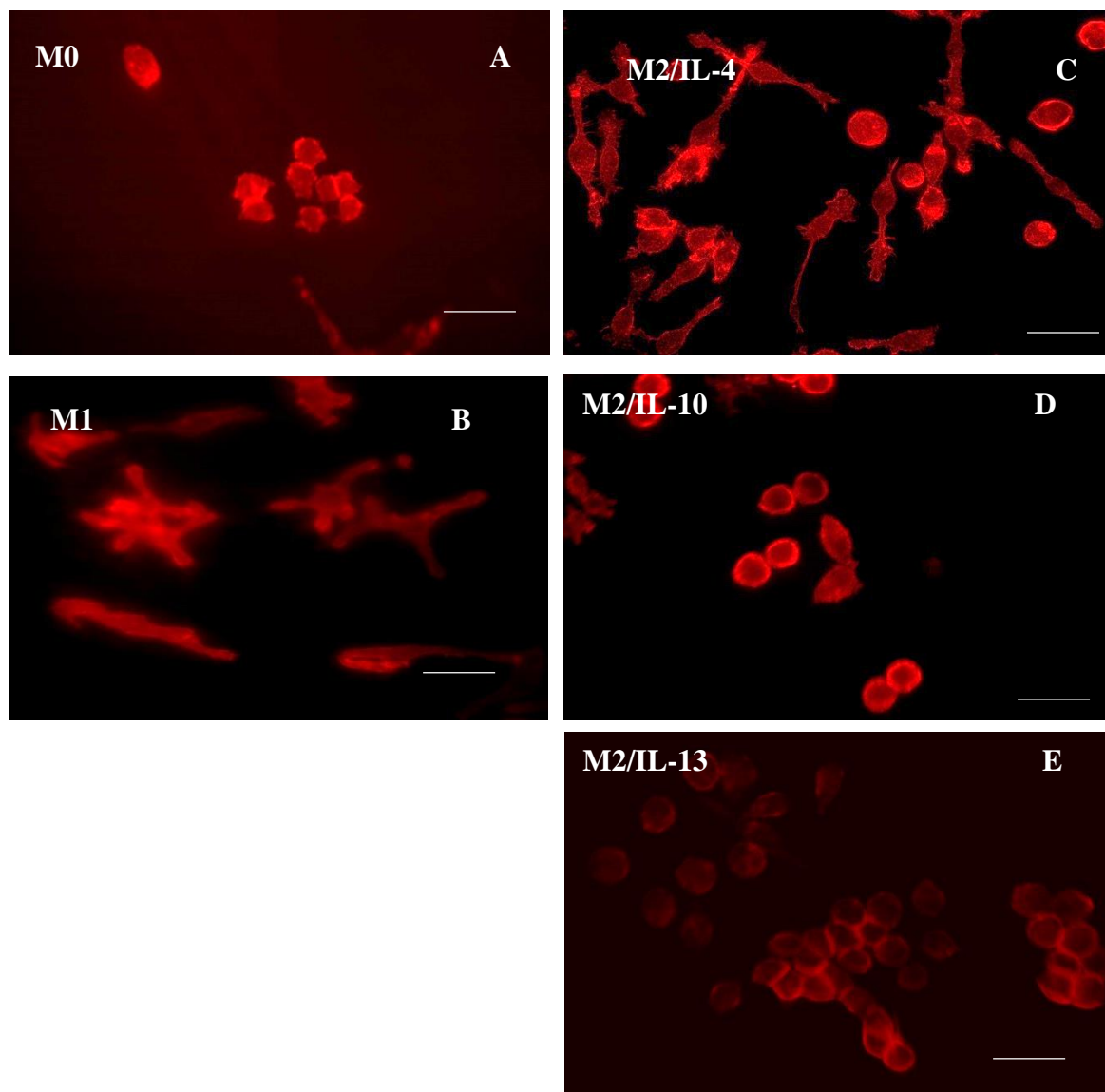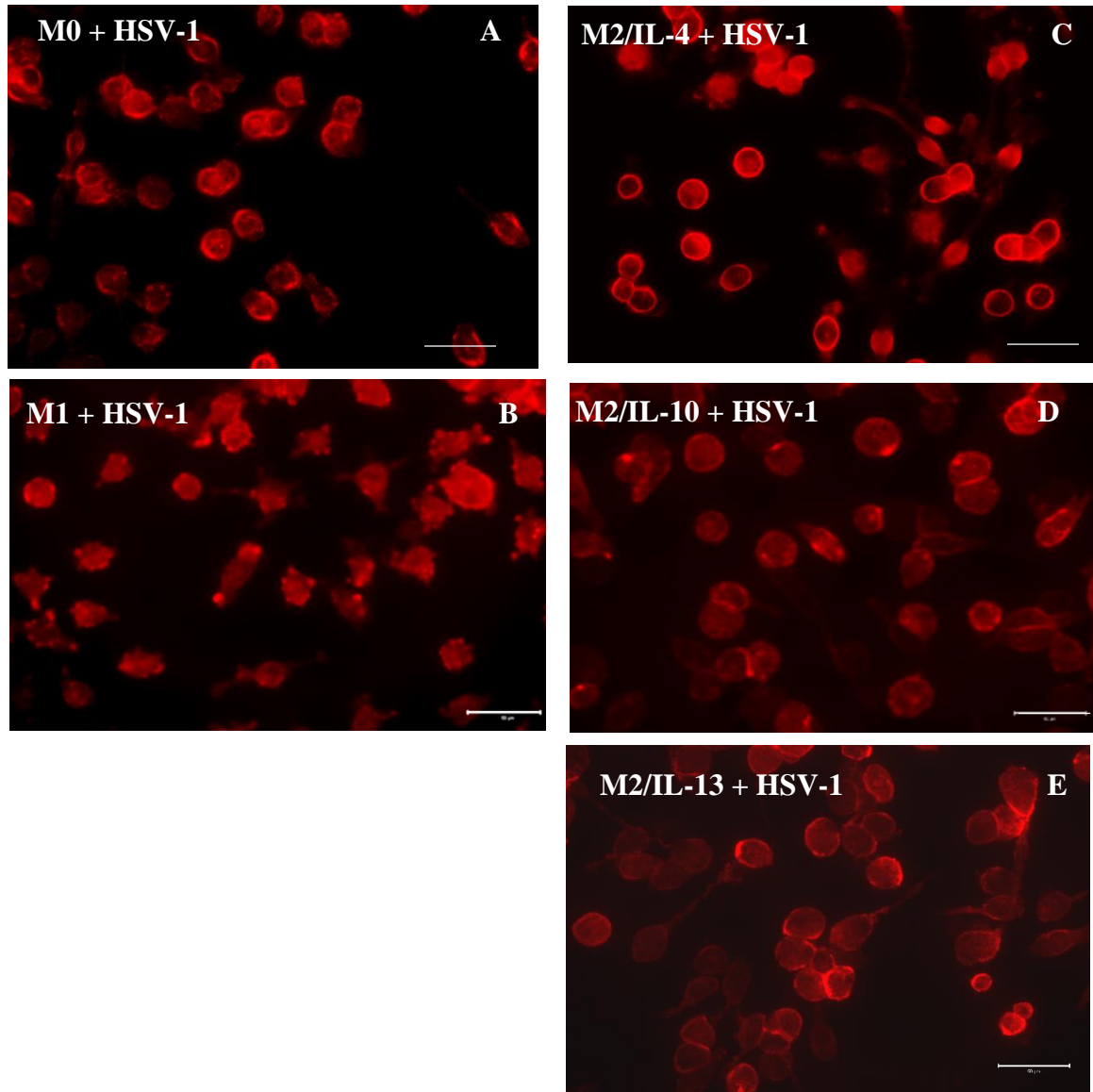
Figure 12: Changes in Cellular Morphology of Polarized and Un-polarized J774A.1 Macrophage 48 hours post- HSV-1 infection. A-E showed HSV-1 infected un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained with phalloidin (for actin arrangement), and then evaluated by immunofluorescence microscopy. A showed infected un-

polarized control (M0), B, infected polarized M1 macrophages by LPS and IFN-γ, and C- F

showed infected polarized M2 macrophages by IL-4, IL-10, and IL-13 respectively. Following

HSV-1 infection, all phenotypes became rounded and aggregated. (Scale bar =50μm).
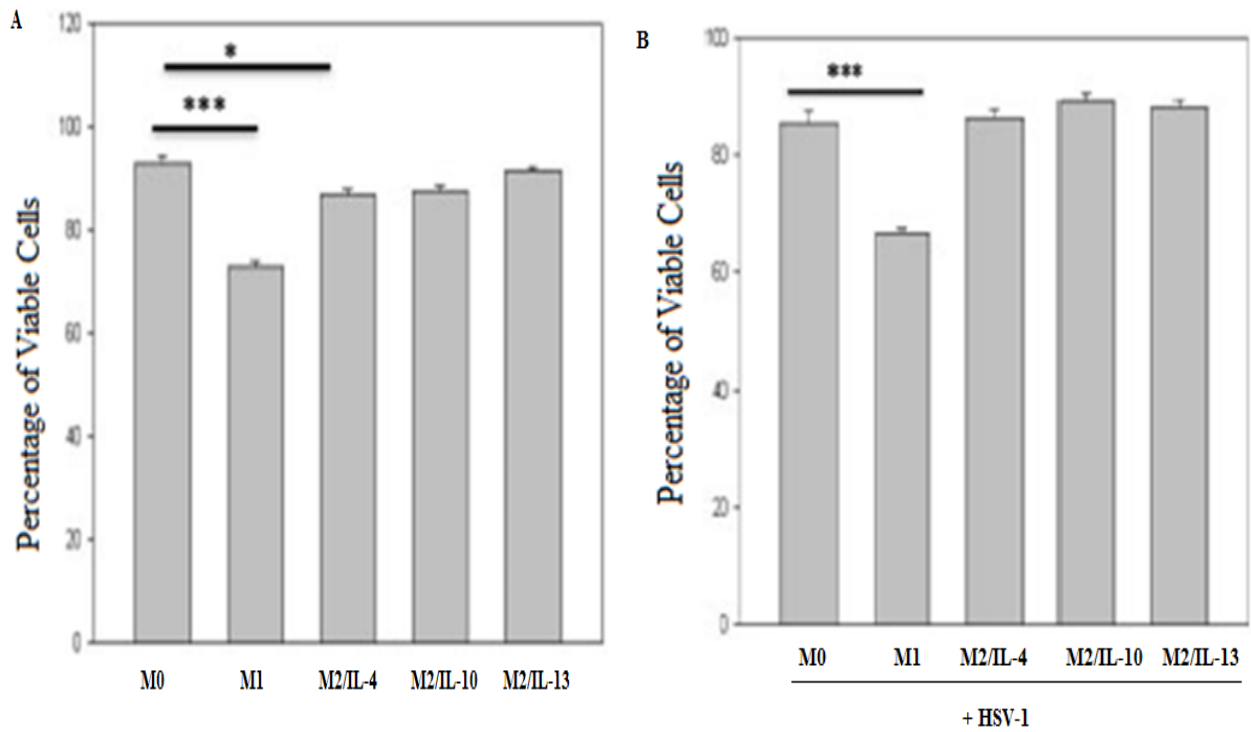
Figure 13: Cell viability of unpolarized (M0) and polarized macrophages (M1, M2) after 18 hours without HSV-1 infection (A) or with HSV-1 infection (B). Percentages of viable cells are shown as the mean and error bars represent standard error. These data represent the mean of three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.
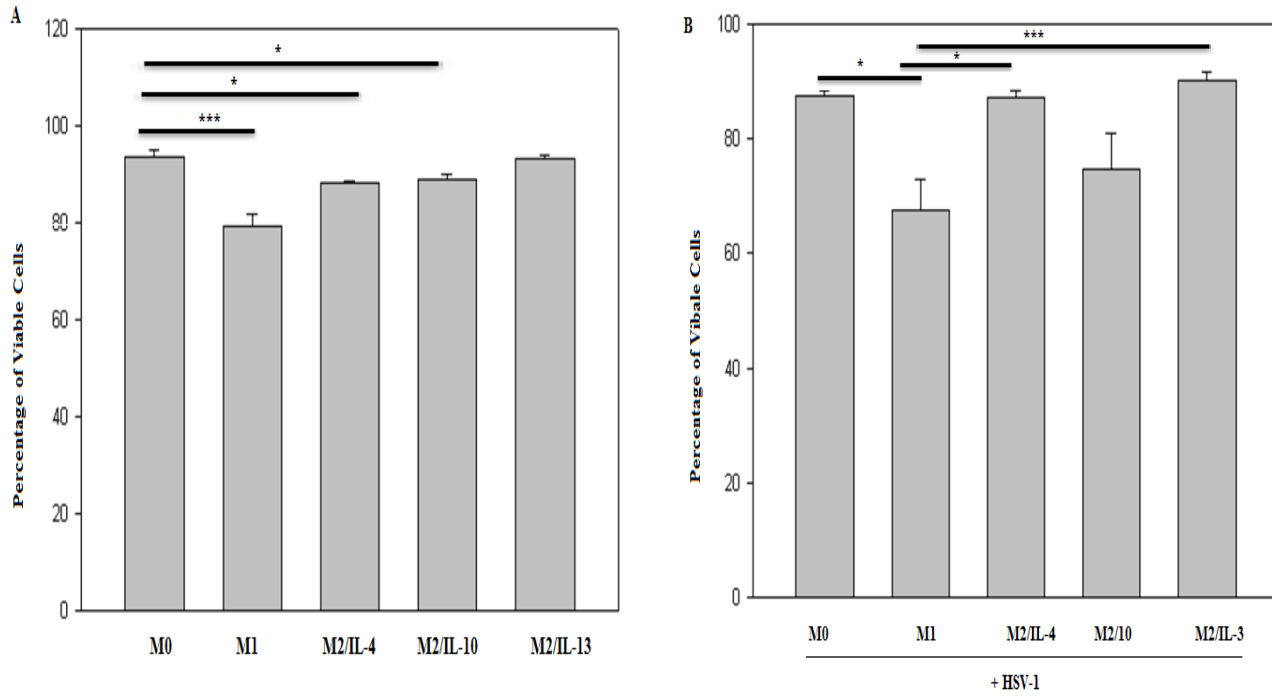
Figure 14: Cell viability of unpolarized (M0) and polarized macrophages (M1, M2) after 24 hours without HSV-1 infection (A) or with HSV-1 infection (B). Percentages of viable cells are shown as the mean and error bars represent standard error error. These data represent the mean of three independent experiments. *, p ≤0.05;***, p ≤0.001.
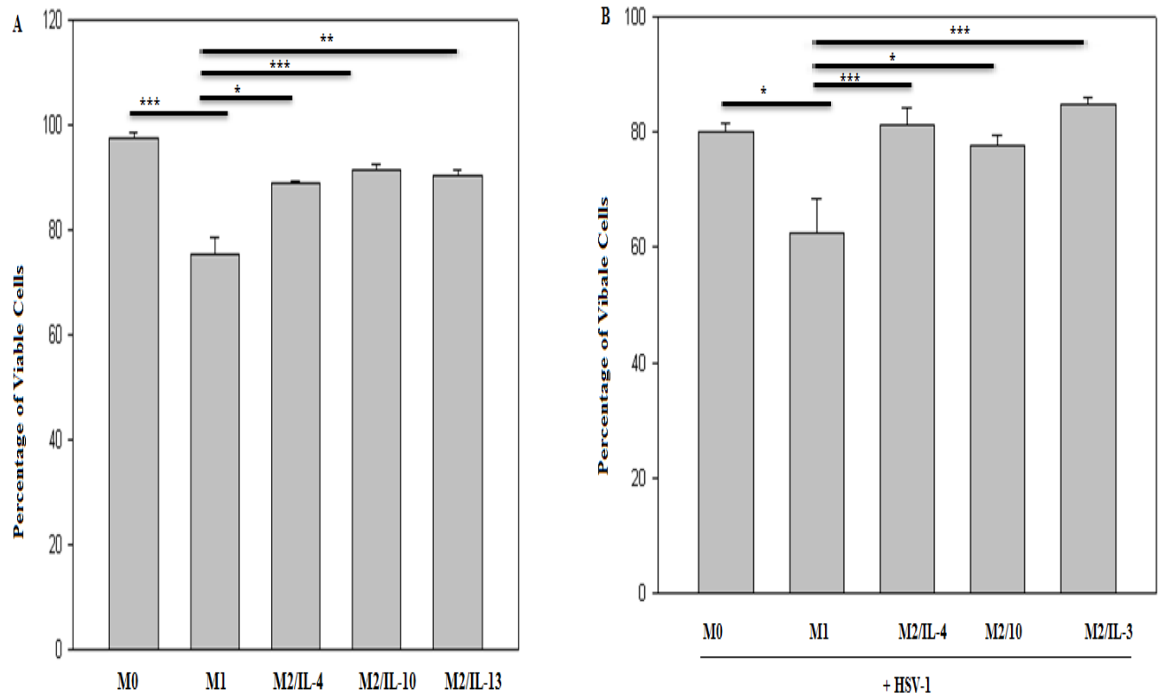
Figure 15: Cell viability of unpolarized (M0) and polarized macrophages (M1, M2) after 48 hours without HSV-1 infection (A) or with HSV-1 infection (B).Percentages of viable cells are shown as the mean and error bars represent standard error. These data represent the mean of three independent experiments. *, p <0.05; **,p< 0.010; ***, p <0.001.

Figure 16: Flow cytometry analysis of CD80 expression on the surface of J774.A1 murine cells. Histograms (A) and bar charts (B) show the expression cell surface markers CD 80 for control and polarized macrophages following 24 hours of cytokine treatment without HSV-1 infection. Red: negative isotype control; black: PE/CY5 -conjugated anti-mouse CD80 antibodies. These results were not statistically significant. There were no statistically significant differences between control M0 macrophages and M1 or M2 macrophages induced by IL-4, IL-10, or IL-13. Three independent experiments were performed with one representative experiment shown for histograms (A). Error bars represent standard error of three trials (B).

Figure 17: Flow cytometry analysis of CD80 expression on the surface of J774.A1 murine cells. Histograms (A) and bar charts (B) show the expression cell surface markers CD 80 for control and polarized macrophages following 24 hours of cytokine treatment with HSV-1 infection. Red: negative isotype control; black: PE/CY5 -conjugated anti-mouse CD80 antibodies. These results were not statistically significant. Three independent experiments were performed with one representative experiment shown for histograms (A). Error bars represent standard error of three trials (B).

Figure 18: CD 80 expression for control and polarized macrophages following 24 hours of cytokines pre- and post-HSV-1 infection. These results were not a statistically significant difference. Error bars represent standard error. Representative experiment of three trials is shown.
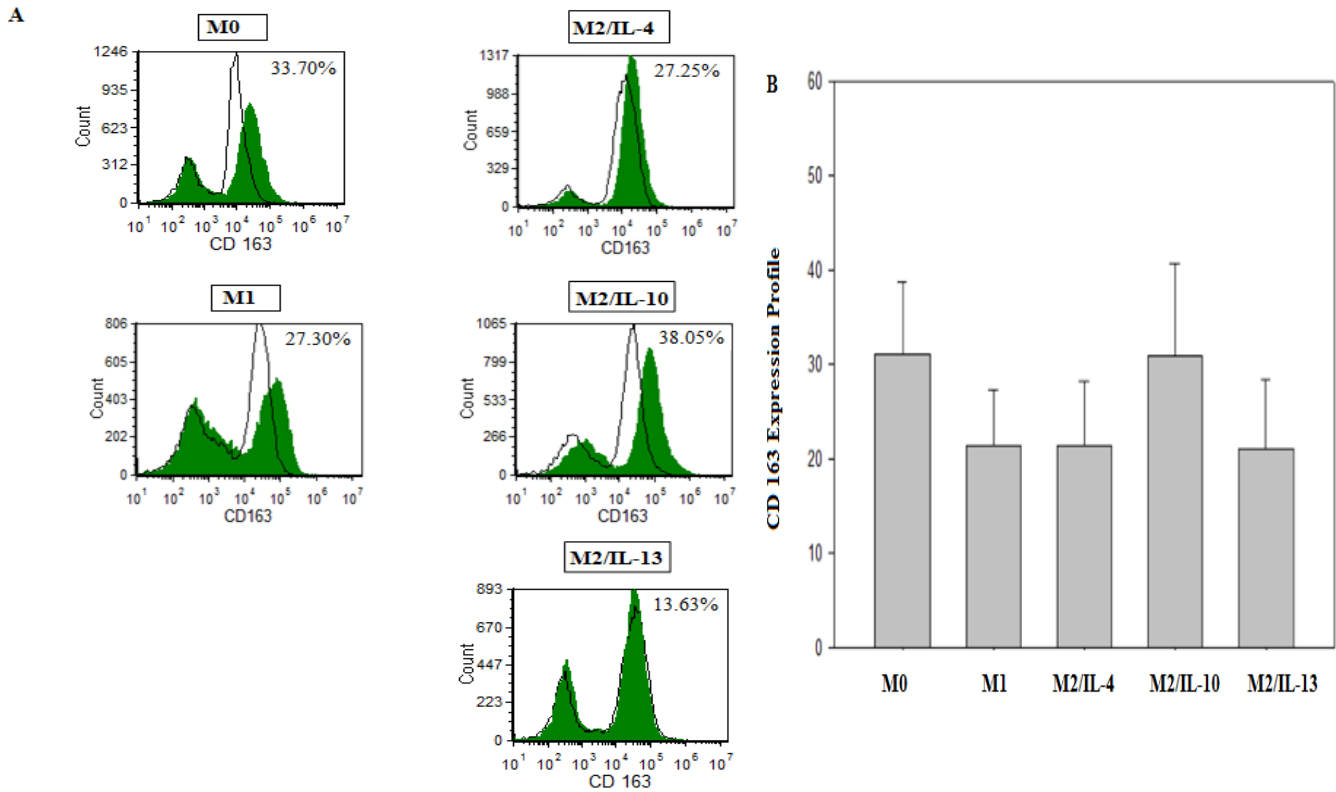
Figure 19 : Flow cytometry analysis of CD 163 expression on the surface of J774.A1 murine cells. Histograms (A) and bar charts (B) show the expression cell surface markers CD 163 for control and polarized macrophages following 24 hours of cytokine treatment without HSV-1 infection. Green: negative isotype control; black: FITC -conjugated anti-mouse CD 163 antibodies. There were not a statistically significant observed, when compared M0 control to M1 and M2 macrophages induced by IL-4, IL-10, or IL-13. Three independent experiments were performed with one representative experiment shown for histograms (A). Error bars represent standard error of three trials (B).
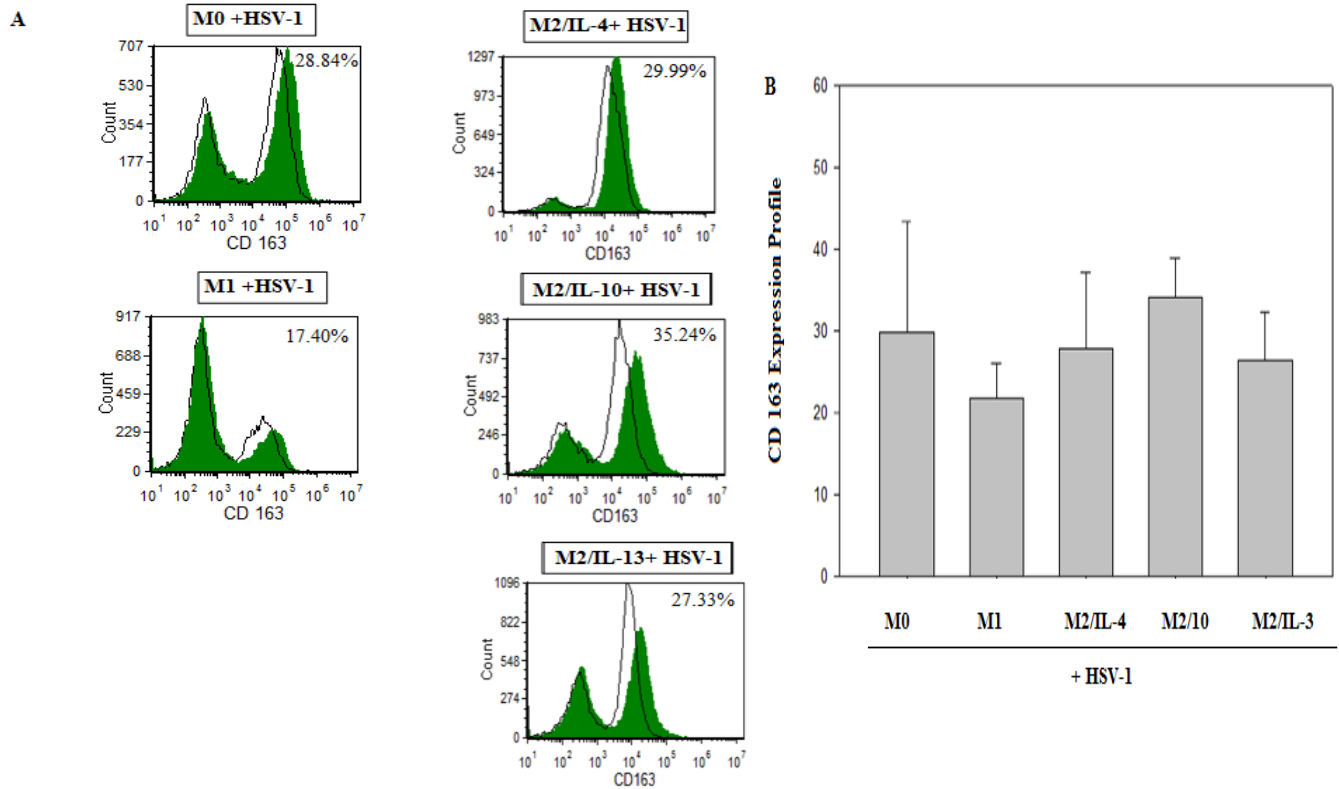
Figure 20: Flow cytometry analysis of CD 163 expression on the surface of J774.A1 murine cells. Histograms (A) and bar charts (B) show the expression cell surface markers CD 163 for control and polarized macrophages following 24 hours of cytokine treatment with HSV-1 infection. Green: negative isotype control; black: FITC -conjugated anti-mouse CD 163 antibodies. These results were not statistically significant. Three independent experiments were performed with one representative experiment shown for histograms (A). Error bars represent standard error of three trials (B).
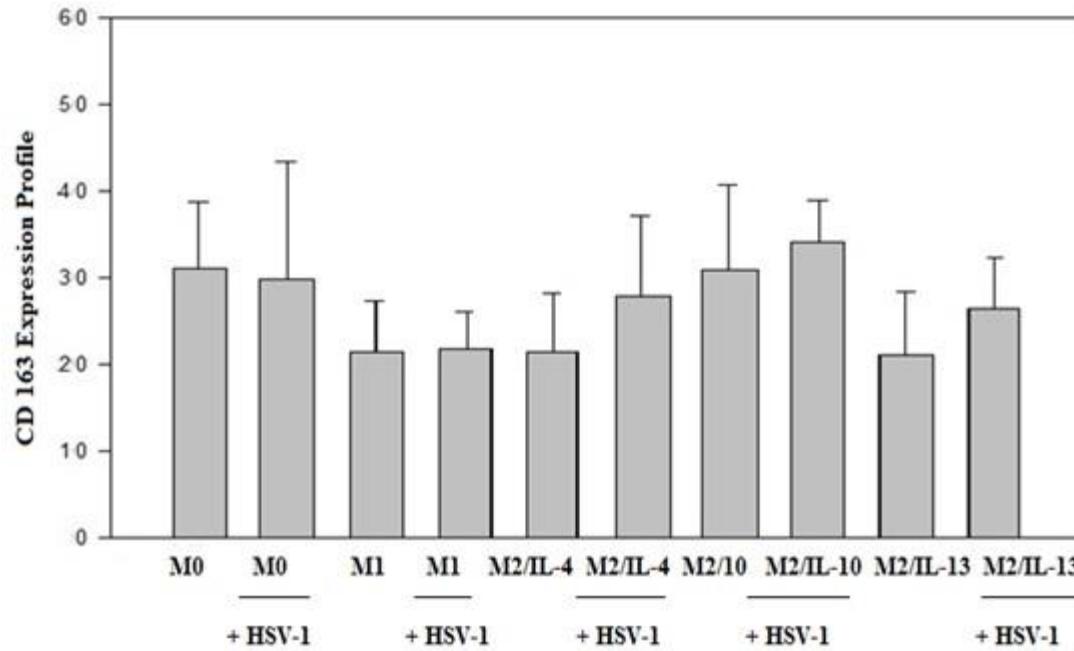
Figure 21: CD 163 expression for control and polarized macrophages following 24 hours of cytokines pre- and post-HSV-1 infection. No statistically significant observed, when compared M0 control to M1 and M2 macrophages induced by IL-4, IL-10, or IL-13. Error bars represent standard error. The experiments were repeated three times.
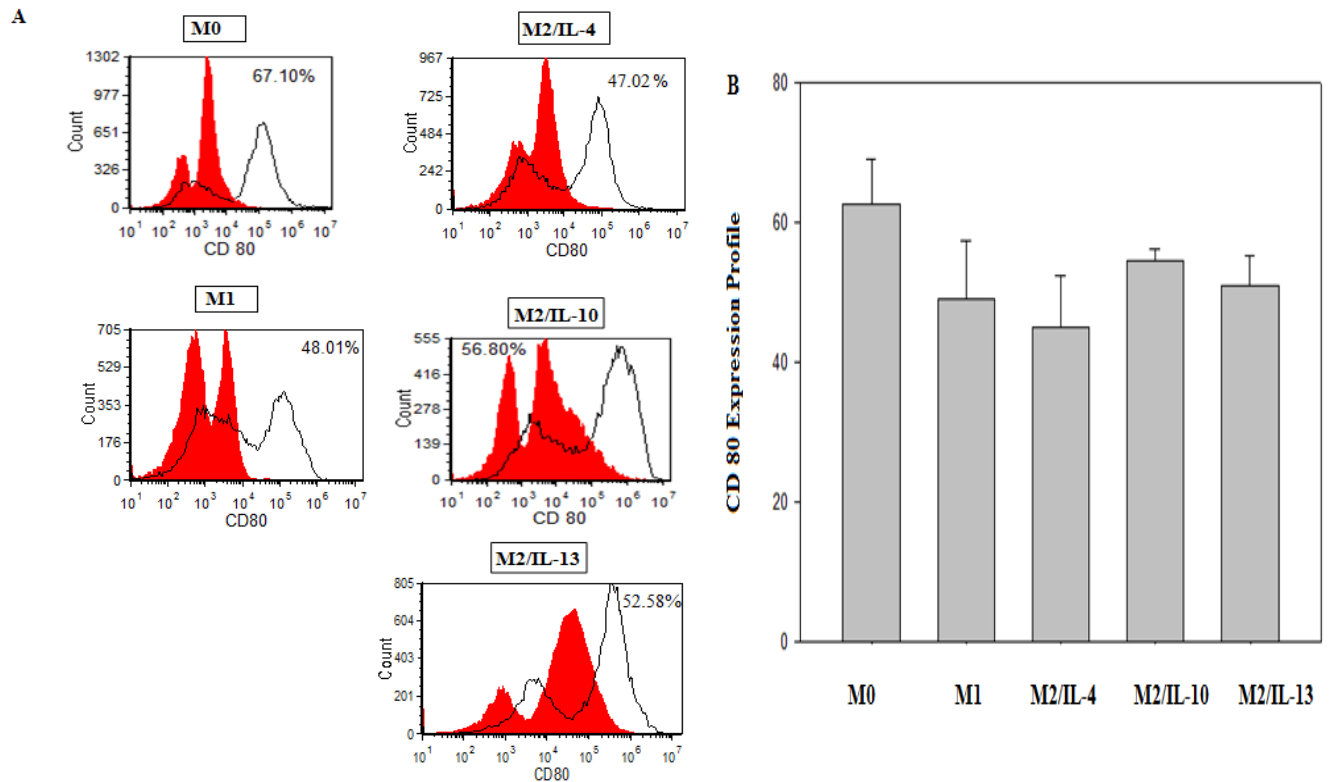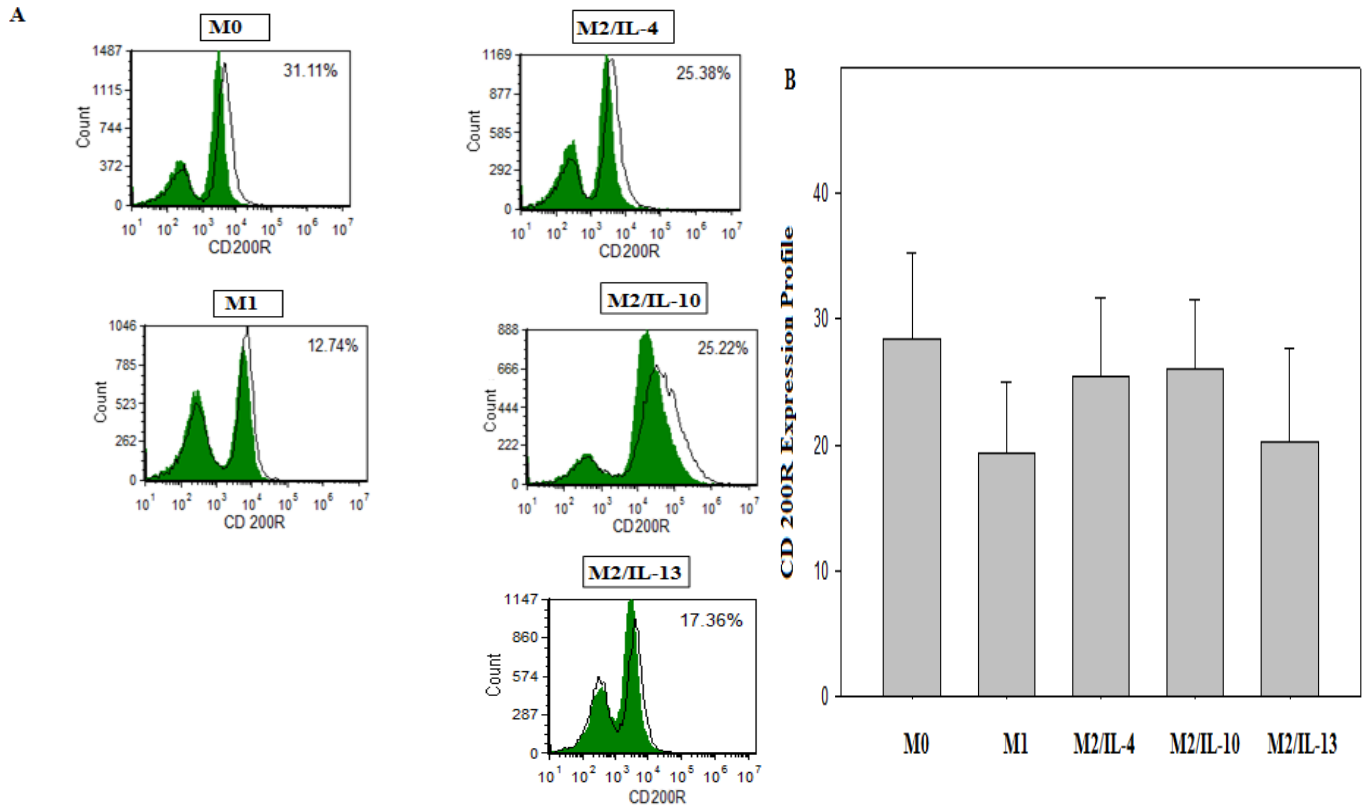
Figure 22: Flow cytometry analysis of CD 200R expression on the surface of J774.A1 murine cells. Histograms (A) and bar charts (B) show the expression cell surface markers CD 200R for control and polarized macrophages following 24 hours of cytokine treatment without HSV-1 infection. Green: negative isotype control; black: FITC -conjugated anti-mouse CD 200R antibodies. These results were not a statistically significant difference. Three independent experiments were performed with one representative experiment shown for histograms (A). Error bars represent standard error of three trials (B).
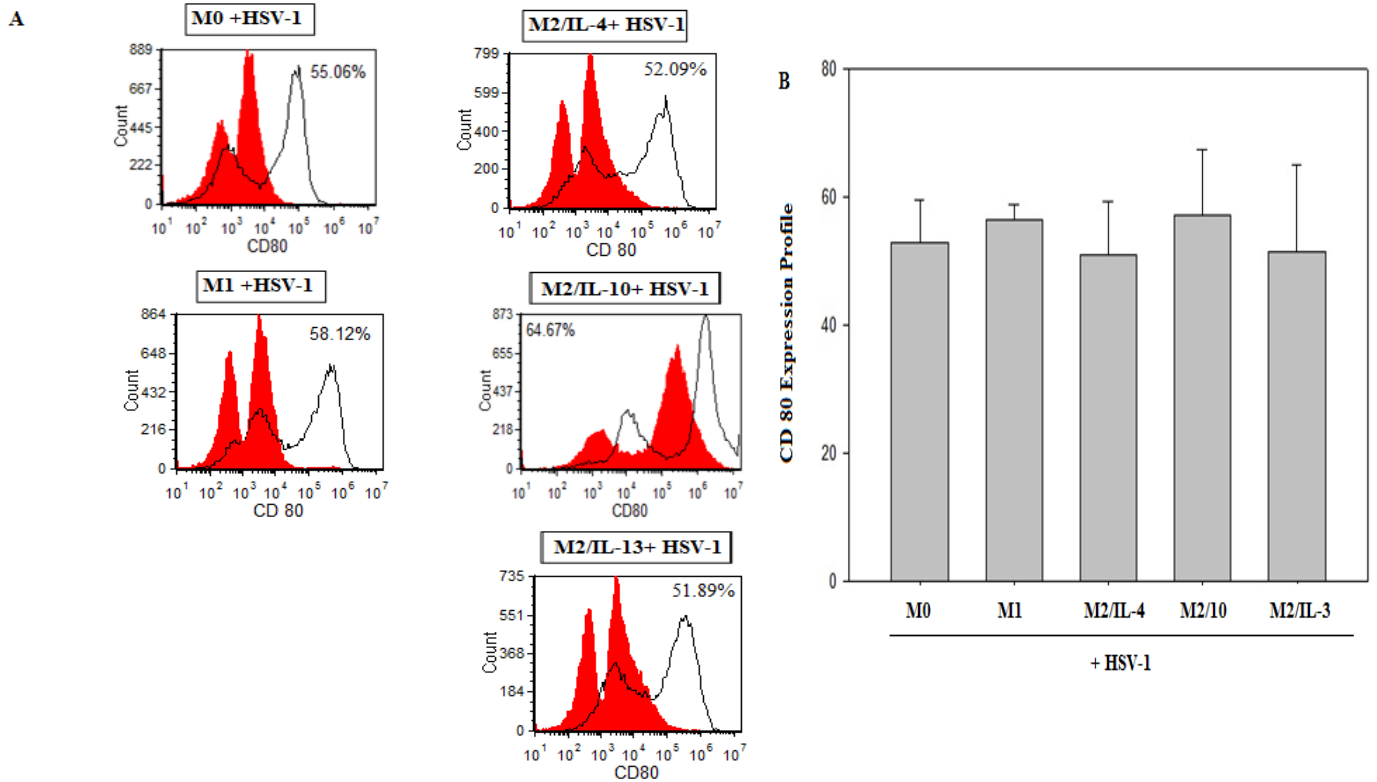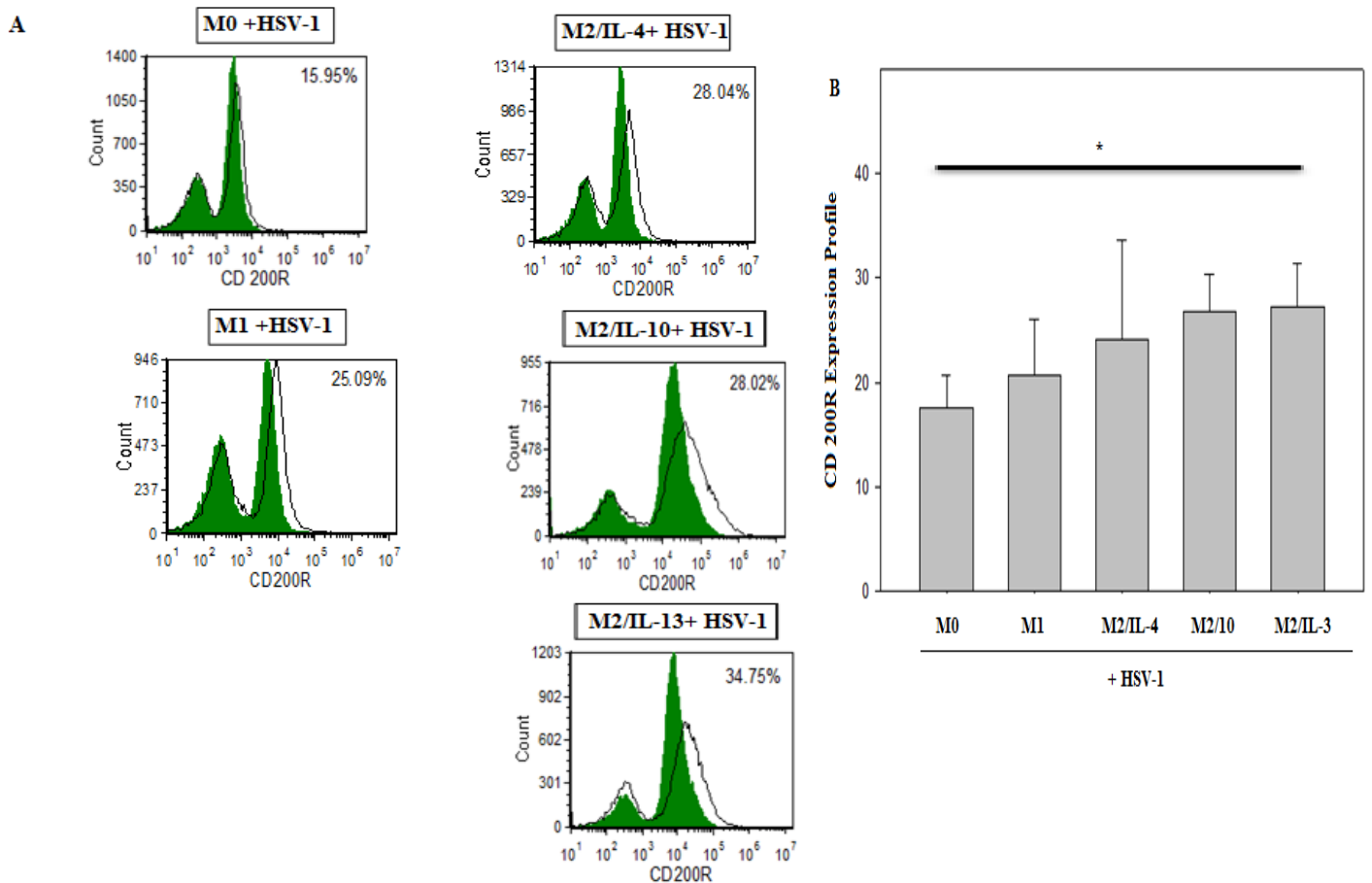
Figure 23: Flow cytometry analysis of CD200R expression on the surface of J774.A1 murine cells. Histograms (A) and bar charts (B) show the expression cell surface markers CD200R for control and polarized macrophages following 24 hours of cytokine treatment with HSV-1 infection. Green: negative isotype control; black: FITC -conjugated anti-mouse CD200R antibodies. Infected M2 induced by IL-13 (*,$p<0.05$) showed a statistically increase in CD200R expression level compared to infected M0 control cells. However, other macrophages population were not statistically significant. Three independent experiments were performed with one representative experiment shown for histograms (A). Error bars represent standard error of three trials (B).
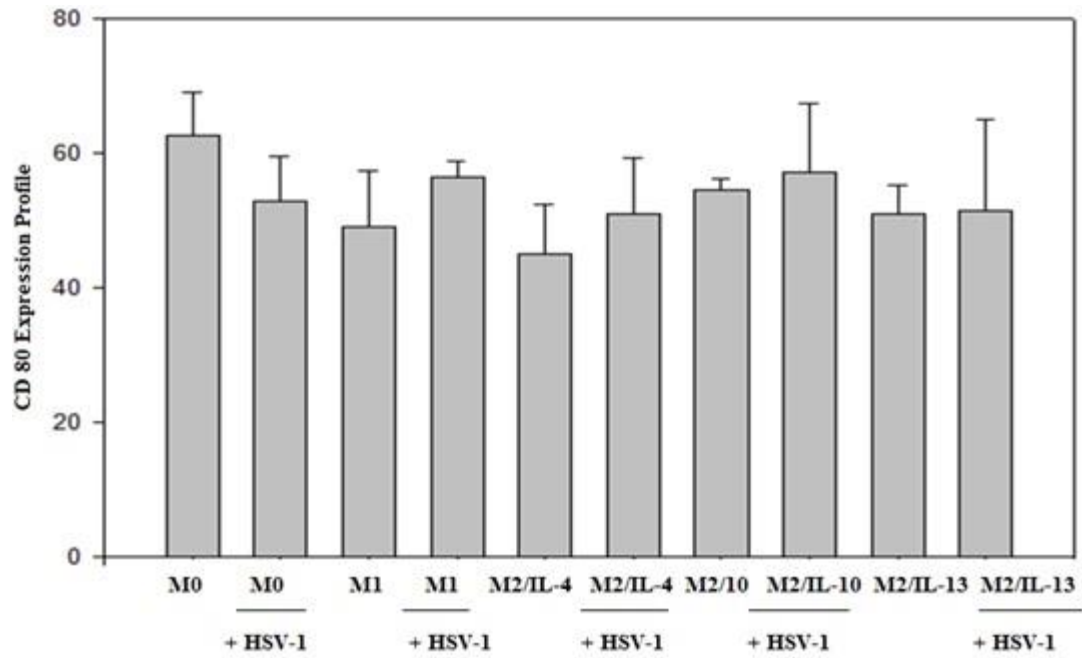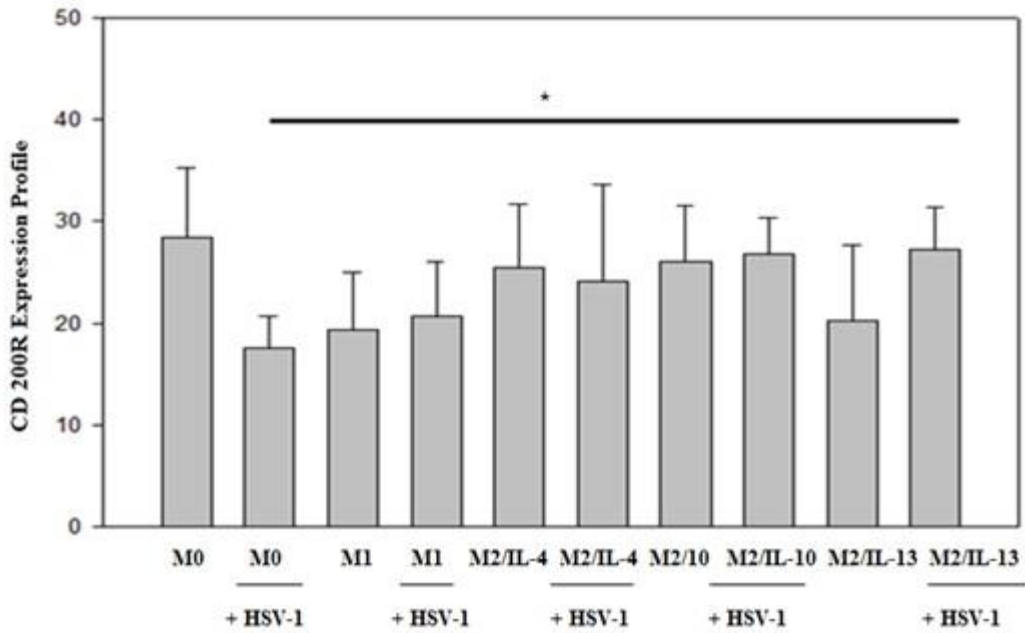
Figure 24: CD200R expression for control and polarized macrophages following 24 hours of cytokines pre- and post-HSV-1 infection. There was a statically significant increase in infected M2 macrophages induced by IL-13 (*,p<0.05) compared to infected M0 control cells. No statistically significant observed, when compared M0 control to M1 and M2 macrophages induced by IL-4 or IL-10. Error bars represent standard error. The experiments were repeated three times.

Table 4: Summary of CD makers' expression on the un-polarized and polarized macrophages pre-

and post- HSV-1 infection

CD Markers Expression	- HSV-1 or +HSV-1	M0	M1	M2/IL-4	M2/IL-10	M2/IL-13
CD80 expression: Unchanged	**-HSV-1**	(63%)	NS reduced (~45%)		NS Slightly reduced (~55%)	
	+HSV-1	(53%)	No difference (~56%)			
	HSV-1 infected cells compared to uninfected	NS slightly reduced	No difference			
CD163 expression: Unchanged	**- HSV-1**	(31%)	NS slightly reduced (~22%)		No difference (31%)	NS Slightly reduced (21%)
	+HSV-1	(29%)	NS reduced (21%)	NS minor reduced (26%)	NS minor increased (34%)	NS minor reduced (26%)
	HSV-1 infected cells compared to uninfected	No difference		NS slightly increased	No difference	NS increased
CD200R expression: changed	**-HSV-1**	(28%)	NS Slightly reduced (19%)	No difference (~26%)		NS Slightly reduced (20%)
	+HSV-1	(17%)	NS slightly increased (21%)	NS increased (~24%)		**increased p<0.05 (27%)**
	HSV-1 infected cells compared to uninfected	NS reduced	No difference			NS up-regulated

* NS: Non Significant
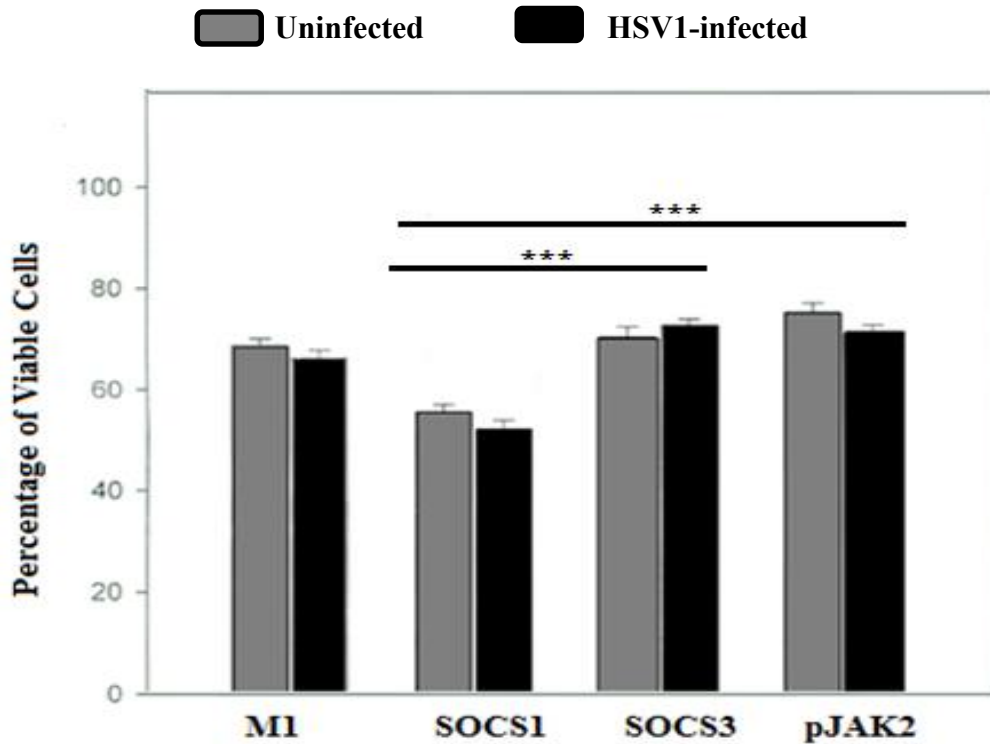
Figure 25: Effects of SOCS1, SOCS3 Peptide Mimetics, and pJAK2 (SOCS1 Inhibitor) on cell viabilities of polarized M1 J774A.1 macrophages. Polarized M1 macrophages are represented as gray bars, and HSV1-infected macrophages are represented in black. ***,p<0.001.

Table 5: Comparisons ratios (pg/ml) of <u>inflammatory cytokine</u> produced in 2 experiments by J774A.1 cells 24 hours after polarization.

Cytokine produced	IL-1β*	IL-6	TNF-α	IL-10
M1 vs. M1 SOCS1	1:1	1:1	5:1	1:2
M1 vs. M1 SOCS3	1:1	33:1	7:1	1:2
M1 vs. M1 pJAK2	1:1	26:1	6:1	1:2

*IFN-γ used to polarized M1 cells inhibits IL-1β expression (Eigenbrod et al. 2013
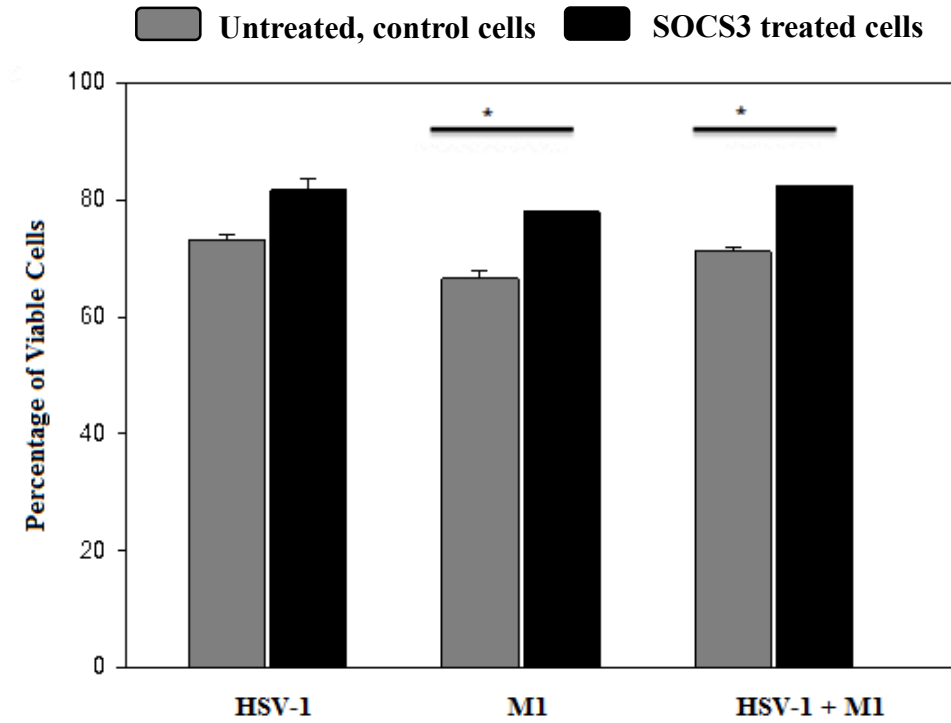
Figure 26: Cell viabilities of RAW 264.7 macrophages 24 hours after M1 polarization. SOCS3 treated cells (black bars) were compared to the untreated, control cells (gray bars). (*,p<0.05).
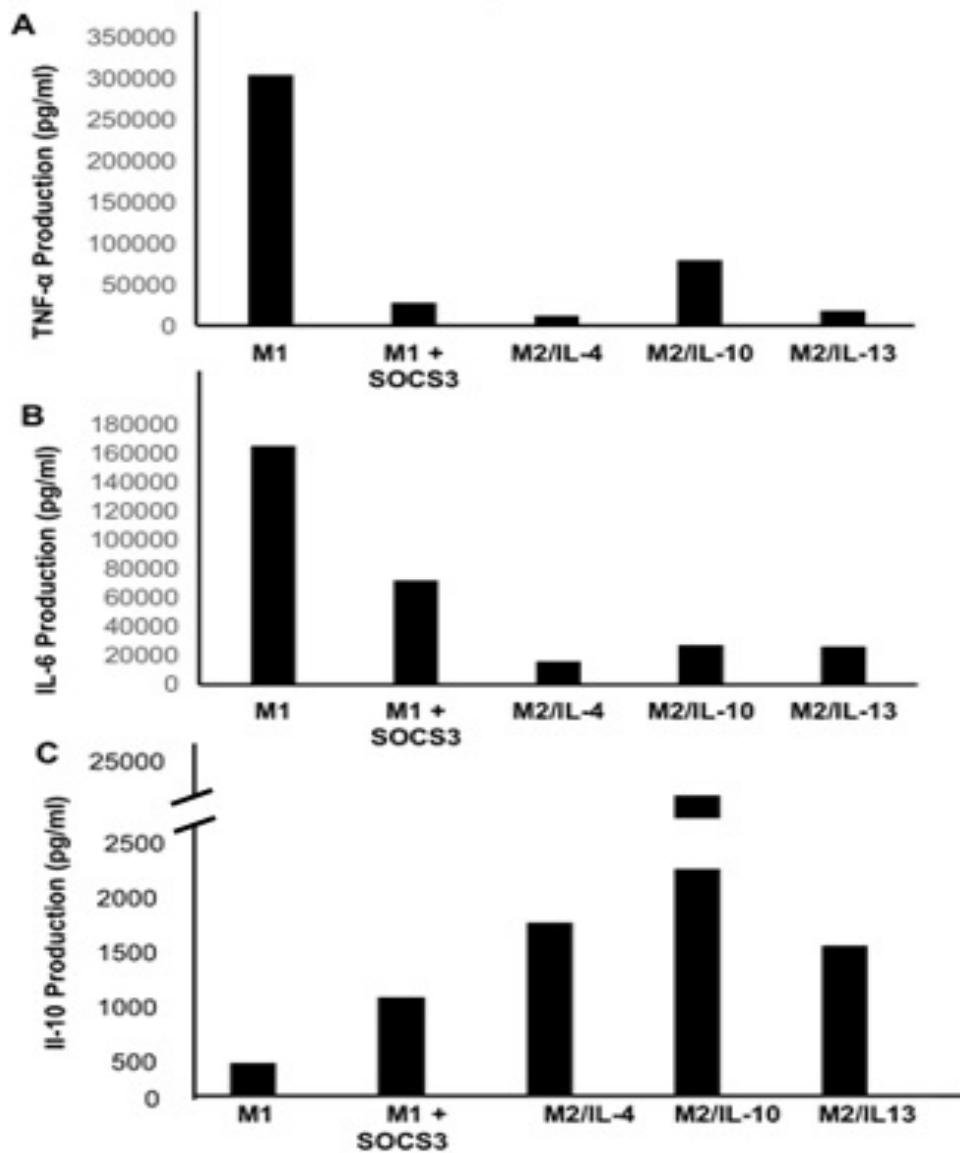
Figure 27: Production of pro-inflammatory A) TNF-α, B) & IL-6 C) and anti-inflammatory IL-10 in 2 experiments by RAW264.7 cells 24 hours after polarization. M1 cells vs M1 cells treated with SOCS3 mimetic vs IL-4, IL-10, and IL-13 polarized M2 cells.
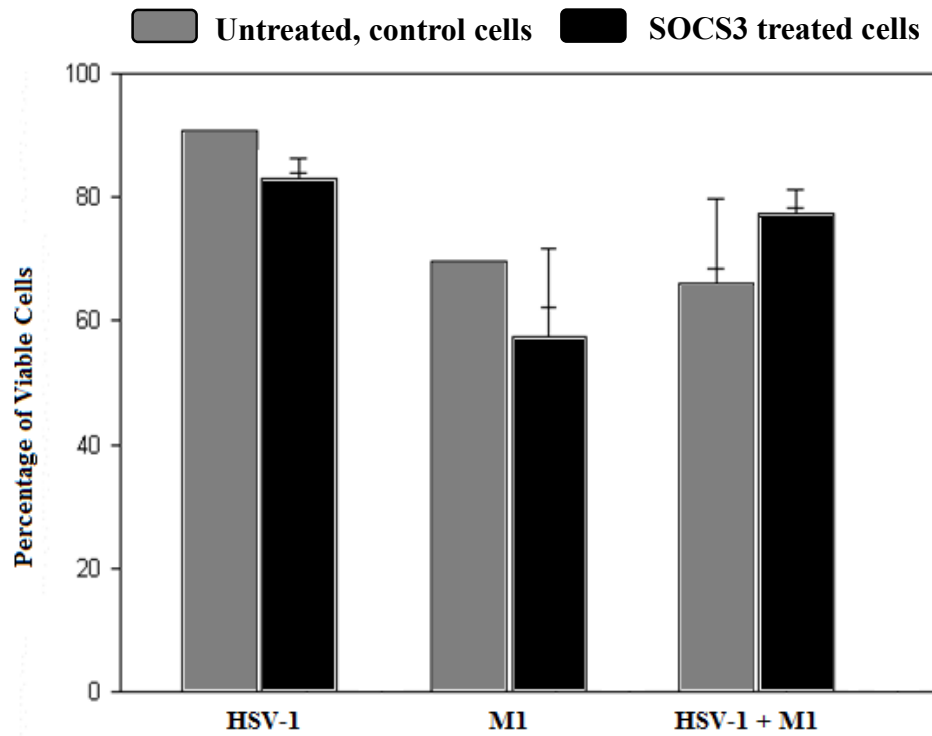
Figure 28: Cell viabilities of J774A.1 macrophages 24 hours after M1 polarization. SOCS3 treated cells (black bars) were compared to the untreated, control cells (gray bars).

DISCUSSION

Macrophage populations are a dynamic group of cells that vary in both phenotype and function depending on the environmental signals and stimuli. Different macrophages can be distinguished by specific molecules expressed on their surface, e.g. CD markers, and will polarize into M1 and M2 phenotypes upon microenvironmental stimulation. This research confirms the findings of a previous study done by Reichard (2012) who found that J774 murine macrophages cells exhibit diversity in cell morphology, cell viability, and cell surface proteins 24 hours following IL-4 or IFN-γ and LPS treatment and /or HSV-1 infection. The two hypotheses around which this research project was built are: 1) There will be differences in various macrophage subtypes in cell morphology, viability, and surface marker expression (CD80, CD163 and CD200R) between M1 and M2 macrophages induced by different stimuli, and 2) the SOCS3 peptide mimetic will alter the cytotoxicity associated with the M1 phenotype or HSV-1 infection.

To test cell morphology hypothesis, microscopy was first performed and images of cells were evaluated. Treatment with IFN-γ/LPS induced J774A.1 macrophage cells from an M0 state to an M1 phenotype, while IL-4, IL-10, or IL-13 induced macrophages from an M0 state to an M2 phenotype. After 18 hours, uninfected M1 macrophages appeared flattened, extended, irregularly shapes, and contained visible intracellular vacuoles and were more strongly adherent compared to M0 and M2 cells. M0 appeared rounded or elongated and M2 macrophages induced by IL-4 and IL-13 showed some degree of cell elongation with the majority of cells appearing rounded. M2 macrophages induced by IL-10 were rounded at 18, 24, and 48 hours, with no evidence of elongation. After 24 and 48 hours of polarization treatment, M1 macrophages demonstrated the same morphological changes described above, although the irregular shape and intracellular vacuoles were more pronounced. Uninfected M1 macrophages could be distinguished from

uninfected M2 macrophages by morphology, while uninfected M2 macrophages induced by either IL-4 or IL-13 could not be differentiated from each other.

These findings agree with those reported in a study by McWhorter et al. (2013) where changes in cellular morphologies in a bone marrow-derived macrophage (BMDMs) cell line were observed when treated with either LPS and INF-γ or IL-4 and IL-13 for 24 hours induced M1 and M2 macrophages, respectively. Similarly, a study by Sheng et al. (2011) found that LPS and IFN-γ treated mouse BV-2 microglial cells and primary microglia displayed alterations in cell shape and increases in cell death (determined using MTT assay). These effects also correlated with the induction of NO after 24 hours of treatment, suggesting that the cytotoxicity was a result of NO induction (Sheng et al., 2011). IFN-γ and TNF-α were also found to stimulate cell death in M1 macrophages (Kyoungho et. al., 2001). The present study also found that cell viability following polarization at different time points was significantly reduced for M1 macrophages compared to M0 macrophages; TNF-α expression was increased although NO was not measured. Additionally, the decrease in viability in M1 macrophages occurred regardless of whether the macrophages were uninfected or infected with HSV-1. M2 macrophages, regardless of method of induction, demonstrated minor, although insignificant, reductions in viability compared with M0 macrophage.

Differences in J774A.1 macrophages cell morphology were noticed between uninfected, polarized cells and uninfected, unpolarized cells, but not between HSV-1-infected M0, M1 and M2 macrophages that all became rounded and aggregated with viral infection, as described by Reichard (2012). These virus-induced morphological changes made it impossible to distinguish between M1 and M2 phenotypes on the basis of morphology alone, since infected M1 and M2 cells appeared identical. However, infected M1 macrophages displayed a different morphology

compared to that of an uninfected M1 macrophages. Uninfected M1 cells were flattened, displayed extended irregular shapes, and contained intracellular vacuoles; HSV-1 infected M1 macrophages were rounded. These morphological changes are likely a result of alterations in the actin cytoskeleton that occur during the normal virus life cycle (Bigley, 2014; Reichard, 2012). Overall, these results for 18 and 48 hours are in agreement with the observations at 24 hours made by Reichard (2012). Additionally, Li et al. (2005) noticed that an HSV-1 infected human cell lines displayed few rounded cells 4 hours post-infection while almost all the cells became rounded with 1 to 3 days of infection. Cell viabilities were further reduced in HSV-1 infected M0 and M1 macrophages, as well as in infected M2 macrophages (induced by IL-4, IL-10, and IL-13) after 18, 24, and 48 hours compared to uninfected polarized macrophages. These observations suggest that the effects of HSV-1 infection supersede the effects of polarization, and that the viral infection itself causes cytotoxicity and changes in cell morphology that are indistinguishable among the macrophage phenotypes (i.e., M0 vs. M1 vs. M2).

A study by Ambarus et al. (2012) used flow cytometry and quantitative RT-PCR to identify CD markers following incubation of human monocyte-derived macrophages without or with cytokines IFN-γ, IL-4, and IL-10 for 4 days. They found that CD80 expression levels were specific (i.e., highest expression compared to other polarized macrophage populations) to M1 macrophages exposed to IFN-γ; however, CD163 and CD200R were up-regulated in M2 macrophages induced by IL-4 and IL-10, respectively. Similarly, IL-13 up-regulated CD200R. CD80 expression was slightly, but not significantly, down-regulated on uninfected M1 macrophages and M2 macrophages activated by IL-4, IL-10, and IL-13 compared to uninfected M0 macrophages. Based on these observation, we expected to observe up-regulation of CD80 expression on M1 murine macrophages and down regulation of CD80 in M2 subsets. Our's was an unexpected result given

that CD80 is antigen stumilation co- factor as is CD86. CD86 expression was up-regulated in uninfected M1 polarized J774A.1 macrophages compared to uninfected M2 polarized cells (Reichard, 2012). Infected M1 macrophages and M2 macrophages stimulated by IL-10 also exhibited a minor increase in CD80 expression.

Reichard (2012) also found that CD86 expression was reduced in HSV-1 infected J774A.1 macrophages, while this study found that CD80 expression was slightly, but not significantly increased in infected J774A.1 cells. While one study using *Toxoplamsa gondii* had found that monocytes quickly up-regulated CD80 and CD86 early during infection (Subauste et al., 1998), others have found that expression of CD80 is difficult to associate with CD86, since the latter is expressed first and at greater levels than CD80 (Lenschow et al., 1994). Therefore, the link between the CD86 observed by Reichard (2012) cannot predict or assume the presence of CD80.

CD163 is used as a marker for the M2 phenotype. IL-10 was found to cause an up-regulation of CD163 on M2 human macrophages (Ambarus et al., 2012) while IFN-γ and LPS were found to down-regulate CD163 expression (Buechlelr et al., 2000). Based on these observation, we predict CD163 expression will be up-regulated on M2 murine macrophages induced by IL-10 and down regulated on M1 macrophages. There was an increase in the level of CD163 in infected M2 macrophages compared to uninfected M2 macrophage cells. Further, a greater abundance of CD163 was detected on infected M2 macrophages induced by IL-10 compared to infected M2 macrophages induced by IL-4 and IL-13 (not statistically significant). This increase may be a result of the ant-inflammatory response of M2 cells associated with HSV-1 infection. This is suggested based on the fact that CD163 expression was increased on the surface of monocytes of human immunodeficiency virus (HIV) patients, indicating that it may play a role in the immune response to limit inflammation (Tippett et al., 2011). Conversely, reducing the

levels of CD163 limits the anti-inflammatory effects, resulting in pro-inflammatory conditions that help to destroy virus infected cells (Tuluc et al., 2014). A reduction of CD163 expression on M1 macrophages uninfected or infected with HSV-1 indicates that CD163 is a marker for M2 macrophages (although not significantly different), specifically in M2 macrophages induced by IL-10. A recent study done by Jaguin et al, 2013 indicated that CD206 macrophage mannose receptor marker expression on human macrophages was not able to discriminate between M1 and M2 populations, similar to observations in murine RAW264.7 cells (Reichard, 2014).

We did not observe the expected results. This may have been due to limited sampling numbers, species-dependent differences (human vs. mice), sensitivity of technique/approach, and sub-culturing of macrophage cell lines that may have led to the loss of gene expression and/or impaired immune function. Therefore, increasing the sample number and isolating fresh cells may have helped to achieve statistically significant results.

The third CD marker evaluated in this study, CD200R, an immune-inhibitory receptor to prevent inflammatory macrophage activation (Hoek et al., 2000). Similar to CD163, CD200R expression induces an anti-inflammatory response that helps to control pro-inflammatory responses by limiting pro-inflammatory cytokine secretion (Jenmalm et al., 2006). We expected to observe up-regulation of CD200R expression on M2 murine macrophages induced by IL-4 or IL-13. In this study, the only statistically significant difference in CD200R expression occurred between HSV-1 infected M0 and HSV-1 infected M2 macrophages induced by IL-13 (17% versus 28% of the population, respectively). Increasing the levels of CD200R expression in M2 RAW264.7 macrophages may halt the pro-inflammatory signaling pathway (Albeshri, 2014). Ambarus et al. (2012) showed that CD200R expression was specific for M2a macrophages induced by either IL-4 or IL-13. When human cells are stimulated with IL-4, IL-13, or IL-10 for 3 days *in*

vitro, an increase (not significant in the case of IL-10) in CD 200R expression occurred with even greater expression of CD200R after 7 days infection by *T. crassiceps* (Koning et al., 2010). The data described here are also in agreement with the observations of Jaguin et al., (2013) that reported that CD200R expression was unique to the M2 polarized human macrophages treated with IFN-γ, LPS and IL-4 also used in this study. These CD markers are summarized in (Figure 29).
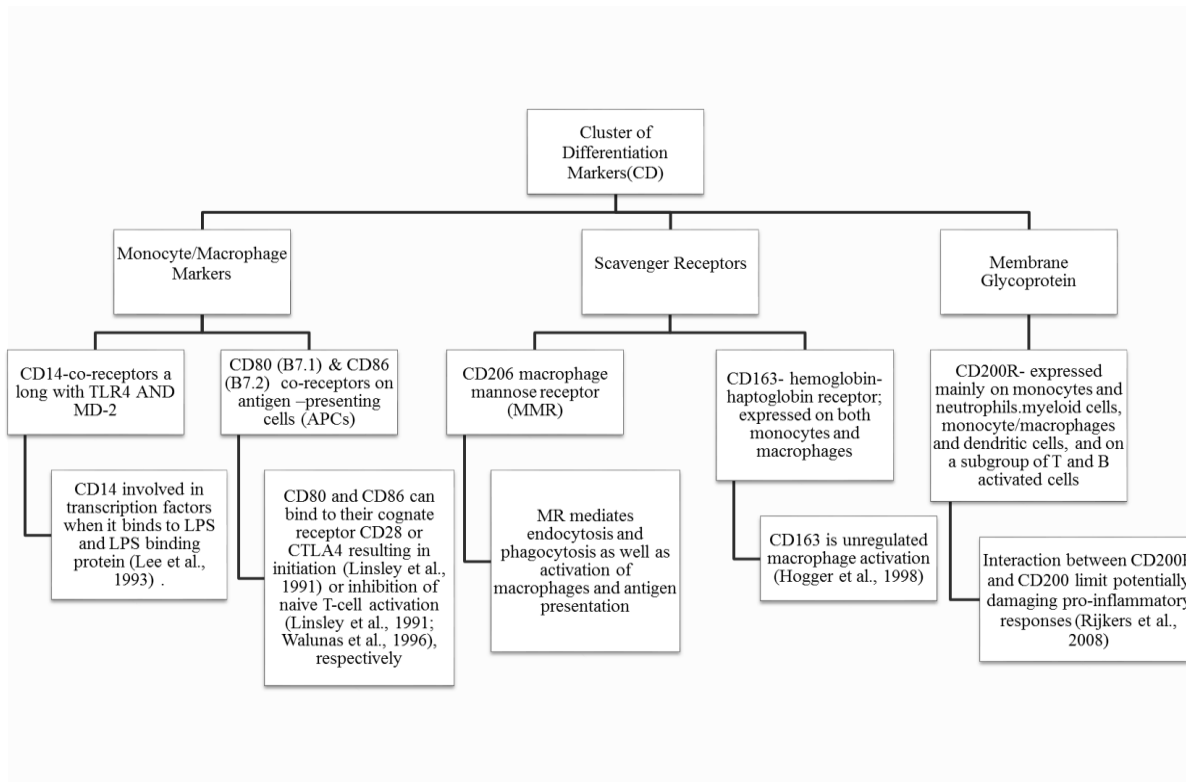
Figure 29: Summary of the CD markers examined in this study (CD80, CD163, CD200R) and that of Reichard (2012) (CD14, CD206, CD86).

SOCS proteins work as feedback inhibitors for cytokines signaling through the JAK/STAT pathway that regulates inflammatory responses in macrophages (Yoshimura *et al*., 2005). Reichard (2012) reported that uninfected J774A.1 M1 cells expressed higher levels of SOCS1 than SOCS3 with a SOC1/SOCS3 ratio of 7:1 at 24 hours after polarization. HSV-1-infected M1 cells expressed a SOCS1/SOCS3 ratio of 1:1 while M2-infected cells exhibited a SOCS1/SOCS3 ratio

of 1:2. SOCS3 represses the M1 pro-inflammatory murine macrophage phenotype, reducing the macrophage inflammatory responses (Qin and others 2012a). Qasimi *et al*. (2006) showed that SOCS3 protein mediates IL-10 inhibition of TNF-α and nitric oxide production. IL-10 was responsible for the anti-inflammatory response to *Borrelia burgdorferi* in this same macrophage cell line (J774A.1) (Dennis *et al*., 2006). Based on these published studies, we hypothesized that the HSV-1-infected J774A.1 M1 macrophages were attempting to counteract the effects of inflammatory molecules induced by polarization by producing SOCS3.

Cell viabilities of polarized M1 and HSV-1-infected M1 J774A.1 macrophages were determined following treatment with SOCS1 and SOCS3 peptide mimetics and pJAK2 for 24 hours. The SOCS3 peptide mimetic and pJAK2 peptide mimetic resulted in increased viability of polarized M1 macrophages compared with SOCS1 treated cells. Production of TNF-α and IL-6 was reduced in polarized M1 macrophage treated with SOCS3 or pJAK2 peptide mimetic compared with polarized M1 untreated cells. There were no differences in the level of IL-1β produced between M1 untreated and M1 treated with either SOCS1, SOCS3, or pJAK2. Eigenbrod *et al*. (2013) provided an explanation for the lack of IL-1 β production. They showed that M1 cells induced by IFN-γ and LPS inhibited IL1β production. This observation correlates well with present results in M1 cells (both RAW and J774A.1) treated with IFN-γ and LPS. IL-10 levels increased two-fold, indicating an anti-inflammatory effect. The same result was observed by Qin et al. (2012) where M1 cells with SOCS3 showed a decrease in the production of pro-inflammatory cytokines TNF-α, IL-6 and an increase in the anti-inflammatory IL-10 compared to cells that were deficient in SOCS3. The anti-inflammatory effect of SOCS3 is further supported by Jo et al. (2005) who used a recombinant cell-penetrating form of SOCS3 (CP-SCS3) to protect mice (C3H/HeJ) from the lethal effects of *Staphylococcus* enterotoxin B and LPS. Within 2 hours after injection, CP-

SOCS3 was distributed in multiple organs, persisted for at least 8 hours, and reduced the production of inflammatory cytokines.

Based on these observations and anti-inflammatory support for SOCS3 from the literature, we tested whether the SOCS3 peptide mimetic could modify the cytotoxicity of the M1 polarization treatment or virus infection. When compared with the untreated controls, the SOCS3 peptide mimetic increased cell viability in the M1 and HSV-1-infected M1 cells, however, SOCS3 did not significantly affect HSV-1-infected M0 (unpolarized) cells. It appeared that the SOCS3 peptide mimetic protected the macrophages from the lytic effects of M1 polarization. The SOCS3 peptide mimetic showed no significant effect on HSV-1 infected, M1 polarized, or HSV-1 + M1-treated J774 cells; however, the SOCS3 peptide mimetic increased cell viability in HSV-1-infected M1 J774A.1 macrophages. No change was observed in IL-1β expression between M1, M1 treated with SOCS3, and M2 macrophages. As discussed previously, IFN-γ used to polarized M1 cells inhibits IL-1β expression (Eigenbrod et al., 2013)

Untreated M1 cells produce high levels of TNF-α and IL-6, while M1 cells treated with SOCS3 peptide mimetic behaved similar to the M2 phenotype by producing the anti-inflammatory cytokine IL-10. This result suggests that SOCS3 mimetic exerts an anti-inflammatory effect by diminishing the lytic effect of M1 polarization. The mechanism behind SOCS3's anti-inflammatory effect likely depend on SOCS3 regulation of the STAT pathway responsible for macrophage activation and M1 polarization (Qin et al., 2012).

Future studies:

Macrophages are pivotal cells in inflammation and host defense; thus, alteration of macrophage activity may prove a useful strategy for the treatment of certain diseases. Many diseases, even those of a non-infectious nature, involve the activity of macrophages in generating an inflammatory response that wreak havoc on the human body. For instance, an imbalance between M1 and M2 phenotypes is related to neurodegenerative diseases (Tang and Le, 2015). Significant inflammation is associated with neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (AD). Changes in microglial phenotypes are associated with stage and severity of these diseases; therefore, switching microglial cells between M1 and M2 phenotypes may provide a useful therapeutic approach.

In order to switch the microglial population to M2 (anti-inflammatory) macrophages, it would be necessary to supply M2-inducing stimuli. It is not feasible to use injected cytokines, given that cytokines do not pass the blood-brain barrier (Tang and Le, 2015). It would be necessary to use certain chemical compounds, such as fasudil to induce M2 polarization or minocycline that inhibits M1 polarization (Kobayashi et al., 2013; Zhang et al., 2013). Given that HSV-1 infection has been shown to be a risk factor for AD later in life (Itzhaki, 2014), I propose to evaluate the combined effect of HSV-1 with the polarizing-modifying chemicals fasudil or minocycline. I would polarize microglial cells via stimulation with IFN-γ and LPS to induce the M1 state and IL-4, IL-10 or IL-13 to induce the M2 state. I would then infect them with HSV-1 and treat them with fasudil or minocycline and observe whether there is a shift in the cell population from the M1 to the M2 phenotype. I would also assess viability (by haemocytometer or MTT assay), cell morphology (observation under the light microscope) and cytokine production (by enzyme-linked immunosorbent assay (ELISA) or Luminex Multiplex Immunoassays) to ensure there is a

reduction in pro-inflammatory cytokines in M1 cells as the population is shifted to the anti-inflammatory M2 state.

A separate line of interesting research also stems from the role of SOCS1 and SOCS3 in the development and progression of AD. The present study showed that a SOCS3 peptide mimetic was successful in reducing the amount of pro-inflammatory cytokines released by M1 macrophages. Therefore, one suggested approach for neurodegenerative diseases therapy is to induce SOCS3 in microglial cells by gene therapy using delivery by a vector in order to decrease the elevated levels pro-inflammatory cytokines and reduce inflammation.

Microtubule reorganization during HSV-1 infection facilitates the virus' movement to and from the host cell nucleus (Bigley, 2014). Therefore, determining the effect the SOCS3 on microtubule structure of virus-infected cells would be an interest. An *in vitro* approach would include inducing M1 macrophages treated with SOCS3 peptide (or leaving them untreated as a control) with or without HSV-1 (MOI of 0.1) for 24 hours. After this time, changes in microtubules would be evaluated. I would use an anti-mouse anti-alpha tubulin antibody as microtubule marker (indirect immunofluorescence). In the SOCS3-treated and the virus-infected cells, I expect to see fewer vacuoles resulting from the anti-inflammatory effect of SOCS3. I would expect that the microtubule network in these macrophages treated with SOCS3 will be more stable (less disrupted) than M1 infected cells.

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SUPPLEMENTAL FIGURES

18 hours **24 hours** **48 hours**
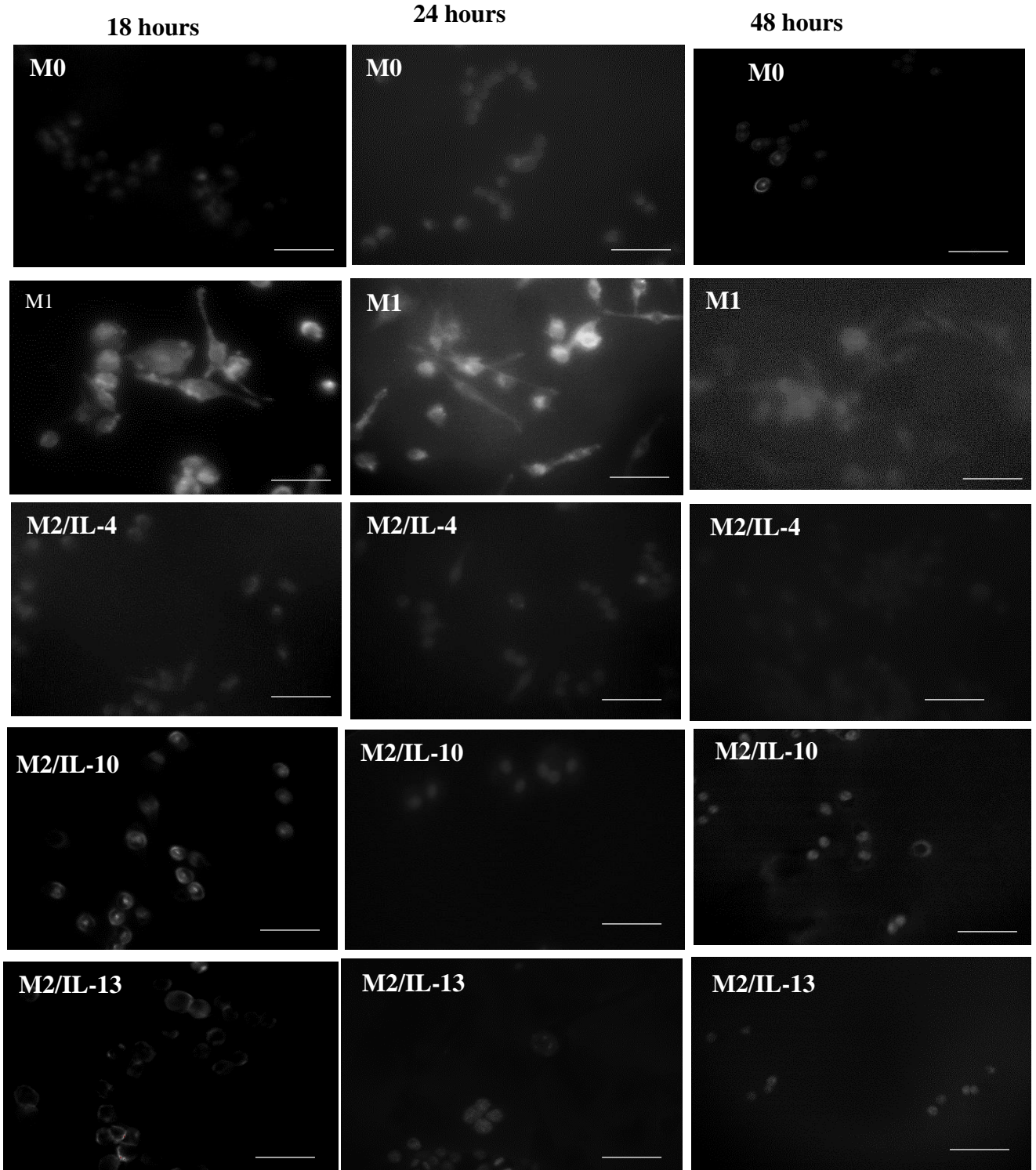
Figure S 1. Changes in Expression of CD80 in Uninfected, Un-polarized and Polarized J774A.1 Macrophage following Cytokines Stimulation for 18, 24, 48 hours. Un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained with Pacific blue conjugated anti-mouse CD80 antibodies and then evaluated by immunofluorescence microscopy. M1 macrophages showed the highest expression levels of CD80 at 18 and 24 hours, whereas it was down regulated at 48 hours. No apparent differences in the CD80 expression between these macrophages at different points in time. (Scale bar =50μm).
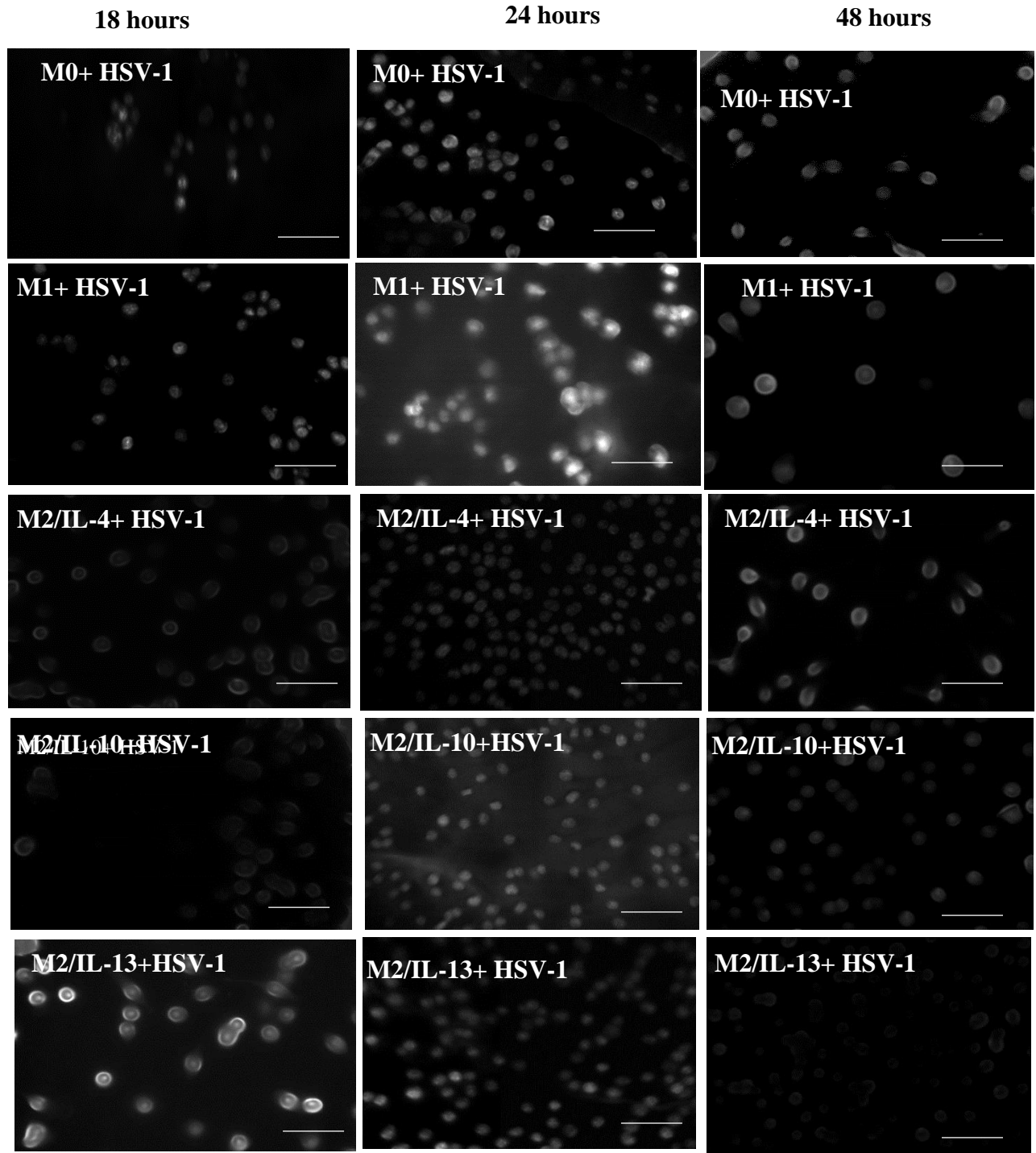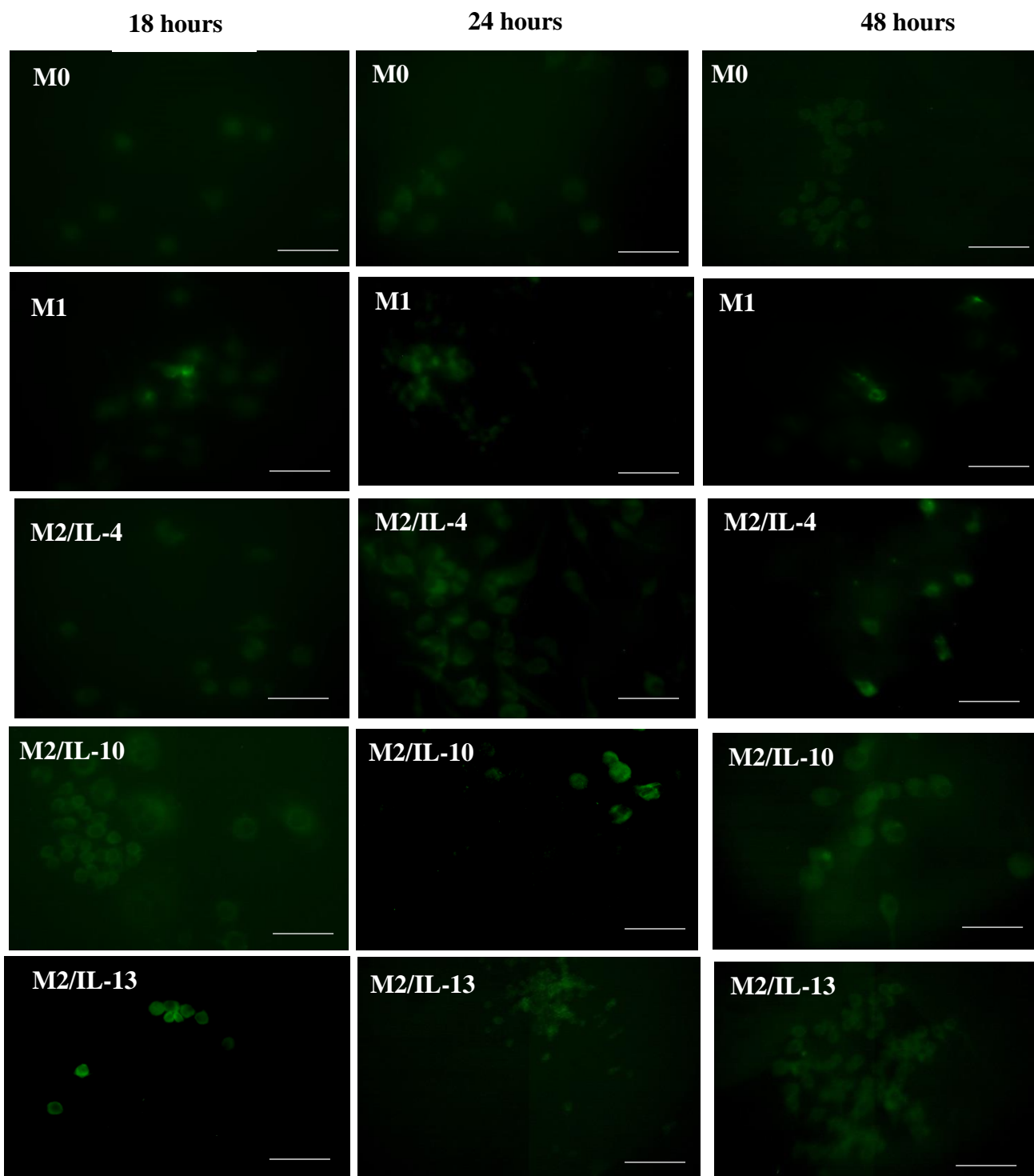
Figure S 2. Changes in Expression of CD80 in Un-polarized and Polarized J774A.1 Macrophage following Cytokines Stimulation and Infection with HSV-1 for 18, 24, 48 hours. Infected, Un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained

with Pacific blue conjugated anti-mouse CD80 antibodies and then evaluated by immunofluorescence microscopy. Infected M1 macrophages showed an increase in CD80 expression at 24 and 48 hours. (Scale bar =50μm).

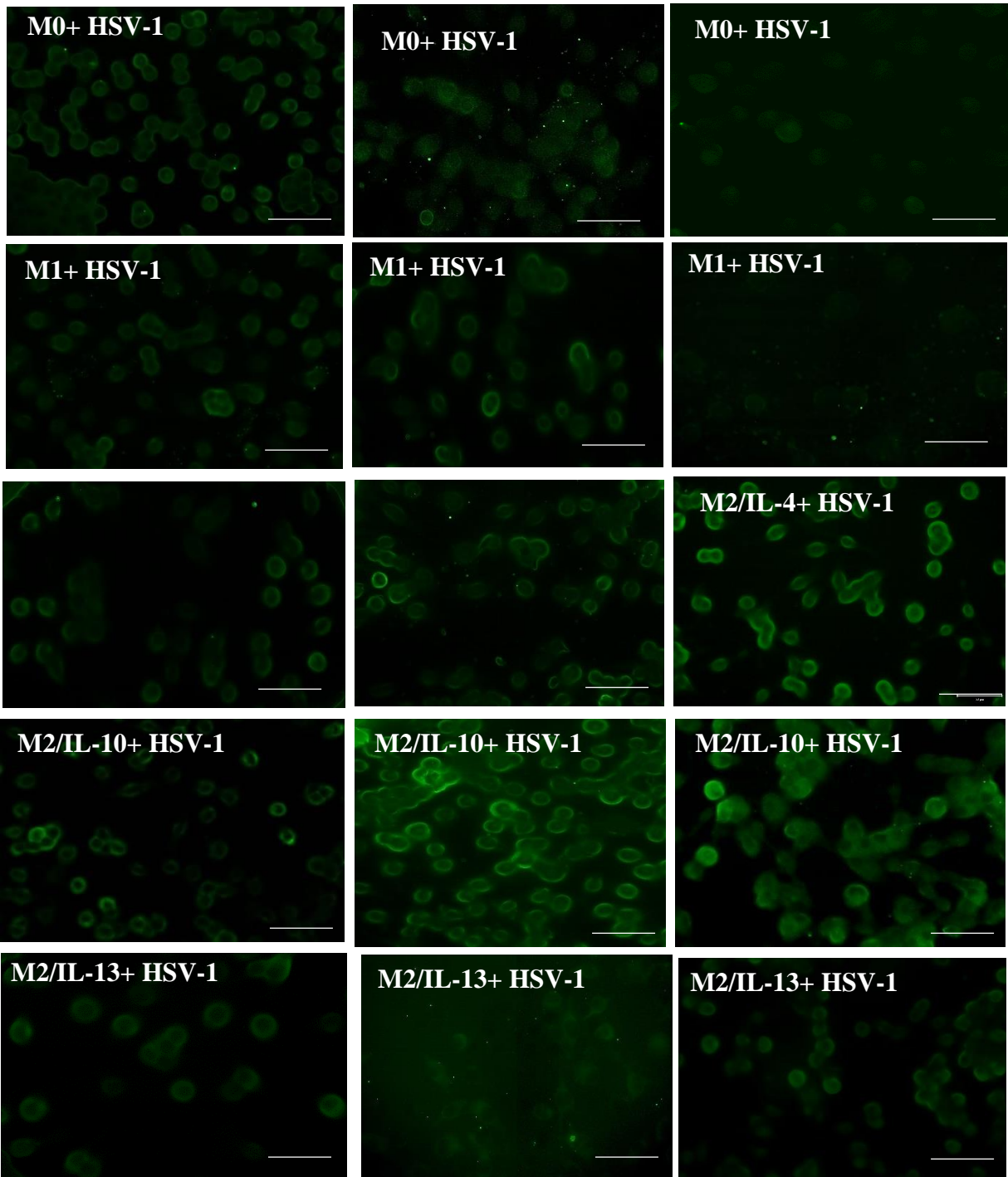|	**18 hours**	**24 hours**	**48 hours**
M0			
M1			
M2/IL-4			
M2/IL-10			
M2/IL-13			

Figure S 3. Changes in Expression of CD163 in Un-polarized and Polarized J774A.1 Macrophage following Cytokines Stimulation for 18, 24, 48 hours. Uninfected**,** Un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained with FITC conjugated anti-mouse CD163 antibodies and then evaluated by immunofluorescence microscopy. M2 macrophages induced by IL-10 or IL-13 showed higher expression of CD163 at 24 (for M2/IL-10) and (18 hours for M2/IL-13). CD163 expression were down-regulated among macrophages. (Scale bar =50μm).

18 hours **24 hours** **48 hours**

M0+ HSV-1

M0+ HSV-1

M0+ HSV-1

M1+ HSV-1

M1+ HSV-1

M1+ HSV-1

M2/IL-4+ HSV-1

M2/IL-10+ HSV-1

M2/IL-10+ HSV-1

M2/IL-10+ HSV-1

M2/IL-13+ HSV-1
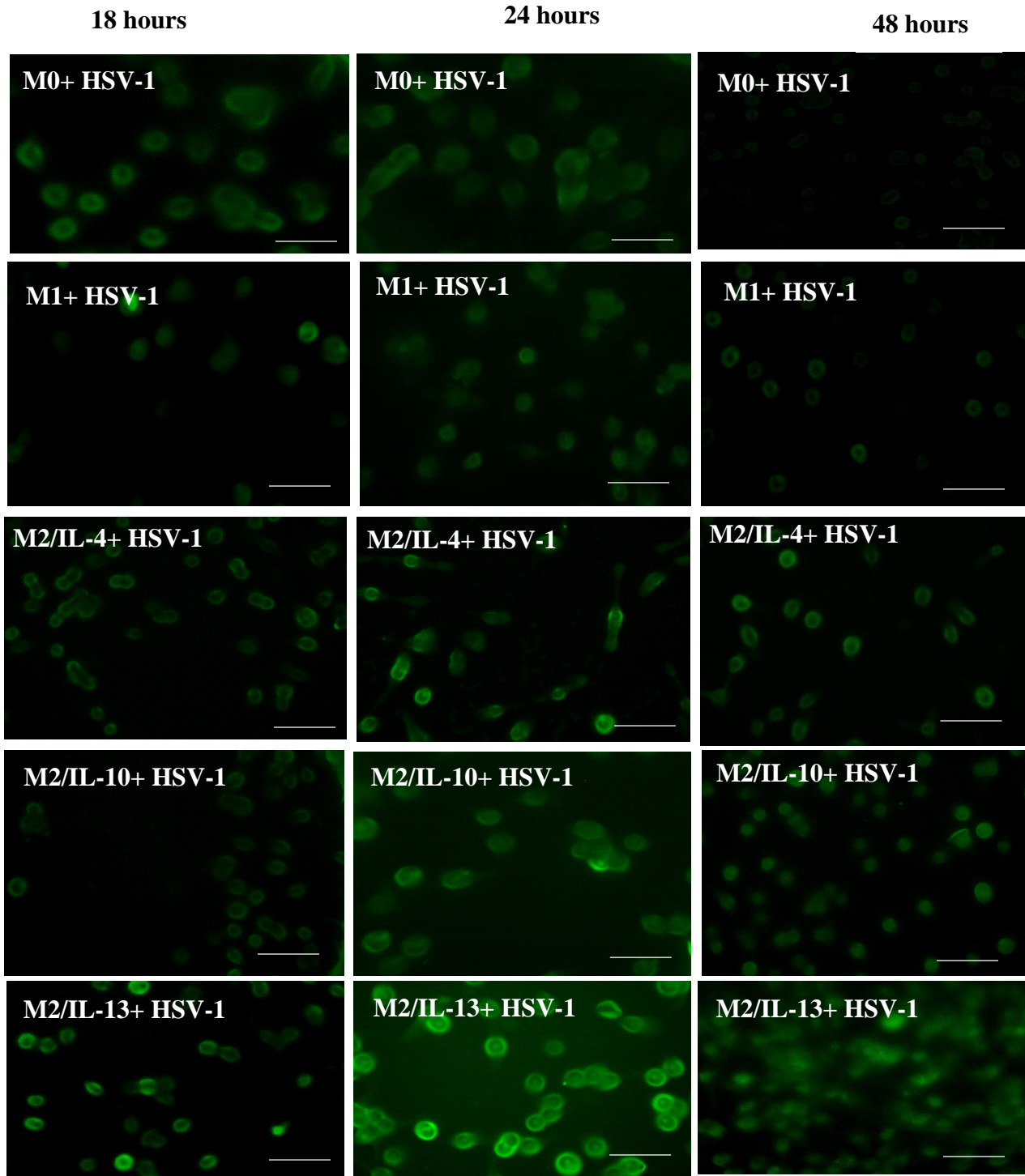
M2/IL-13+ HSV-1

M2/IL-13+ HSV-1

Figure S 4. Changes in Expression of CD163 in Un-polarized and Polarized J774A.1 Macrophage following Cytokines Stimulation and Infection with HSV-1 for 18, 24, 48 hours. Infected**,** Un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained with FITC conjugated anti-mouse CD163 antibodies and then evaluated by immunofluorescence microscopy. Infected M2 macrophages polarized by IL-10 or IL-4 showed an up-regulation of CD163 expression at 24 and 48 hours CD163 expression were down-regulated among macrophages. (Scale bar =50μm).

| | **18 hours** | **24 hours** | **48 hours** |
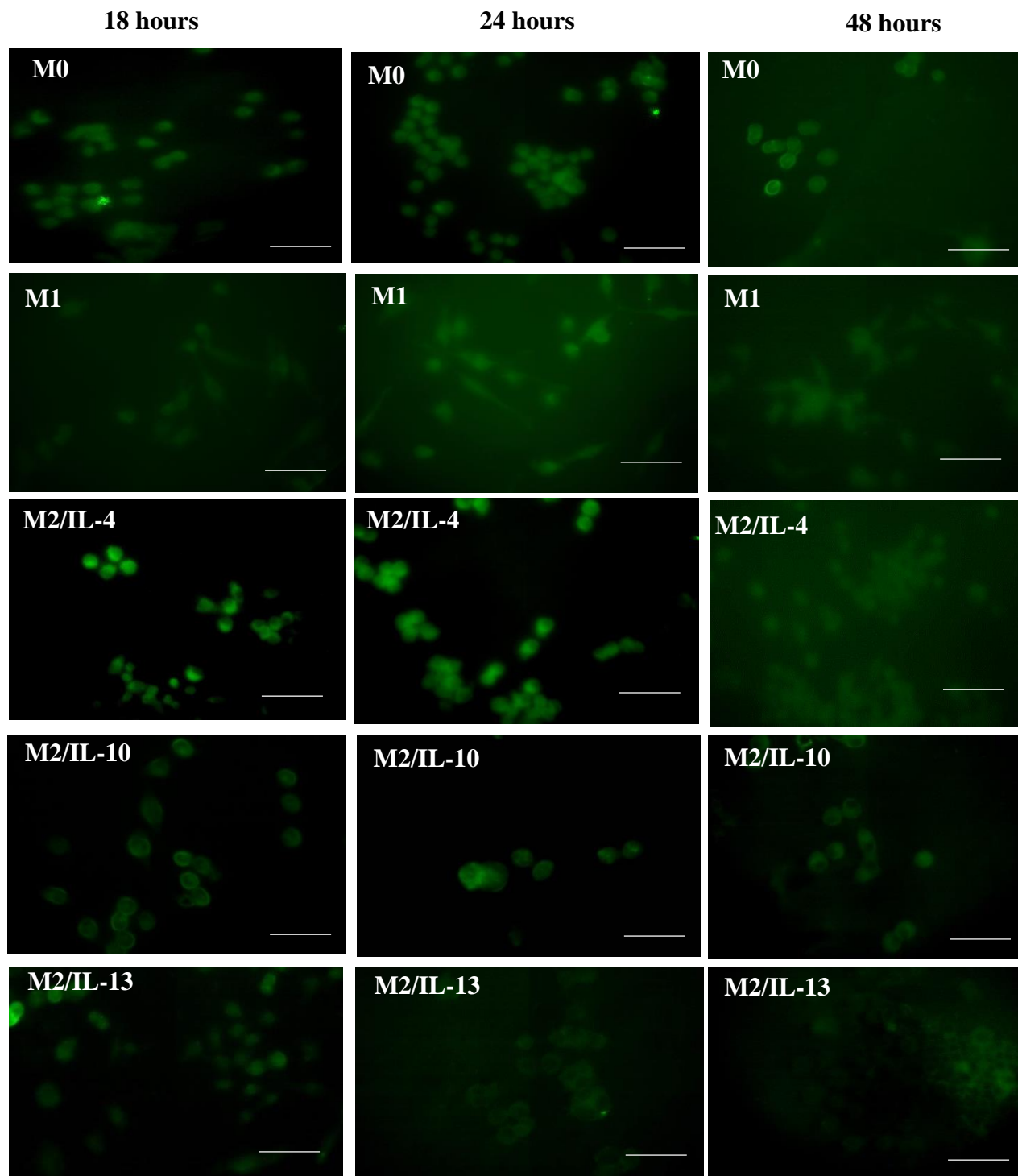
Figure S 5. Changes in Expression of CD200R in Un-polarized and Polarized J774A.1 Macrophage following Cytokines Stimulation for 18, 24, 48 hours. Infected, Un-polarized and

cytokine-stimulated (polarized) macrophages were fixed and stained with Alexa Fluor 488 conjugated anti-CD200R antibodies and then evaluated by immunofluorescence microscopy. Uninfected M2 macrophages polarized by IL-4 showed an up-regulation of CD200R expression at 18 and 24 hours. No difference in CD200R expression were observed among macrophages. (Scale bar =50μm).

Figure S 6. Changes in Expression of CD200R in Un-polarized and Polarized J774A.1 Macrophage following Cytokines Stimulation and Infection with HSV-1 for 18, 24, 48 hours.

Infected, Un-polarized and cytokine-stimulated (polarized) macrophages were fixed and stained

with Alexa Fluor 488 conjugated anti-CD200R antibodies and then evaluated by immunofluorescence microscopy. CD200R expression was highly up-regulated in infected M2 macrophages induced by IL-13 at 24 hours, and slightly high in infected M2 macrophages induced by IL-4 and IL-13 at 24 hours. (Scale bar =50μm).