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The Effect of HSV-1 Infection on Differentiated and Polarized U937 cells

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The Effect of HSV-1 Infection on Differentiated and Polarized U937 cells

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

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY ALLOLO ALDREIWISH ENTITLED THE EFFECT OF HSV-1 INFECTION ON DIFFRENTIATED AND POLARIZED U937 CELLS BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Aldreiwish, Allolo. M.S. Department of Microbiology and Immunology, Wright State University, 2013. The effect of HSV-1 Infection on Differentiated and Polarized U937 Cells.

Herpes simplex virus type 1 (HSV-1) challenges the host immune system through several mechanisms (Frey, et al., 2009). In vitro, U937 cells (human macrophage-like precursor cell line) are not susceptible to HSV-1 infection when they are not differentiating (López-Guerrero and Alonso, 1997). Differentiation of these cells' resistance can abrogates their resistance to HSV-1 (Tenney and Morahan, 1991). In this study, we examined the effect of HSV-1 infection on differentiated and polarized U937 cells. U937 cells are differentiated to M0 cells. Then, M0 cells are polarized to distinct phenotypes, M1 or M2. M1 are pro-inflammatory macrophages while M2 are anti-inflammatory cells. We examined the effect of polarization and HSV-1 infection on cellular viability, morphology and the expression levels of CD14 and CD86. This study showed that differentiation and polarization of U937 cells decreased their viability more than the HSV-1 infection. M2 macrophages showed a major decrease in cell viability compared to M0 and M1, possibly due to IL-4-inducing apoptosis. Differentiation and

polarization of U937 cells up-regulates their CD86 expression levels and down-regulates their CD14 expression levels. Furthermore, M1 showed the greater $CD14^{+}$ / $CD86^{+}$ cell population. HSV-1 infection induced some morphological changes in M0, M1 and M2 cells. M0-infected cells appeared more rounded while M1-infected cells lost their defined shape and became irregular. Interestingly, HSV-1 infection induced M2 CD86 expression ($p<0.002$), but did not induce CD86 expression in M1cells. CD206 expression levels remained unchanged in infected and uninfected cells. Differentiation and polarization of U937 cells induced more changes on these cells than HSV-1 infection.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BSA = Bovine serum albumin

CD4= Cluster of differentiation 4

CD8= Cluster of differentiation 8

CD14 = Cluster of differentiation 14

CD86 = Cluster of differentiation 86

DMEM = Dulbecco's Modified Eagle's Medium

FCS = Fetal calf serum

HSV-1 = Herpes simplex virus type 1

HIV= Human immunodeficiency virus

IFN-α= Interferon-alpha

IFN-β=Interferon-beta

IFN-γ = Interferon-gamma

IL-4 = Interleukin-4

IL-10=Interleukin-10

IL-12=Interleukin-12

IL-13 = Interleukin-13

IL-23=Interleukin-23

iNOS = inducible nitric oxide synthase

JAK=Janus Kinase

KIR = Kinase inhibitory region

LPS = Lipopolysaccharide

MOI = Multiplicity of infection

MyD88= Myeloid differentiation primary response 88

NF-κB= Nuclear factor kappa-light-chain-enhancer of activated B cells

NK= Natural Killer cell

PBS = Phosphate buffered saline

pDCs= Plasmacytoid dendritic cells

PFU = Plaque forming units

PMA= Phorbol 12-myristate 13-acetate

RPMI= Roswell Park Memorial Institute medium

RSV= Respiratory Syncytial Virus

STAT= Signal Transducer and Activator of Transcription

SOCS1 = Suppressor of cytokine signaling-1

SOCS3 = Suppressor of cytokine signaling-3

TLR = Toll-like receptor

TNF-α = Tumor necrosis factor-alpha

Acknowledgement

I would like to thank Dr. Nancy Bigley for her endless support, guidance, and for being my thesis advisor who always contributes to my work. Also, I would like to thank Dr. Barbara Hull, the program director, for her time, suggestions and guidance over my entire masters' years. I would like to thank Dr. F. Javier Alvarez-Leefmans for being in my committee and appreciate his time and suggestions to my research. I'm very Grateful for having my beloved supporting father and family in Saudi. Lastly, many thanks to my government, my colleagues and friends.

Dedication

I would like to dedicate my thesis study to my dad (Mr. Dreiwish Aldreiwish), and my siblings for their endless support and encouragement. Also, I would like to dedicate my thesis study to my deceased mother (Amal Aldreiwish).

Introduction

Herpes simplex virus type 1 (HSV-1) is a double stranded DNA virus. It belongs to the Herpesvirade family and replicates in the nucleus of infected cells (Frey, et al., 2009). HSV-1 is a common human pathogen (Melchjorsen, et al., 2002), which infects approximately up to 80 percent of world population (Frey, et al., 2009) and causes many diseases such as orofacial infections and enciphalitis (Melchjorsen, et al., 2010). Once an individual becomes infected with HSV-1, he becomes a carrier for life. The primary infection is often asymptomatic (Frey, et al., 2009). Initially, HSV-1 enters the mucosal surfaces through epidermal or other epithelial cells. After the virus initiates an infection at a peripheral site, it migrates to the sensory neurons, replicates and causes a latent infection in a few of these neurons, (Kodukula, et al., 1999). HSV-1 infection induces the immune system to produce significant increase in cytokines, specifically, type I interferons (IFNs) (Melchjorsen, et al., 2010).

Both the innate and adaptive immune system contribute to control of HSV-1 infection (Zheng, et al., 2012). The innate immune response is modulated by recruitment of several immune cells such as natural killer cells (NK), plasmacytoid dendritic cells (pDCs), production of type I interferon (IFN-α and IFN-β)(Chew, et al., 200), and proinflammatory cytokines (Melchjorsen, et al., 2010). Additionally, the adaptive immune system manages the infection through activation of $CD4^+$ and $CD8^+$ T

cells, particularly through production of IFN- γ by NK cells and by T helper 1 cells (Kodukula, et al., 1999).

Macrophage recruitment is critical for controlling HSV-1 invasion (Melchjorsen, et al., 2002). Macrophages exhibit an essential role in host resistance in response to HSV-1 infection by two mechanisms. Intrinsic resistance is provided by IFN-α/ IFN-β which prevents intracellular virus replication, and extrinsic resistance of IFN-γ-activated macrophages which inactivates virus, suppresses replication and destroys HSV-infected cells (Cheng. et.al., 2000). Moreover, these phagocytes manage the HSV-1 infection not only through presenting viral antigens to responding T cells but also by producing several inflammatory cytokines and chemokines (Melchjorsen, et al., 2002). Particularly, In early stages of HSV-1 infection macrophages express IFN-γ, TNF-α, iNOS, and IL-12 which work together to inhibit HSV-1 replication (Kodukula, et al., 1999).

Macrophages are noted for their plasticity and capability to alter their function in response to different microenvironments. M1 and M2 polarized cells, in particular, are two activated macrophage phenotypes (Mantovani, et al., 2004). The classical activation pathway induces macrophage maturation to M1 cells. Interferon (IFN-γ), tumor necrosis factor (TNF-α) or bacterial lipopolysaccharide (LPS) triggers macrophage differentiation to M1 subtype through the classical pathway. In contrast, M2 population can be induced by IL-4 or IL-13 via the

alternative activation pathway (Wang, et al., 2010). M1 macrophages are considered pro-inflammatory phagocytes and produce many cytokines such as interleukin 12 (IL-12), (Mantovani. et.al., 2004), which induces other cytokines production from NK cells and T lymphocytes in response to pathogens invasion (Trinchieri, 1995). Nitric oxide and Il-23 are also produced by M1 cells in response to HSV-1 to promote host defense against this virus (Mantovani, et al., 2004). M2 macrophages are anti-inflammatory cells and produce cytokines such as IL-10 that contribute to tissue remodeling and angiogenesis (Kigerl, et al., 2009).

M1 and M2 macrophages express several receptors that enable them to recognize and then respond to external signals Both M1 and M2 cells express different levels of CD14 and CD86 (Bonato, et al., 2001). Macrophage surface protein (CD14) helps TLR4 along with MD2 to recognize LPS signals, thus stimulating the macrophage activation to a distinct phenotype with a significant inflammatory cytokine response (Parham, 2009). CD86, however, is expressed on resting monocytes, rapidly upregulated in macrophages activation (Bonato, et al., 2001) and plays a critical role in T-cell response stimulation after pathogen invasion Activation of macrophages by different stimuli such as LPS and IFN-γ (M1), or IL-4 (M2) to a distinct phenotype affects their expression levels of CD14 and CD86, especially in response to HSV-1 infection.

Hypothesis: HSV-1-infected U937 cells differentiated to M0 and polarized to M1 or M2 phenotypes exhibit distinct changes in cell surface proteins, cell morphology and viability.

Aims of the current Study:

- Examine the effect of HSV-1 infection on differentiated and polarized U937 cells viability and morphology.

- Examine the effect of HSV-1 infection on expression levels of CD14 and CD86 in M0, M1 and M2 macrophages.

Literature Background

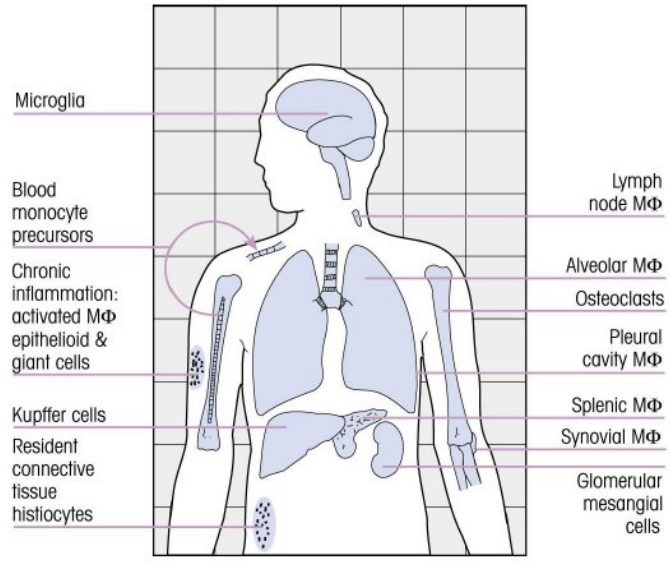
U937 CELLS:

U937 is a human macrophage-like precursor cell line (Tenney and Morahan, 1991). It was established from a 37 year-old male patient with histiocytic lymphoma (ATCC).This cell line is used frequently because it exhibits many monocytic characteristics, including monocyte-like antigens, functions and receptors (Tenney and Morahan, 1991). It has been universally used as an in vitro model of macrophages to investigate and examine the effect of several human pathogens such as poliovirus, paravirus, HIV and HSV at the cellular level (López-Guerrero and Alonso, 1997). For example, In 1992, a study showed that an HSV-1 infection of U937cells resulted in a significant increase in HIV replication (Feng, et al.,) Additionally, another study conducted in 2000, showed that an RSV infection of U937 cells caused a depression in their production of TNF-α, inflammatory cytokine, while it enhanced their production of IL-10, an anti-inflammatory cytokine (Barr, et al.). Interestingly, in previous studies, undifferentiated U937 cells showed a strong resistance to HSV-1 infection. U937 are not susceptible to (HSV-1) infection when they not treated with any differentiating stimuli (López-Guerrero and Alonso, 1997).

Moreover, such resistance was abrogated by treating the U937 cells with several inducers to influence their differentiation and alter their resistance to the virus (López-Guerrero and Alonso, 1997). Treatment of these cells with phorbol 12-myristate 13-acetate (PMA) induced their differentiation and thus raised their permissivity to HSV-1 infection (Tenney and Morahan, 1991). Other treatments such as vitamin D3 or mezercin (protein kinase C activator) can cause macrophage maturation as well; hence, increase their susceptibility to HSV-1. After treatment, U937 cells have shown changes in their permissiveness to HSV-1 infection; however, the exact mechanisms for this susceptibility is unclear (Tenney and Morahan, 1991).

Macrophages:

Macrophages are large mononuclear phagocytes (Yu, et al., 1990) derived from promonocyte precursors. Bone marrow promonocytes differentiate to mature blood monocytes which eventually reside in tissues as macrophages (Delves, et al., 2011) (Fig.1).



Delves *et al. Roitt's Essential Immunology, 12th ed.*
© 2011 Delves *et al.* Published 2011 by Blackwell Publishing Ltd.

Figure1: The system of macrophages as mononuclear phagocytes.
Bone marrow promonocytes mature to circulating monocytes which eventually forms tissue macrophages. (Adapted from Delves et al., 2011).

Macrophages play a significant role in the immune system exhibiting activities that depend on the inducing agents (Biswas, et al., 2012). Their uniqueness relies on their continuous activation; interestingly, they have opposing activities, for example, anti-inflammatory versus pro-inflammatory functions. Also, macrophages exhibit tolerogenic and immunogenic activities in addition to tissue destructive and tissue remodeling abilities (Stout, et al., 2005).

Macrophages express a variety of cell membrane receptors which enable them to recognize and act against foreign antigens. Scavenger receptors, for instance, bind to a variety of foreign as well as endogenous molecules (Peiser and Gordon, 2001). Macrophages are the most critical immune cell acting in response to virus invasion. They release several pro-inflammatory chemokines and cytokines, besides directing other inflammatory cells to the infection site (Lannello, et al., 2011). HSV-1 interacts with macrophages in several ways. In humans, the differentiation state of these phagocytes influences their permissiveness to the virus, although the exact mechanism has not been fully defined (Kemp, et al., 1990). When macrophages are activated in response to viral attack, they ingest the pathogen and, as antigen presenting cells, present the virus antigens to responding T cells (Lannello, et al., 2011). They are also able to kill the viruses nonspecifically. Sometimes these mononuclear phagocytes cannot kill the viruses and, instead, allow them to replicate and produce a lethal infection (Roitt, 1999). In contrast to epithelial cells which go through necrosis after HSV invasion, macrophages undergo apoptosis. Unfortunately, the mechanism of this apoptosis is not yet clear (Lannello, et al., 2011). Ultimately, human macrophages are able to recognize the initial HSV infection and produce inflammatory cytokines, interferons (IFNs), and tumor necrosis factor (TNF-α) (Melchjorsen, et al., 2010).

Macrophages polarization:

Macrophages play several anti-viral roles. They have intrinsic resistance to control the virus replication via type I IFN. This resistance undergoes many changes depending on the differentiation states of the macrophages, which can be altered either naturally or by in vitro inducers (Tenney and Morahan, 1991). Interestingly, macrophages function alternatively in response to their microenvironment (Stout, et al., 2005). In their protective role, they minimize inflammation and trigger tissue repair (Varin and Gordon, 2009).

Polarizing of macrophages by LPS and IFN-γ, a Th1 cytokine, generate an activated subtype, M1,17, characterized by enhancement and suppression of several secreted and membranous proteins (Yu. et al., 1990). This path of activation is known as the classical pathway (Gordon and Martinez, 2010). M1 cells exhibit antimicrobial properties and up-regulate expression of certain anti-microbial enzymes. Inducible nitric oxide synthase (iNOS) is one of those linked enzymes that produce nitric oxide from L-arginine (Wang, et al., 2010). Additionally, the classical activated M1 macrophages are distinguished by their antigen-presenting capacity and production of increased levels of IL-12 and IL-23 (Mantovani, et al., 2004). LPS -stimulation of the M1 macrophages' Toll like receptor 4 (TLR4) causes a downstream signaling pathways through MyD88 and TRIF. Each of these adaptor molecules leads to activation of several transcription factors. For example, MyD88 activates IRF5 and NF-κB which is also activated by TRIF signaling cascade (Biswas, et al., 2012) (Fig.2).

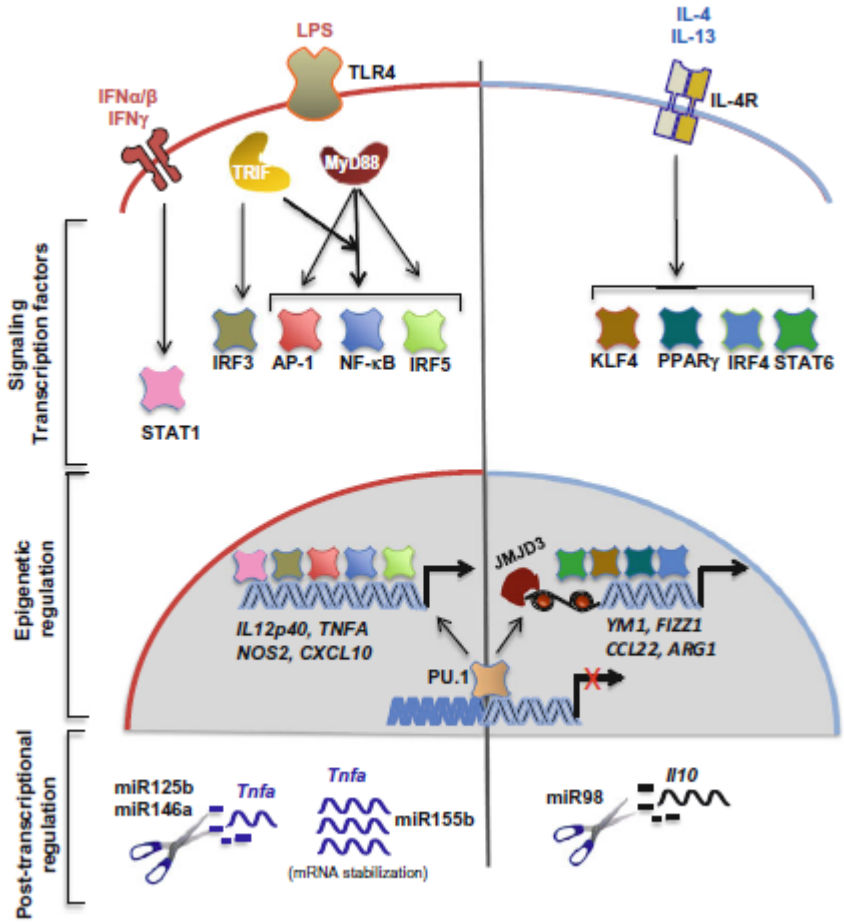


Figure 2: Macrophage Polarization

LPS stimulation of NF-κB, IRF5, AP-1 and IRF3 via MyD88 and TRIF (left) versus IL-4 stimulation of STAT6, IRF4 and KLF4 through IL-4R (right).

(Adapted from Biswas, et al., 2012).

M2 is the other phenotype of polarized macrophages. The Th2 cytokine IL-4 in addition to IL-13, mediates the M2 alternative activation pathway (Gordon and Martinez, 2010). During selected pathogen and parasitic infections, IL-4 is released as a Th2 response to work against such invasion. The production of IL-4 stimulates differentiation of macrophages to M2 cells (Varin and Gordon, 2009). Gratchev, et al., (2001) showed that IL-4 induced alternative macrophages overexpress extracellular matrix protein (ECM). This overexpression of ECM suggests a critical role of M2 in tissue reconstruction. ECM is deposited during acute and chronic inflammation, particularly during the healing period. High levels of non-opsonic receptors such as the mannose receptor, CD206 are critical markers for M2 human macrophages (Mantovani, et al., 2004).

Besides the molecular differences between M1 and M2, the production levels of certain cytokines is a critical distinguishing tool for each phenotype (Wang, et al., 2010). Whereas high levels of IL-10 and low levels of IL-12 are markers for M2, M1 cells produce a low level of Il-10 and a high level of IL-12 (Mantovani, et al., 2004) (Fig.3). While M1 macrophages are involved in tumoricidal, microbicidal, and pro-inflammatory activities, M2 macrophages engage in anti-inflammatory, immunoregulatory and tissue-remodelling activities (Biswas, et al., 2012).

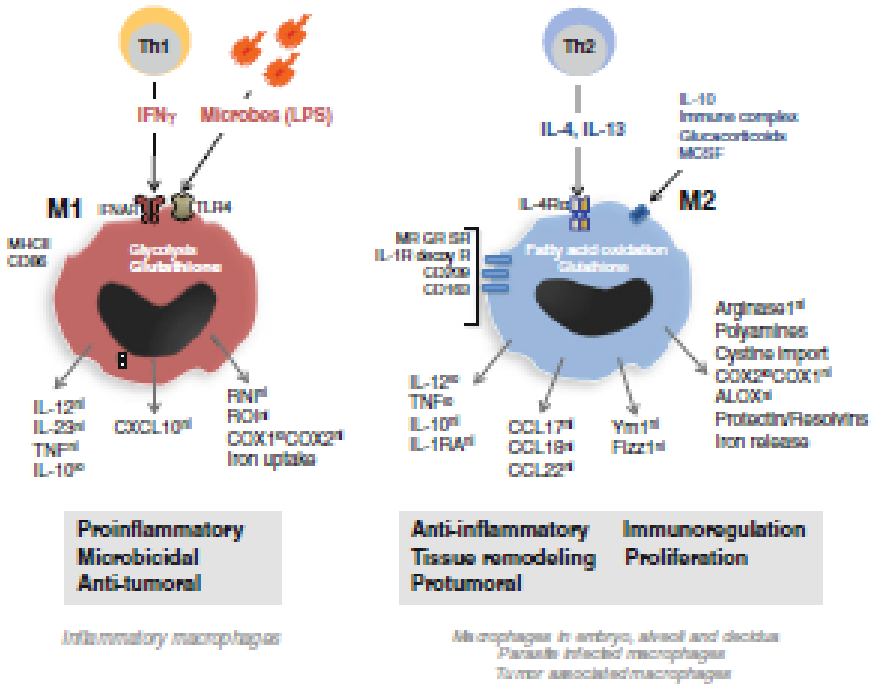


Figure 3. Macrophages polarized phenotypes, M1 cells versus M2 cells.

M1 mirror Th1 response (left). IFN-γ, a Th1- cytokine, and lipopolysaccharides (LPS) polarize Macrophages to M1 cells via classical activation pathway. These M1 macrophages secrete a high level of IL-12, IL-23 and TNF and a low level of IL-10. In contrast, M2 macrophages mirror Th2 response (right). Th2 cytokines IL-4 and IL-13 polarize macrophages to M2 phenotype via the alternative pathway. M2 macrophages produce high levels of IL-10 and low levels of IL-12 and TNF. (Adapted from Biswas. et. al., 2012)

HSV-1:

HSV-1 belongs to the α-herpesviridae subfamily (Lannello, et al., 2011). As a member of the Herpesviridae family, its unique structure is composed of an envelope, tegument protein, an icosahedral capsid that surrounds the core and a linear double- stranded DNA genome resides within the core (Kinpe, et al., 2007)(Fig.4). The HSV-1 envelope is composed of many oligomeric glycoproteins. These glycoproteins exert several functions. Some participate in viral invasion of host cells, others are involved in disseminating the virus while other glycoprotins are responsible for immune evasion. gB and gC have been found as homodimers while gI-gE and gL-gH are heterodimers (Handler, et al., 1996).

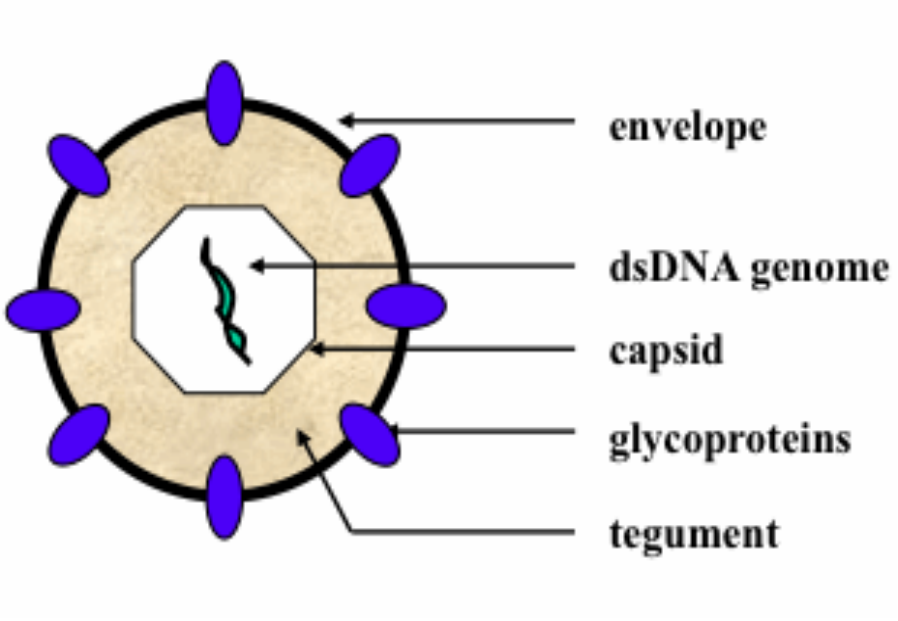


Figure 4. Structure of Herpes simplex virus type I. HSV-I is composed of envelope, tegument, and an icosahedral capsid with a ds-DNA genome. (Adapted from www.mdpi.com).

The bulky HSV-1 genome requires approximately 18 hours to be replicated in the nucleus (Peri, et al., 2008) (Fig.5). HSV-1 is more widespread than HSV-2, and mostly transmitted through non-sexual contacts in childhood and adolescence (Smith and Robinson, 2002). There are several infection forms in humans (Peri, et al., 2008). Following initial HSV infection, an individual becomes a carrier of the virus for life. The primary infection is usually mild or symptomless (Lannello, et al., 2011). Moreover, the virus needs a mucosal surface to generate an infection (Kinpe, et al., 2007). Contact of HSV-1 with debraded skin or a mucosal membrane (Lannello, et al., 2011) results in its adsorbtion to the host cells through its envelop glycoproteins. Specifically, cellular heparan sulphate receptors interact with virus glycoproteins gB or gC results. Membrane fusion and release of viral particles into the cell occurs subsequently, initiation of primary infection (Ellermann-Eriksen , 2005).

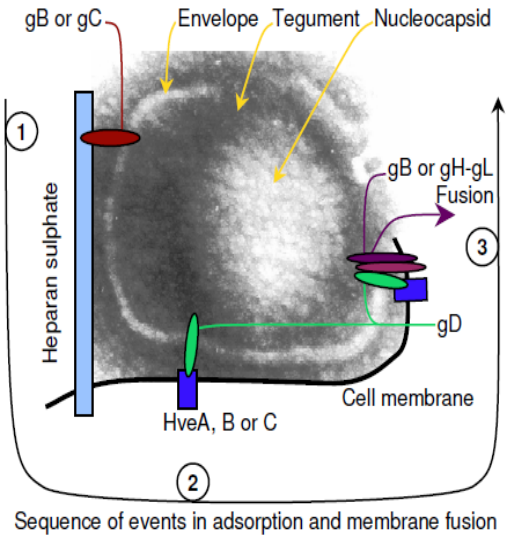


Figure 5. HSV-1 entrance to the host cell and glycoproteins participation. Main virus composition elements.1) and 2) during virus fusion to the cell membrane after binding to it. 3) process of infecting the host cell. (Adapted from Eriksen-Ellermann, 2005)

Later, HSV-1 attached to neuron receptors on epithelial cells migrates through neuron axons to the body of sensory nerves where it resides and turns into a latent virus (Lannello, et al., 2011). It stays in dormant in a very few of the host sensory neurons until subsequent activation (Peri, et al., 2008).

Stress, physical trauma, immunosupression or exposure to radiation reactivate the HSV-1 from its latent phase (Lannello, et al., 2011). The infection initiation process along with the primary infected cells type determine whether virus will be a lytic or latent (Ellermann-Eriksen, 2005). Usually, when a latent virus becomes reactivated, infected people develop painful blisters or cold sores on their lips (Lannello, et al., 2011). In many cases, skin lesions appear either at the same sites of primary infections or near to them (Peri, et al., 2008). Although more common in neonates and immunocompromized people, infection of the cornea results in keratits and encephalitis in reactivated infections. HSV infection is also considered as the primary cause of sporadic infectious encephalitis in healthy people (Lannello, et al., 2011). Pattern recognition receptors (PRRs), including toll like receptors (TLRs), mediate the primary recognition of HSV-1. In particular, TLR2 and TLR9 recognize HSV-1 surface structure and its DNA, respectively. These PRRs promote the expression of pro-inflammatory cytokines and IFNs (Melchjorsen, et al., 2010), which activate the primary phagocytes, macrophages and other immune system cells (Ellermann-Eriksen, 2005).

In murine cell line J774A.1, the cytokines signaling within macrophages microenvironments have the major effects on cells viability and surface marker

expression than the HSV-1 infection. As human cell line, U937 cells response to differentiation treatments and become more permissive to HSV-1. (López-Guerrero and Alonso, 1997). Here, we examined the effect of polarization treatments and HSV-1 infection on these cells as in vitro model of human macrophages.

Materials and Methods

Cell lines and Cells culture:

The U937 human-like macrophage cell line originated from a histiocytic lymphoma. U937 cells were cultured in 1640 RPMI media (Fisher Scientific) (Aderka, et al., 1989). The 1640 RPMI was supplemented with 10% heat inactivated fetal bovine calf serum (FCS), 50µl/ml gentamicin, 0.01ml/ml pyruvate, 2 mm l-glutamine, and 10 mm HEPES. Cells were plated in 25 cm^2 vented cap culture flasks (Fisher Scientific) and incubated in a 37^oC -humidified incubator supplied with 5% CO_2. U937 cells were sub-cultured using fresh 1640 RPMI media two times weekly.

For culturing the HSV-1 and determining the virus titer (plaque assay), Vero cells were used. Vero cells (CCL-81, ATCC) were cultured in DMEM (Fisher Scientific) with 10% FSC. 100 cm^2 petri dishes were used to grow Vero cells. Cells were incubated at 37^oC and 5% CO_2 (Frey, et al., 2009). Cell culture flasks, FCS, and cell strippers were purchased from Fisher Scientific.

Polarization treatments:

U937 cells were grown in RPMI on plates to 50% confluency. For cell polarizing, different treatments were added to the culture media. To induce differentiation of U937 to M0 macrophages, cells were treated with PMA (200 ng/ml) and incubated at 37^oC for 24 hours. After 24 hours, another dose of PMA was added. For M1 induction, cells were treated with recombinant human interferon gamma, (IFN-γ) at 20ng/ml (Peprotech,NJ) ,and lipopolysaccharide,

(LPS) at 20ng/ml. Recombinant human interleukin 4, (IL-4) at 100ng/ml (Peprotech,NJ) was added to induce M2 phenotype differentiation. The day following treatments, the cells were centrifuged and resuspended in 1 ml of fresh media for analysis (Frey, et al., 2009).

Cell Viability:

After reaching 50% confluncy in culture plates, U937 cells were differentiated. For control cells, IFN-γ and LPS (M1) or IL-4 (M2) was applied without the virus. For the other group of cells, administration of HSV-1 (MOI of 0.1) along with polarization treatments was done (Frey, et al., 2009). After 24 hours, cells were transferred to centrifuge tubes and centrifuged for 5 minutes. The supernatants were discarded and pellets were resuspended in 1 ml of fresh media. Trypan blue dye (Fisher Scientific) was used to perfourm the trypan blue exclusion test and viable cells were counted using hemocytometer.

Immunofluorescent staning

U937 cells were cultured in cm^2 plates in RPMI enriched media. When cells were 50% confluent, different treatments were introduced. Cells were treated with PMA for 24 hours. The next day, IFN-γ and LPS were added for (M1) cells, while IL-4 was added for (M2) cells. HSV-1 was added along with these treatments, while the control cell group was not exposed to the virus. After 24 hours of treatment with or without the virus, cells were washed three times with 1% BSA, (0.5 gm of bovine serum albumin was dissolved in 50ml of 1X phosphate buffered saline). Then cells were fixed with 4% paraformaldehyde for

15 minutes at room temperature. Another washing with 1% BSA followed the fixation step. Then, cells were permeabilized with iced acetone and the plates were then placed in -20^{o}C freezer for 10 minutes. After that, cells were blocked with 3% BSA for an hour at room temperature. Sample staining was done following the blocking procedure. Dilutions of antibodies were made using the blocking buffer (3% BSA).

Table1. (Antibody Concantrations for Immunofluorescent staning)

Stain/ Antibody	Concentration	Company
Red-Phalloidin X	3 µl/million cells	Life Tech.
FITC Anti-human CD14	5 µl/million cells	BioLegand
BV Anti-human CD86	5 µl/ million cells	bioLegand

After adding antibody dilutions, cells were incubated overnight at 4^{o}C. 24 hours later, cells were rinsed gently three times with 1% BSA. Coverslips were added carefully to the slides along with a mounting medium (Vector Laboratories). Slides were analyzed by Fluorescence microscopy.

Flow cytometry analysis:

U937 cells were cultured on plates. At 50% confluncy, cells were exposed to different treatments. As control, cells were treated with PMA for 24 hours. Next day, some cells were polarized with LPS and IFN-γ (M1) or IL-4 (M2). The other group of cells were treated as well and infected with HSV-1 at the same time. Then cells were incubated at 37^{o}C for 24 hours. Then, cells suspensions were centrifuged and pellets were re-suspended in 1 ml fresh RPMI media. Numbers of cells were determined for each sample in the hemocytometer using trypan blue exclusion method. After that, cells were washed three times with 1% BSA/PBS. Following each washing step, cells were centrifuged for 5 minutes. Cells were blocked with 3% BSA for an hour at room temperature to prevent any non-specific binding of antibodies. Flourochrome-conjugated primary antibodies (BioLegand) were diluted in the blocking buffer (0.1-10 μg/ml). Then, cells were incubated with the antibody dilutions for one hour at 4C. After that, cells were rinsed three times with 1% BSA. Pellets were then suspended in 500μl - 1ml of a solution, which was made of 1.5 ml of FCS and 0.15 sodium azide dissolved in 15 ml of 1X PBS, following the Abcam.com recommendation.

Table.2 (Antibody concentration for Flow Cytometry Analysis)

FITC anti-human CD-14	5μl/million cells	BoiLegend
PYC-F anti-human CD-86	5μl/million cells	BioLegend
CD-206	5μl/million cells	BioLegend

Virus titer determination:

After the cells were treated and infected, cell lysate was prepared to determine virus titer. Cells were centrifuged for 5 minutes, and fresh media (500µl-1 ml) was added to the pellets. Suspensions were placed at -20^{o}C for 24 hours. Next day, cells were thawed and frozen again at -20c^{o} for another day. The process of thawing and freezing was repeated two to three times. Suspensions were then centrifuged and supernatants were used to infect monolayers of vero cells in six-well plates. Plaque assay was done using serial dilution factors (10^{-1}- 10^{-6}) to determine virus plaque forming unit (PFU).

Statistics:

Sigma Plot.12 software was used to analyze the data. Data are represented as mean ± SEM.

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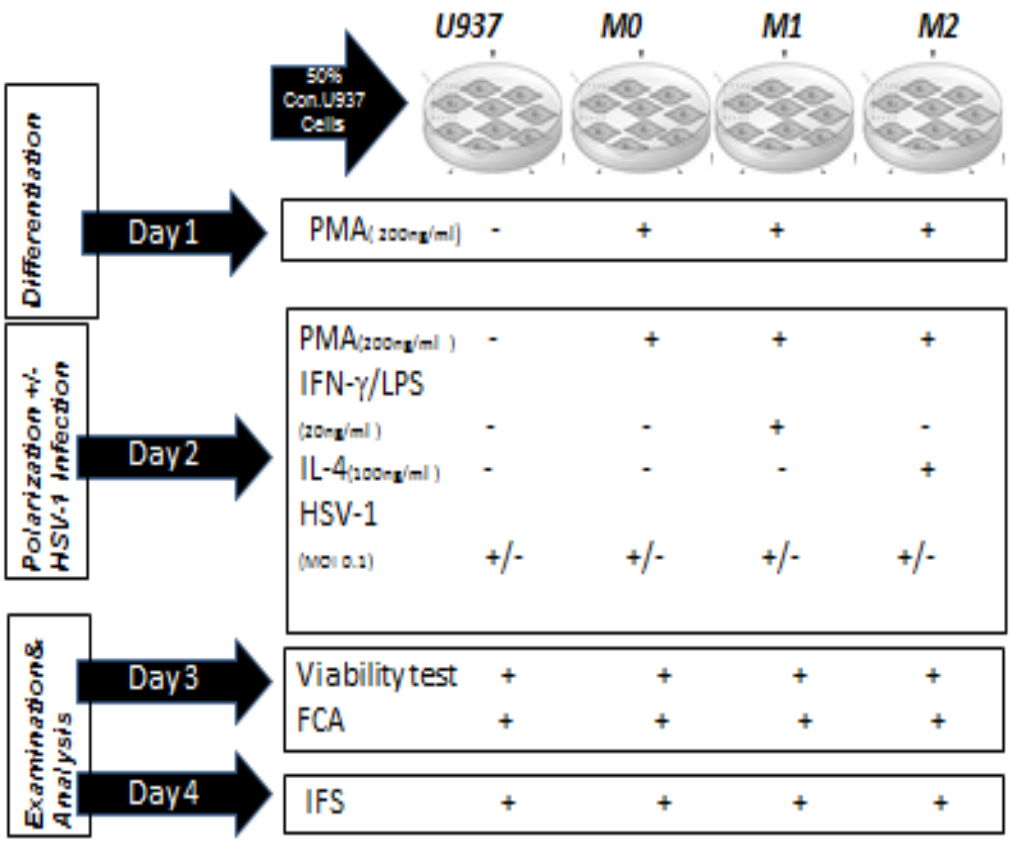


Figure 6.Summary of In vitro Differentiation and Polarization steps.

U937 cells are treated with PMA differentiated to M0 cells. M0 cells treated with IFNγ /LPS to be polarized to M1 cells. M0 macrophages treated with IL-4 to be polarized to M2 cells.

Results

Differentiation and Polarization of U937 cells Decreases their Viability

Differentiated and polarized cells exhibited a significant decrease in cell viability in comparison to the untreated U937 control cells (Fig.8). Following treatment with PMA for 24 hours, M0 cells showed a 59% decrease in cell viability compared to control cells($p < 0.001$). M1 polarized macrophages showed a 54% significant decrease in cell viability after IFN-γ and LPS treatments ($p < 0.001$). IL-4 treated M2 macrophages displayed a 75% decrease in their viability ($p < 0.001$). Interestingly, the differences in cell viability between M0 and M1 were significant ($p<0.05$); the differences between M1 and M2 were also significant ($P < 0.05$).

PMA-Stimulation of U937 Monocytes Enhances their Differentiation and Causes their Adherent, M0 Macrophages

U937 cells are not adherent cells. When cultured, they do not adhere to the plates or flasks surfaces. After treated with PMA for 24 hours, they attached to the surfaces and differentiated to M0 macrophages (Fig.9).

Cytokines microenvironment causes Polarization of M0 macrophages to activated phenotypes, M1 and M2

M0 cells, when treated with IFN-γ and LPS for 24 hours, activated to M1 macrophages. M1 cells were flattened, and developed some pseudopodia. Following treatment with IL-4 M0 cells polarized to M2 macrophages. M2 macrophages were rounded as M0 cells (Fig.10).

PMA-Induced Differentiation of U937 Cells Down-regulates their CD14 Expression Level and Up-Regulates their CD86 Expression Level

26.5% of undifferentiated -U937 cells expressed surface protein CD14. When differentiated with PMA (200ng/ml) for 24 hours to M0 cells, their CD 14 expression level significantly decreased to approximately 2% ($p<0.001$). Also, the PMA differentiation altered the expression level of CD86 in U937 cells. While undifferentiated-U937 cells had a low level of CD86 with a mean value of 0.04%, M0 had an increased level of CD86 expression, of 18.5%. The increase in CD86 expression level between undifferentiated-U937 cells and differentiated M0 cells was significant ($p<0.001$) (table.4).

Polarization of Diffrentiated-U937 Cells (M0 macrophages) to M1 and M2 Phenotypes Alters their CD86 and CD14 Expression levels

(Table.3) polarization of M0 macrophages to M1 phenotype significantly increased the CD86 expression level ($p<0.015$) while decreased their CD14 expression level ($p<0.008$). 18.53% of M0 cells expressed CD86 while 23.52% of M1 macrophages expressed CD86. On the other hand, M0 cells had a higher level of CD14 expression, 1.08%, than M1 cells at 0.44%. Also, IL-4 polarization

of M0 cells to M2 activated phenotype significantly increased their CD86 expression level ($p<0.020$) and decreased their CD14 expression level ($p<0.015$). While 18.53% of M0 cells were $CD86^+$, 22.43% of M2 cells were $CD86^+$. For CD14 expression, M0 showed 1.08% expression level of CD14, while M2 macrophages had a lower level of CD14, 0.63%. Interestingly, M1 exhibited the highest population of $CD14^+/CD86^+$ cells with a percentage of 23.71 in comparison to M0 and M2. Additionally, there were not any significant differences in expression level of surface protein CD206 between M1 and M2 polarized phenotypes.

Herpes Virus Type 1 Infection:

HSV-1 infection of polarized Macrophages insignificantly decreases their viability

HSV-1 infection did not affect U937 cells viability. Virus-infected U937 cells exhibited an increase in viability (Fig.12/13). PMA-treated M0 cells showed a non-significant decrease in their viability compared to uninfected-M0 cells ($p>0.05$). HSV-1 infected-M1 macrophages also had an insignificant decrease ($p>0.05$) of their viability compared to M1 uninfected control cells. Additionally, IL-4 polarized-M2 macrophages showed no significant change in their viability when infected with HSV-1 for 24 hours. However, infected- M0 macrophages showed a 82% decrease in their viability. This reduction was significant (p-value <0.001) compared to U937-infected cells. HSV-1 infected -M1 macrophages

showed 75% decrease (p-value<0.001) and IL-4 treated M2 macrophages also showed a 88% decrease (p-value <0.001) in their viability compare to virus-infected U937 cells. Collectively, differentiation and polarization of U937 cells decrease their viability, not the HSV-1 infection.

HSV-1 Infected M0, M1and M2 Macrophages Exhibit Morphological Changes Compared to Uninfected M0, M1 and M2 Cells.

Control uninfected M0 macrophages were rounded. HSV-1 infection altered M0 morphology in which they became slightly irregular and the infection also caused them to cluster (Fig.23). HSV-1 infection also changed the morphology of M1 cells. While uninfected-M1 cells appeared to be elongated with defined shape and developed some pseudopodia, infected-M1 macrophages became irregular and slightly enlarged, although no significant increase in their diameter was determined (Fig.25). Also, HSV-1 Infection of IL-4-treated M2 cells changes their morphology. HSV-Infected M2 cells exhibited slightly flattened shape in comparison to uninfected M2 (Fig.27). Collectively, HSV-1 infection caused most of M0, M1 and M2 cells to cluster and to be attached to each other.

HSV-1 infected Undifferentiated U937 Cells and PMA-differentiated M0 Cells Exhibit no Significant Alterations of their CD14 and CD86 Expression levels Compared to Uninfected cells

U937 cells and M0 macrophages express both CD14 and CD86. HSV-1 infection insignificantly changed the expression levels of CD14 and CD86 in

undifferentiated U937 and PMA-treated M0 macrophages ($p>0.05$). Uninfected-U937 cells had 26.5% of CD14 expression level while infected cells had a 28.5%. Also, uninfected U937 cells had a 0.04 % of CD86 and HSV-1 infected cells showed an expression level of 0.05. While uninfected M0 cells had a 1.07 and 18.5 expression levels of CD14 and CD86 respectively, infected-M0 cells had 1.22 of CD14 and 23% CD86 (Fig.15/16).

HSV-1 Infection Induces CD86 expression level in M2 polarized macrophages but not in M1phenotype

Uninfected M1 cells had 23.52 % expression level of CD86. When M1 cells were infected with HSV-1, their CD86 expression level did not change, while expression level of CD86 in M2 macrophages significantly increased ($p< 0.001$) after the HSV-1 infection compared to uninfected M2 cells (Fig.17/18). HSV-1 infected M2 cells showed a significant increase in their CD86 expression compared to infected-M0 ($P <0.016$) or infected-M1 ($p <0.002$) cells. However, HSV-1 infection of M1 and M2 cells did not significantly alter their CD14 expression levels compared to uninfected M1 and M2 cells (Fig.15/16).

DISCUSSION

HSV-1 infects human cells, induces the immune system and causes production of inflammatory cytokines (Melchjorsen, et al., 2010). Macrophages play a crucial role in response to viruses. Their microenvironment influences their activation into two phenotypes M1 and M2. Classically activated M1 macrophages are induced by IFN-γ/ LPS and exhibit an antimicrobial features with the ability to trigger Th1 responses. M2 macrophages are induced by IL-4 or IL-13 through the alternative activation pathway and promote Th2 functions (Lolmede, et al., 2009). M1 cells were characterized by their high production of IL-12 and iNOS in response to pathogens (Mantovani, et al., 2004), while the anti-inflammatory M2 cells were characterized by their high production of IL-10 (Gratchev, et al., 2001). Variation of cytokine production among M1 and M2 macrophages distinguishes them from other immune cells (Mantovani. et.al., 2004). In vitro, the human-like macrophages cell line, U937, is highly resistant to the HSV-1 infection. Differentiation of these promonocytes with various treatments such as PMA removes the primary block of virus replication, and disturbs their resistance thus, increasing their permissiveness to the virus (Tenney and Morahan, 1991). In this study we examined the effect of HSV-1 in differentiated U937 cells. Interestingly, previous studies showed that undifferentiated U937 cells have resistance to HSV-1 (Tenney and Morahan, 1991). Here, we differentiated the U937 cells to M0 macrophages then, polarized them to enhance their maturation to M1 or M2 phenotypes. When we

differentiated The U937 cells with PMA (200ng/ml) for 24 hours, the monocytes matured to M0 macrophages and then cells became adherent. Further treatment of M0 cells with LPS (20ng/ml) and inflammatory cytokine IFN-γ (20ng/ml) causes their maturation to M1 proinflammatory phenotype. In contrast, IL-4 treatment of M0 leads to anti-inflammatory M2 cells. After differentiation cell viability decreased in comparison to control untreated-U937 cells. M0 viability significantly decreased by 59%, and M1 viability decreased by 54% while more reduction was found in M2 cells in which their viability decreased by 75%. U937 lymphoma cells expressing PPARγ (peroxisome proliferator-activated receptor γ), which works as an apoptotic-inducers (Konopleva, et al., 2004). IL-4 is one of PPARγ ligands (Huang, et al., 1999). Since we used IL-4 as polarization treatment for M2 phenotype, the decrease of their viability could result from the interaction between apoptotic receptor PPARγ and its ligand IL-4. So, we induced apoptosis on these cells by using IL-4 treatment. Other polarization treatments such as IL-33 and IL-10 should be tested to support this explanation. Interestingly, although mouse macrophages J774A.1 express the PPARγ (Kao, et al., 2009) as U937 cells, IL-4 treatment exhibited greater effect on U937 cells than those mouse macrophages. When we infected the differentiated and polarized macrophages (M0, M1 and M2) with HSV-1 (MOI=0.1) we found that HSV-1 infection decreased viability of M0, M1, and M2 cells in comparison to control uninfected U937 cells. M0 viability decreased by 82%, M1 decreased by 74% and M2 cells by 88%. Furthermore, the viability of infected- differentiated and polarized macrophages did not significantly decrease when compared to

uninfected M0, M1 and M2 cells suggesting that the differentiation and polarization process exhibit greater effect on these U937 cells than the HSV-1 infection. We concluded that polarization, rather than HSV-1 infection caused the significant alteration on these macrophages similar to what had been found in murine macrophages. Interestingly, we also found that HSV-1 infection caused morphological changes to these polarized macrophages (M0, M1, and M2). After infection, M0 cells became irregular compared to their prior-infection rounded shape. While uninfected M1 macrophages expressed pseudopodia on their surfaces, we found that after HSV-1 infection, M1 cells became irregularly shaped with fewer pseudopodia. In M2 macrophages, HSV-1 infection alters their oval shape to rounded cells with no vacuoles. Collectively, Infection with HSV-1 caused M0 cells as well as M1 and M2 to cluster together and appeared in groups. M1 and M2 macrophages express different levels of surface proteins CD14 and CD86 (Bonato, et al., 2001). Similar to Lolmede, et. al (2009), we found that M1 macrophages express relatively higher amount of CD86 in comparison to M2 cells. Also, we found that the HSV-1 did not alter the expression levels of CD14 and CD86 in M0 and M1 over what has been observed in uninfected M0 and M1 cells. Interestingly, CD86 expression level on M2 macrophages dramatically increased after HSV-1 infection suggesting that HSV-1 induced their surface protein expression and caused their further maturation like human macrophages. M1 macrophages exhibited the larger proportion of cells expressing both CDs ($CD14^{+}/CD86^{+}$) before and after HSV-1 infection in comparison to M0 and M2 cells. Mannose receptor CD206 is a

marker for M2 macrophages since they express it more than other immune cells (Choi, et al., 2010). Here, we examined CD206 expression level in polarized macrophages prior to HSV-1infection and after the infection. There was not a significant alteration in CD206 expression between polarized -uninfected cells and polarized-HSV-infected cells suggesting that HSV-1 infection neither up-regulates nor down-regulates macrophages' CD206 expression. Collectively, HSV-1 infection up-regulated CD86 expression in differentiated U937 cells and polarized to M2 while it did not alter their CD 206 expression. Moreover, CD206 expression levels on infected cells were similar to control uninfected cells, with M2 macrophages showing the highest expression level of 54%. However, previous studies suggested no role of CD206 in pathogen invasion. Ultimately, our data suggest that differentiated and polarized U937 cells exhibit permissiveness to HSV-1 although future study of the HSV-1 mRNA levels in these differentiated and polarized cells will provide critical hints about their interaction with the virus.

Ultimately our data show the possibility of developing an in vitro model resembling human macrophage polarized phenotypes (M1 and M2) from U937, promonocytes, lymphoma cells and observing their response to HSV-1 infection. Differentiation and polarization of these U937 cells not only allow the herpes simplex type 1 virus to change their morphology but also surface markers expression levels, especially CD86 expression level on M2 macrophages, moreover, the polarization process had a greater effects on cells than HSV-1 infection.

In future, studying of the effect of other polarization treatments would provide a better understanding of the polarization process and its effect on these U937 lymphoma cells. Study of M2 macrophages would be a beneficial measurement to determine the accuracy of in vitro macrophages activation. Specifically, to examine the possibility of IL-4-induced apoptosis on M2 cells, polarization of these cells with IL-33 would be beneficial on clarifying the reason for the decrease in M2 cells viability because IL-33 signals through nuclear receptor, PPARγ as IL-4 (Fig.7). Other polarization treatments could be also used in vitro to stimulate M2 phenotype activation such as immune complex (IgG) and IL-10, which have been found in vivo to stimulate activation of macrophages to M2 cells through alternative signaling pathways. Use of cytokine IL-10 or immune complex (IgG), which signal through specific receptors and do not stimulate PPARγ, would be beneficial on polarization of M2 cells and examine their viability with or without the HSV-1. Additionally, in vitro stimulation of cytokines production in differentiated and polarized U937 cells (M0, M1 and M2) such as IL-12 for M1 or IL-10 for M2 would help in determining to which extent these U937 cells could be used as an in vitro model of human macrophages.

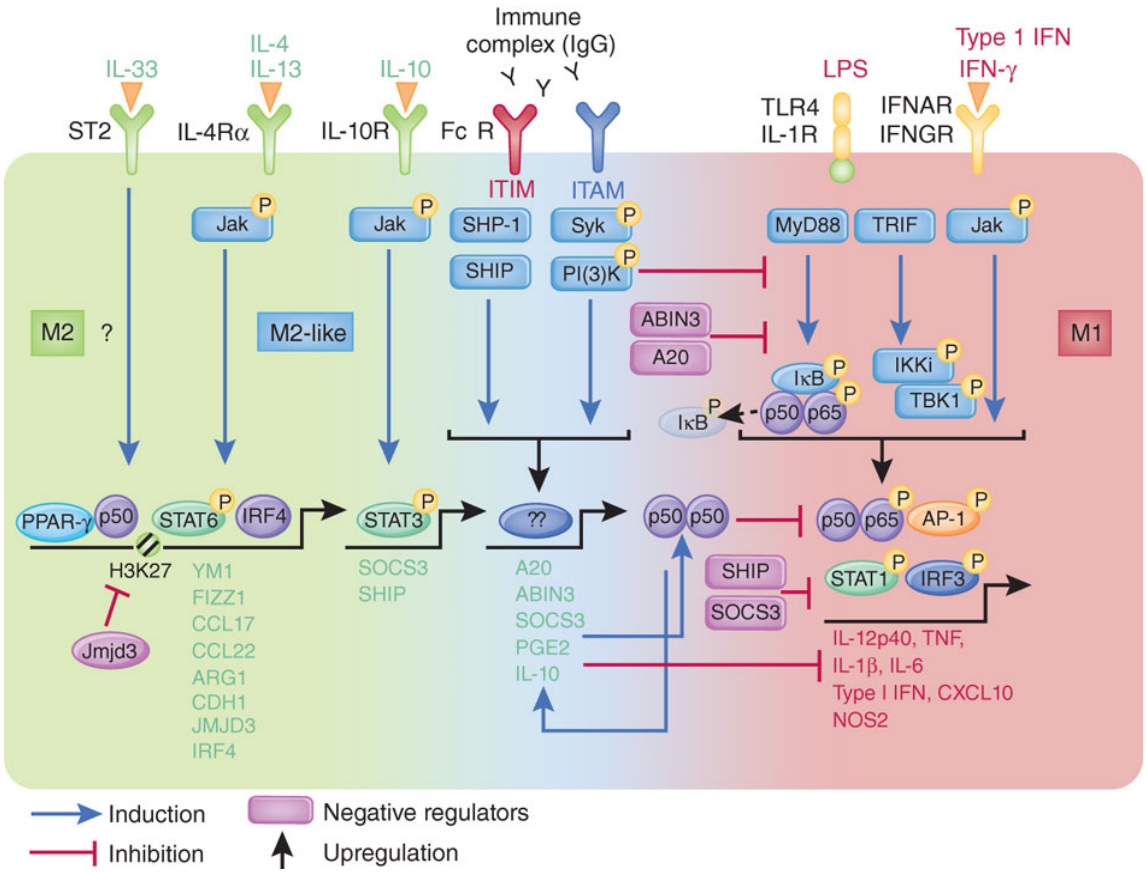


Figure.7.Macrophages polarization via multiple signaling pathways. IL-10 activation of M2 phenotype through specific receptors IL-10R via JAK/STAT signaling pathway. IL-33 activation of M2 phenotype through ST2 receptor and PPARγ nuclear receptor. Alternative activation pathway for M2 cells through Immune complex (IgG) via its ITIM receptor and SHIP-1 or ITAM receptor and its Syk /PI signaling pathway. (Adapted from www.nature.com)

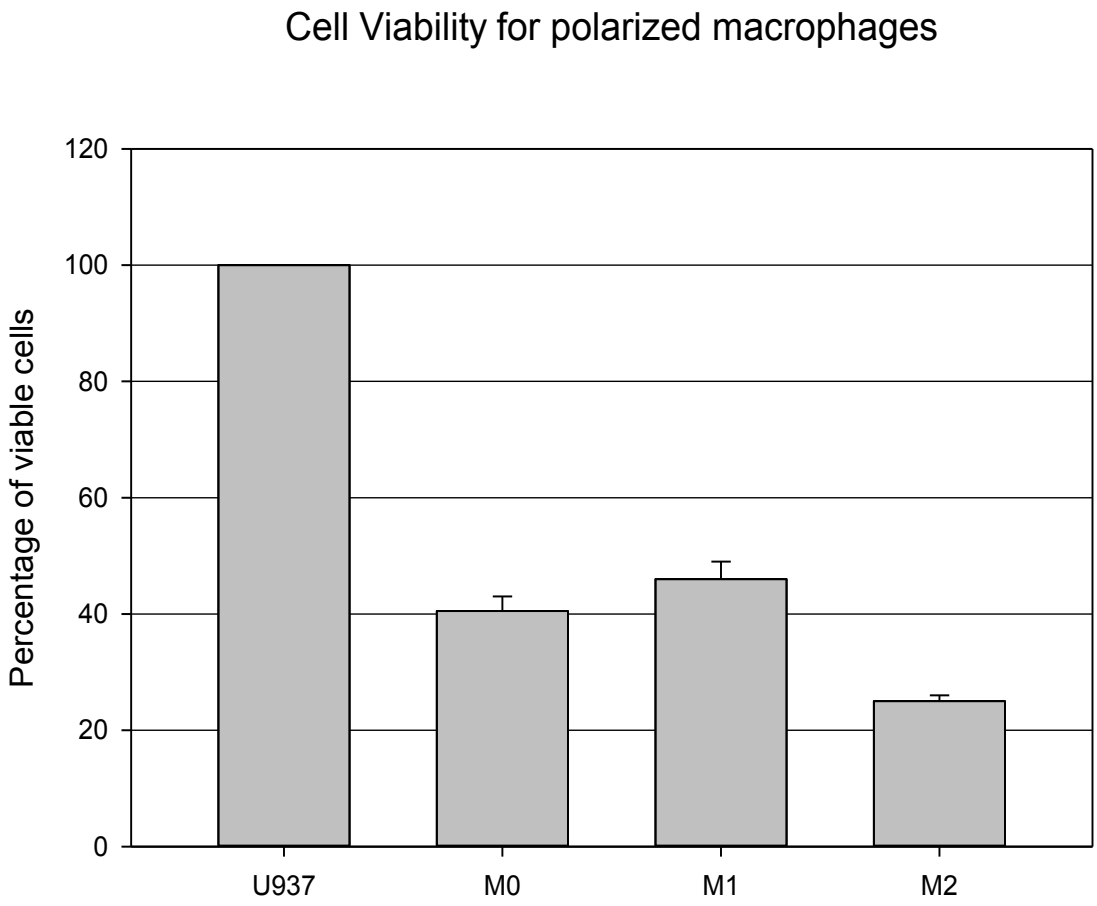


Figure 8. The effect of differentiation and polarization treatments in cells viability in comparison to control untreated U937 cells. M0 macrophages showed a 59.5% decrease (p-value <0.001) in cell viability after PMA treatment. M1 cells showed a significant decrease in cells viability (54 %, p-value<0.001) following 24-hour treatment with LPS + IFN-γ. M2 macrophages exhibited a significant decrease in cell viability (75.0%, p-value <0.001) after 24 hours of IL-4 treatment. Data are represented as SEM.

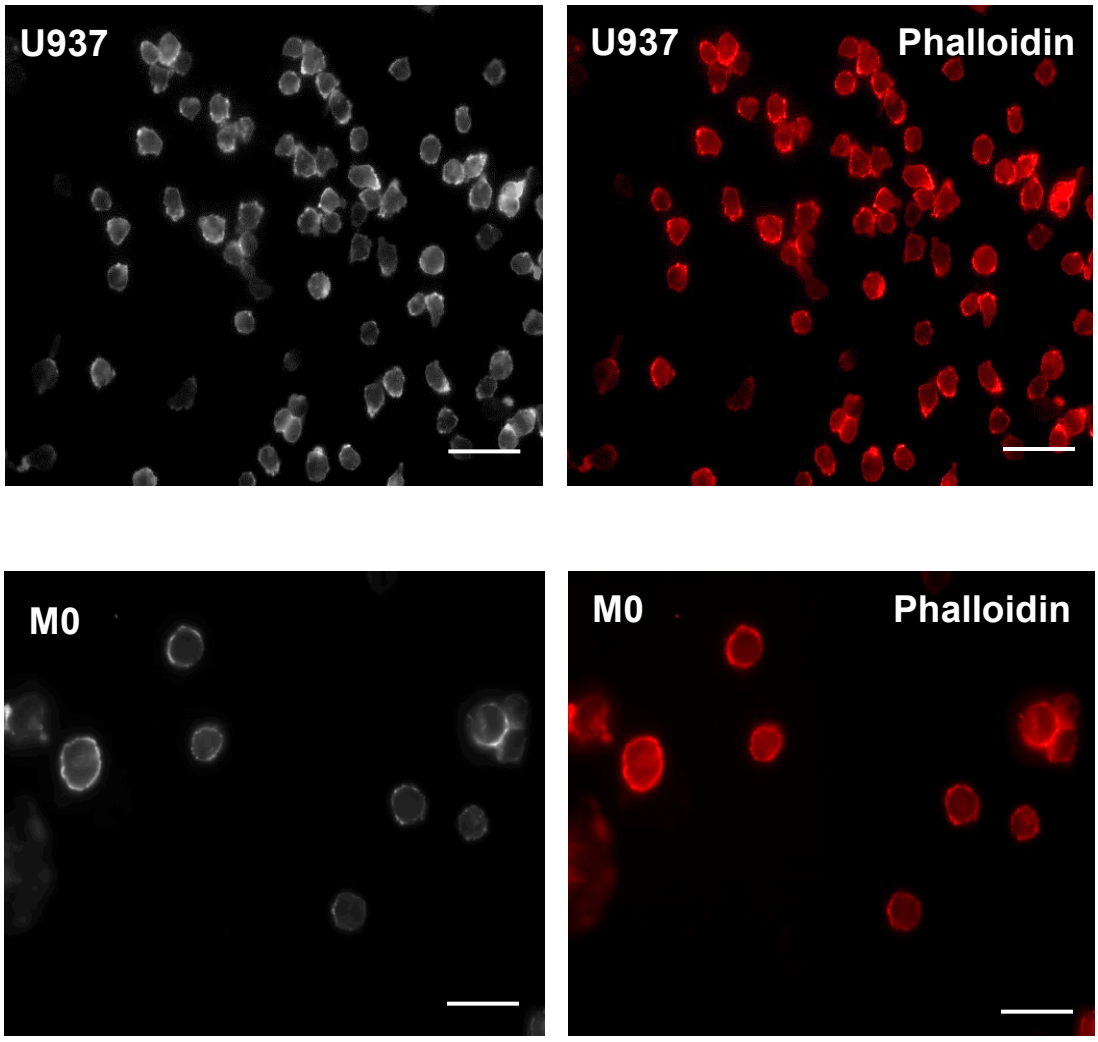


Figure 9. U937 cells and PMA-differentiated M0 cells. U937 non-adherent cells stained with Texas-Red Phalloidin. M0 cells differentiated with PMA for 24 hours stained with Texas-Red Phalloidin. M0 cells were rounded compared to undifferentiated U937 cells (Scale bar = 50 μm).

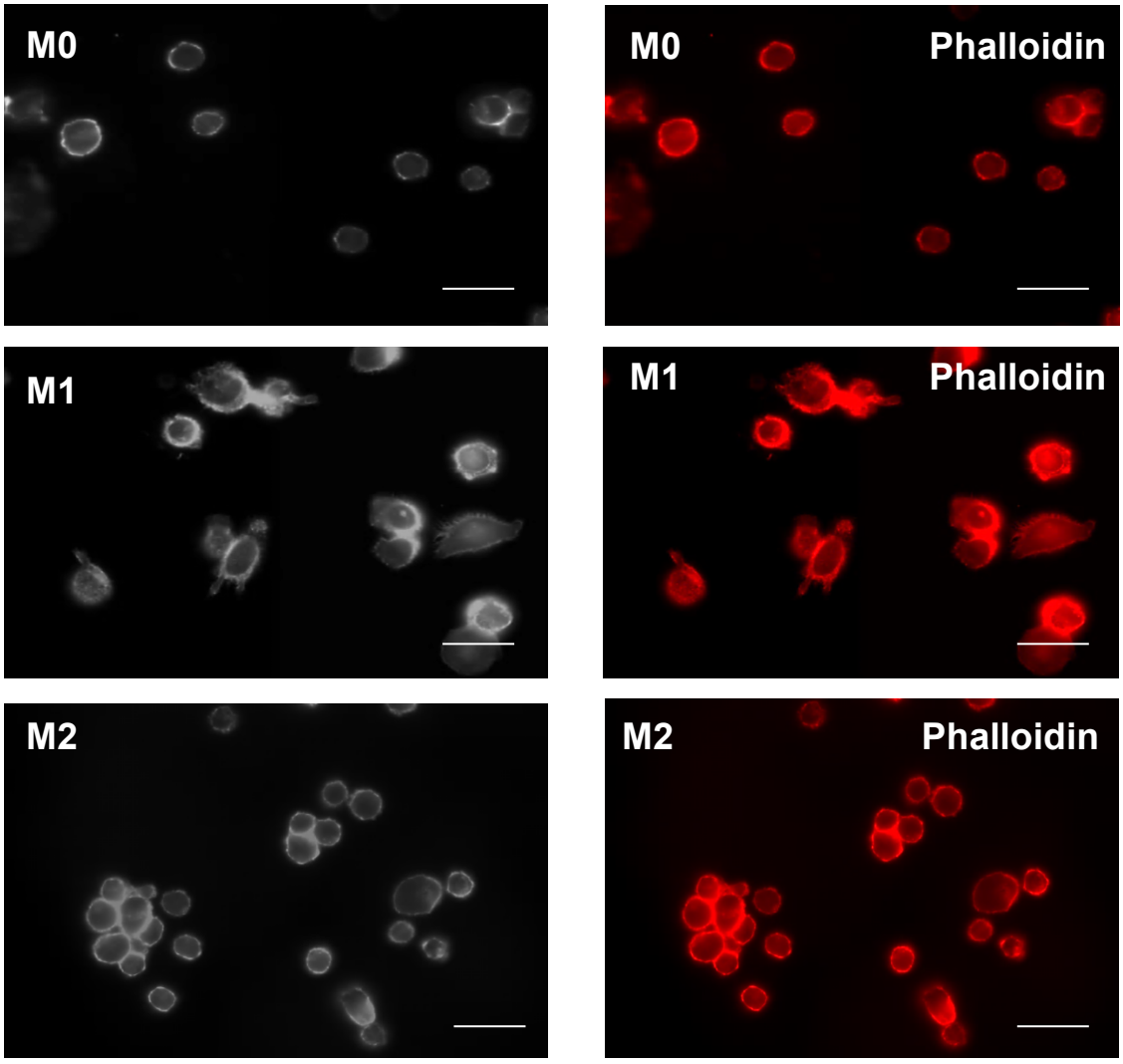
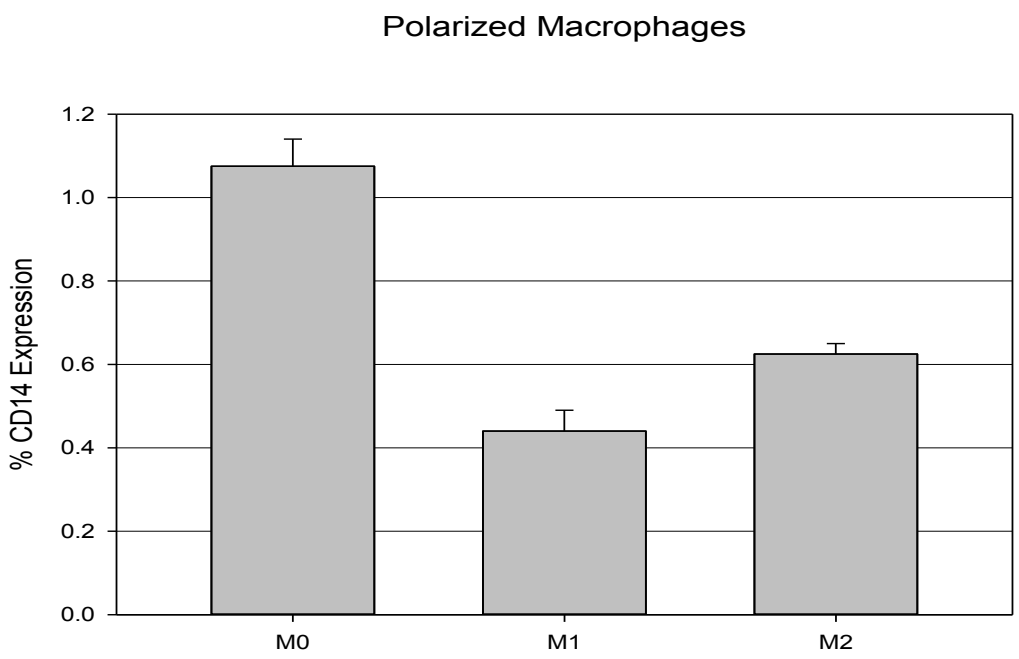
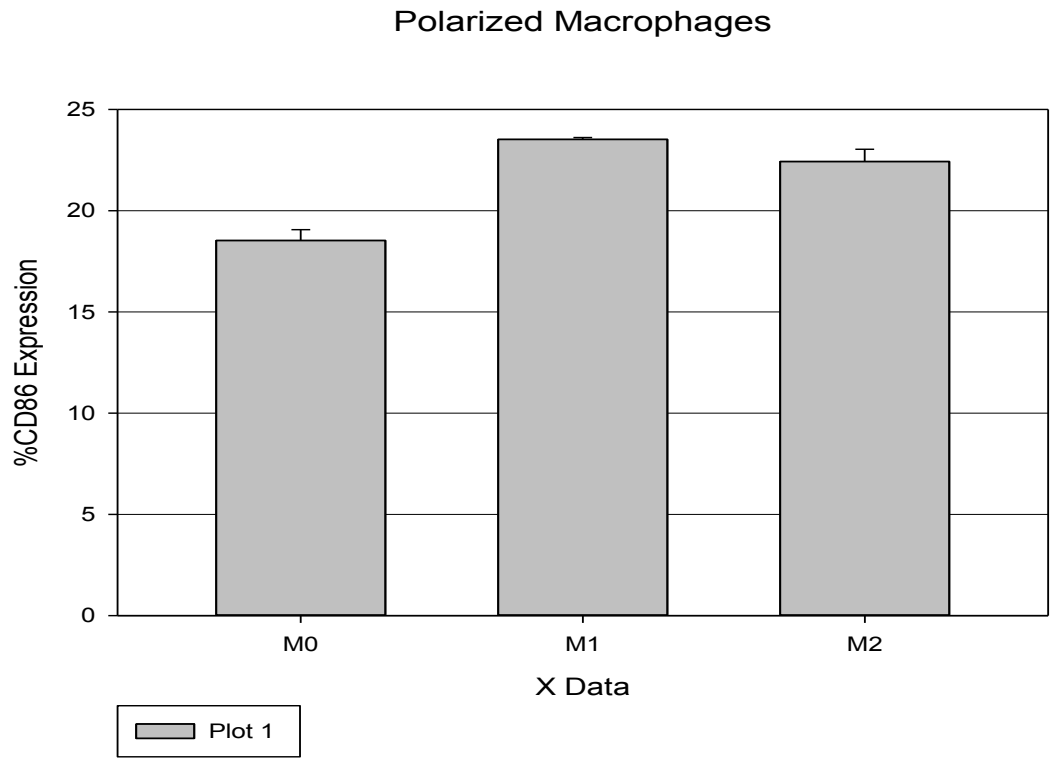


Figure 10. Differentiated M0 cells and Polarized M1 and M2 phenotypes.
M0 cells differentiated with PMA for 24 hours stained with Texas-Red Phalloidin were rounded in shape. M1cells treated with LPS/IFN-γ for 24 hours, stained with Texas-Red Phalloidin exhibited flattened defined shape and developed some pseudopodia . M2 cells treated with IL-4 for 24 hours, stained with Texas-Red Phalloidin were rounded as M0 cells (Scale bar = 50μm).

A)


Polarized Macrophages
% CD14 Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
M0
M1
M2

B)


Polarized Macrophages
%CD86 Expression
25
20
15
10
5
0
M0
M1
M2
X Data
Plot 1

C)
Table 3) Summary of Expression levels of CD14 and CD86 in Differentiated and Polarized Macrophages

Marker	M0	P-Value M0 VS M1	M1	P-value M1 VS M2	M2	P-value M2 VS M0
CD14	1.08	0.008	0.44	**NS**	0.63	0.015
CD86	18.53	0.015	23.52	**NS**	22.43	0.020

Figure 11. Expression levels of CD14 and CD86 in differentiated and Polarized Macrophages. A) CD14 expression in M0, M1 and M2 cells. B) CD86 expression in M0, M1 and M2 cells. C) Table4. Summary of Expression levels of both CD14 and CD86 showing significant differences between M0 and polarized M1& M2 phenotypes. Data are represented as ± SEM.

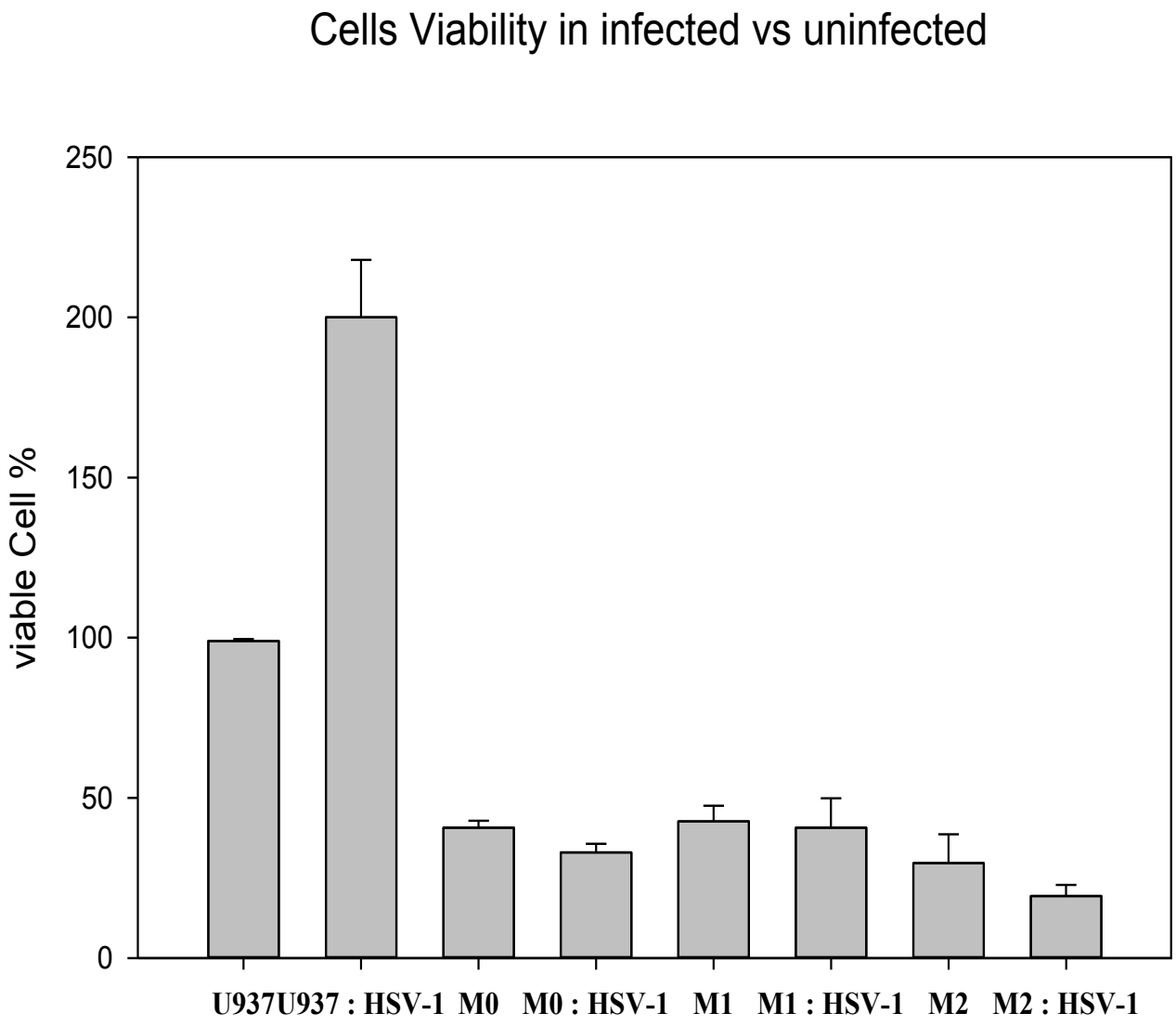


Figure 12.Percentage of viable cells following differentiation, polarization and HSV-1 Infection. M0, M1 and M2 cells showed a significant decrease in cell viability compare to U937 control cells (p-value<0.001). Following HSV-1 infection, M0, M1 and M2 cells showed insignificant decrease in cell viability compare to uninfected control M0, M1 and M2 cells (p-value>0.05).

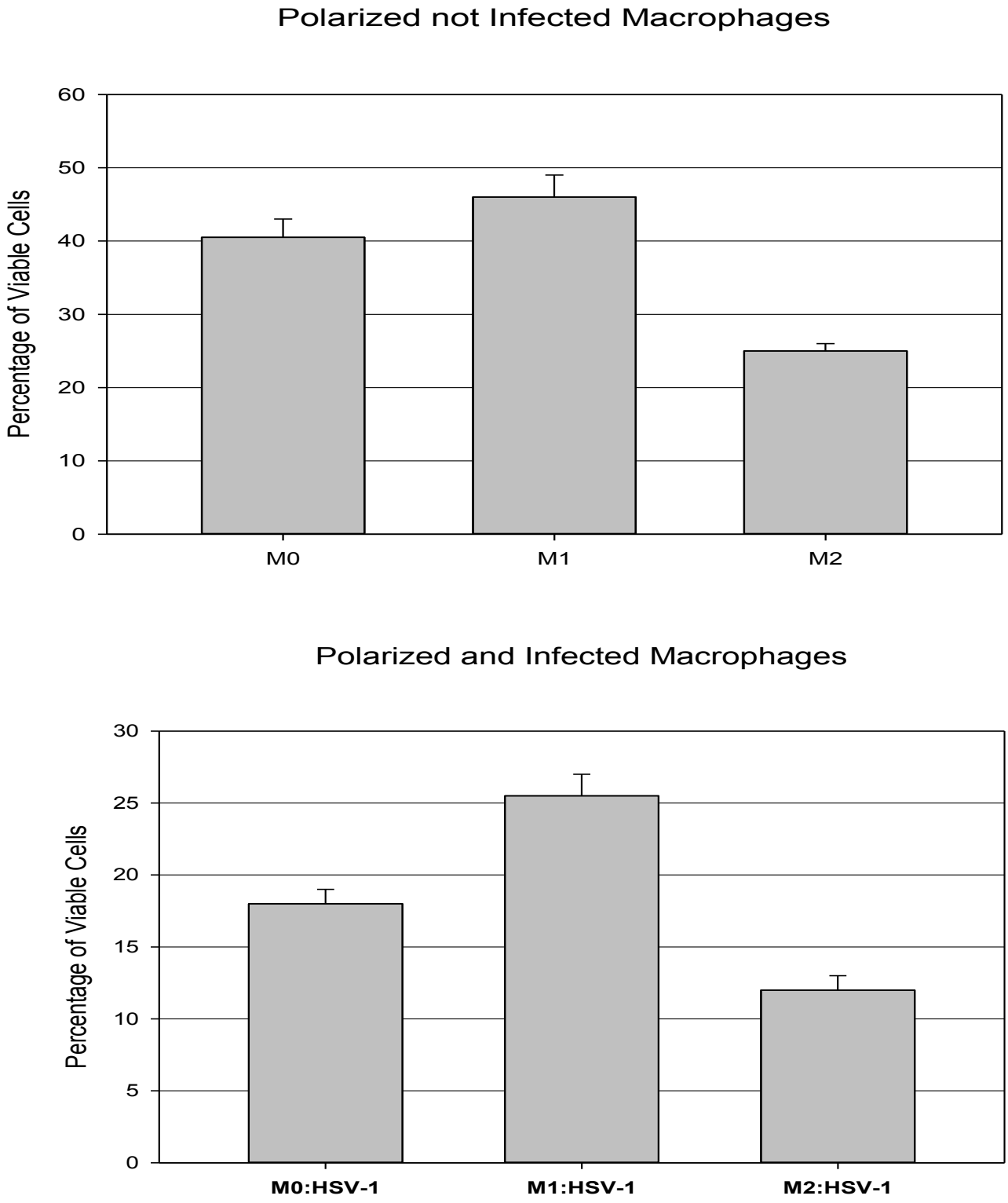


Figure 13.Percentage of Viable Cells following Polarization Treatments. A) Cell viability of differentiated and polarized phenotypes. M2 cells showed significant decrease ($p < 0.036$) compare to M0 &M2. B) Cell viability of infected and polarized macrophages. Data are represented as ± SEM.

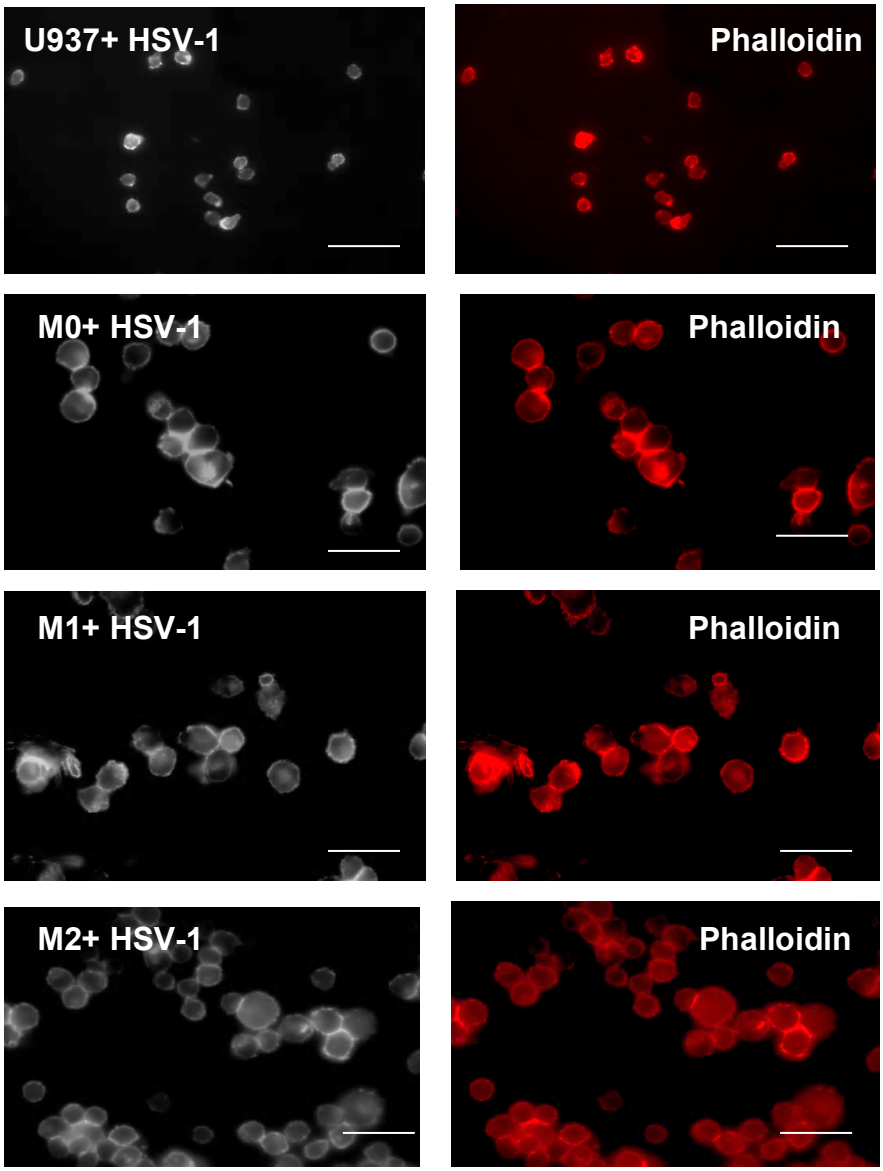


Figure14. **U937 Cells Differentiated, Polarized and HSV-1 Infected.** U937 cells undifferentiated, stained with Texas-Red Phalloidin and infected with HSV-1. M0 cells differentiated with PMA for 24 hours, stained with Texas-Red Phalloidin and infected with HSV-1 were rounded and clustered. M1 cells treated with LPS/IFN-γ for 24 hours, stained with Texas-Red Phalloidin and infected with HSV-1 M1 cells were irregular and flattened. M2 cells treated with IL-4 for 24 hours then stained with Texas-Red Phalloidin and infected with HSV-1. M2 cells appeared rounded and clustered (Scale bar = 50μm).

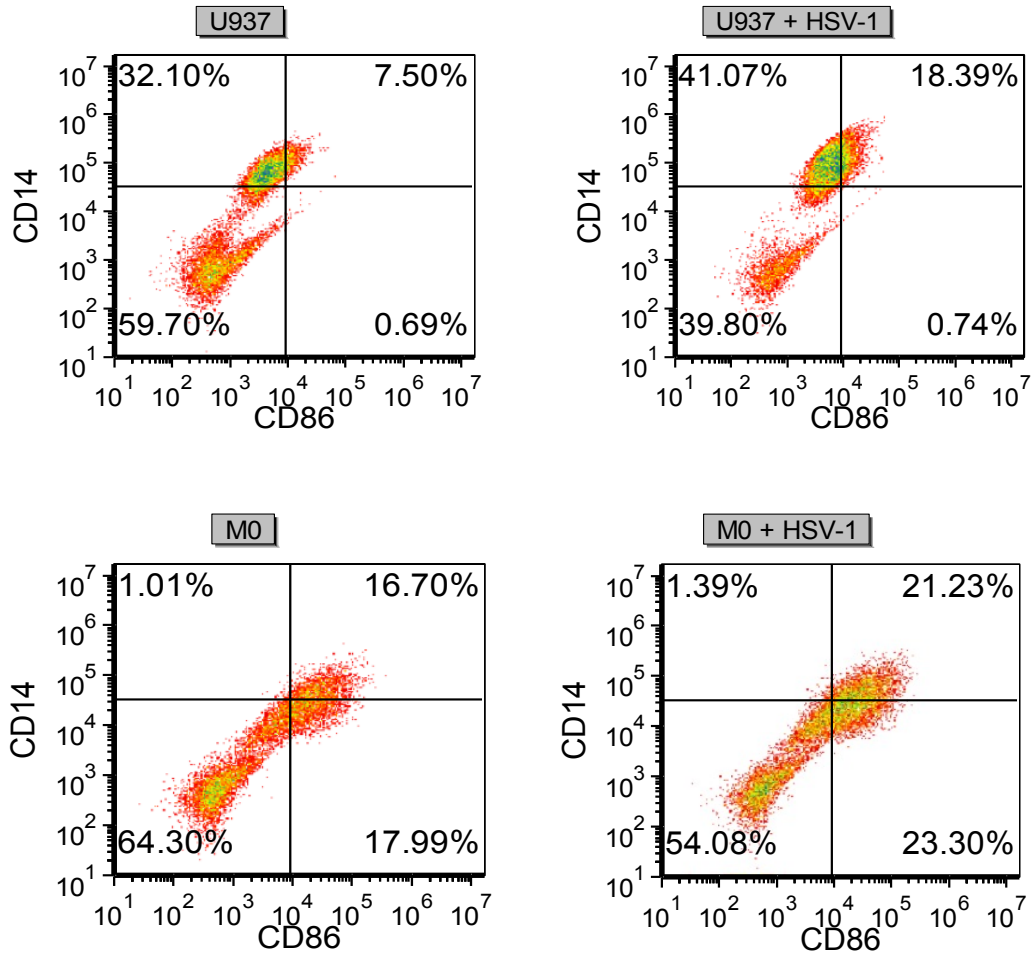
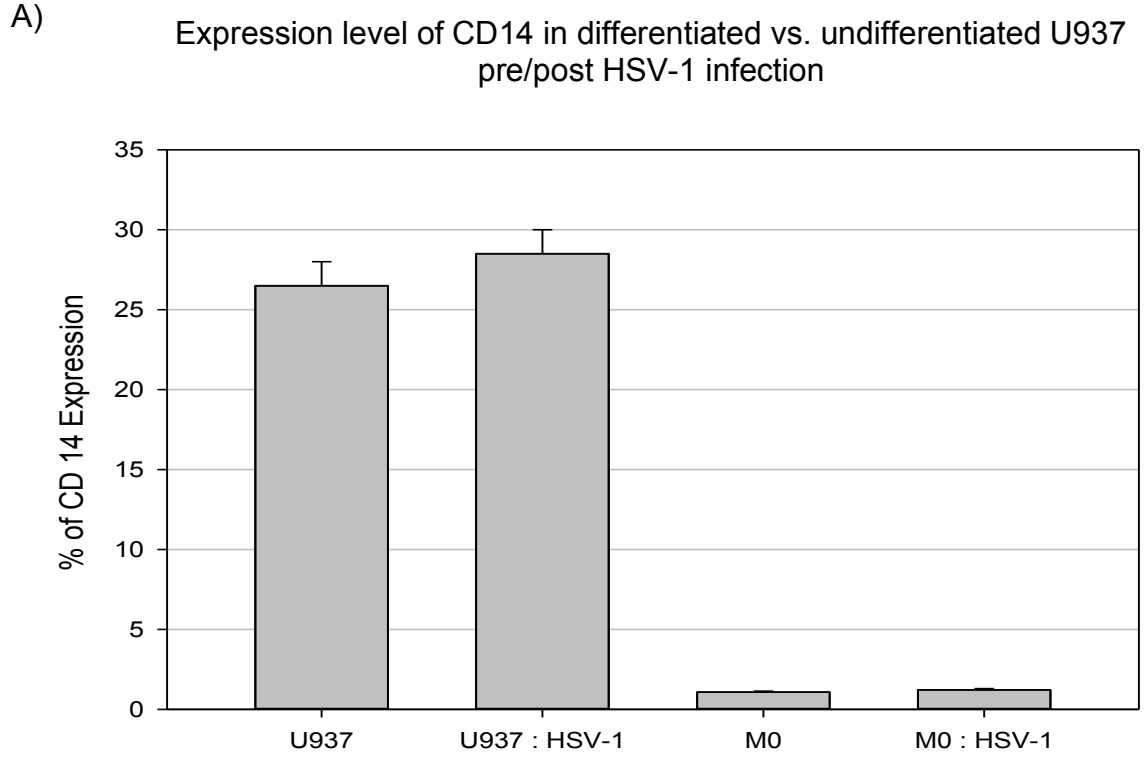


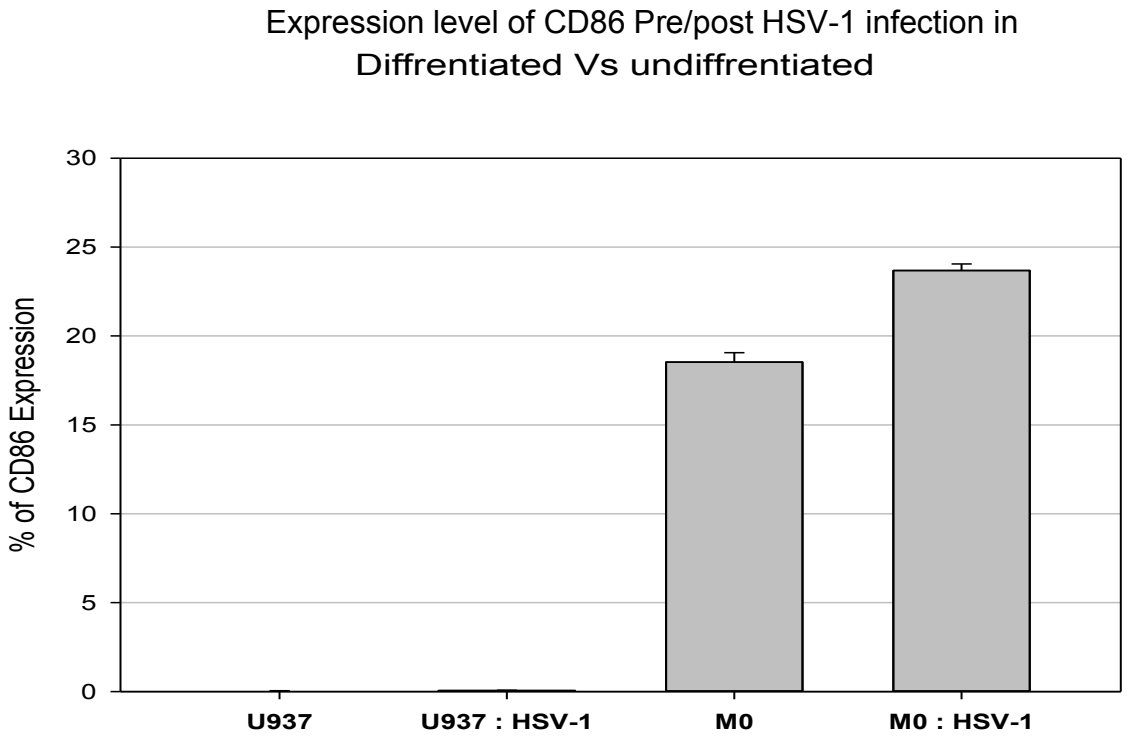
Figure 15. Expression levels of CD14 and CD86 in Undifferentiated (-PMA) and Differentiated (+PMA) U937 Cells pre / post HSV-1 Infection.

Flow cytometric analysis of CD14 and CD86 expression levels in U937 and M0 cells before (Left) and post HSV-1infection (right). Undifferentiated U937 cells showed increased levels of CD14 expression before and after HSV-1 infection ($p<0.001$). 41.07% of Infected-U937 cells expressed CD14 while only 1.39% of infected- M0 cells expressed CD14. M0 cells showed increased levels of CD86 expression. Infected-M0 had a 23.30% CD86 positive cells. Figures above represent a single experiment. Negative Isotope control was used to set experiment quadrants.

A)


Expression level of CD14 in differentiated vs. undifferentiated U937 pre/post HSV-1 infection
% of CD 14 Expression
35
30
25
20
15
10
5
0
U937
U937 : HSV-1
M0
M0 : HSV-1

B)


Expression level of CD86 Pre/post HSV-1 infection in Diffrentiated Vs undiffrentiated
% of CD86 Expression
30
25
20
15
10
5
0
U937
U937 : HSV-1
M0
M0 : HSV-1

C)

Table. 4) Comparison of Significant Differences in Expression of CD14 & CD86 between Polarized Infected and Polarized Uninfected Macrophages.

HSV-1	Surface Marker	-PMA CM / SD	+PMA CM / SD	*P-Value <0.050
-	**CD14**	26.5 2.12	1.07 0.09	< 0.001*
	CD86	0.04 0.014	18.5 0.75	< 0.001*
+	**CD14**	28.5 2.11	1.22 0.11	<0.001*
	CD86	0.05 0.021	23.6 0.70	< 0.001*

Figure 16.Expression levels of CD14 and CD86 in Undiffrentiated-U937 Cells and PMA-differentiated M0 cells before and after HSV-1 Infection. A) Expression level of CD14 in infected and uninfected M0 cells in comparison to U937 cells. B) Expression level of CD86 in U937 cells and M0 cells infected and uninfected. Uninfceted-M0 showed a significant increase in CD86 expression compare to uninfected-U937. C) Comparison of significant differences in expression of CD14 & CD86 between polarized infected and polarized uninfected macrophages. Data are represented as ± SEM.

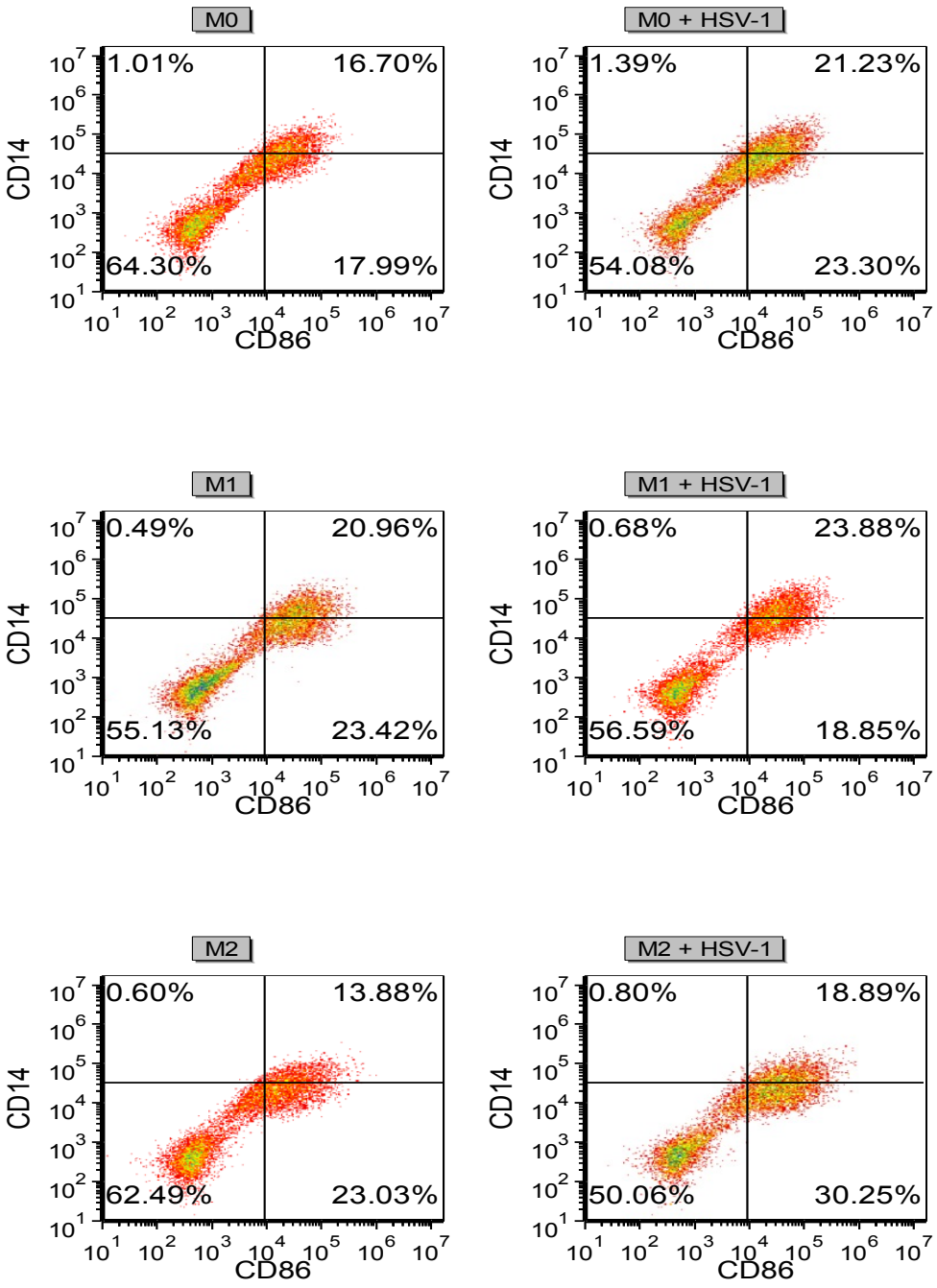
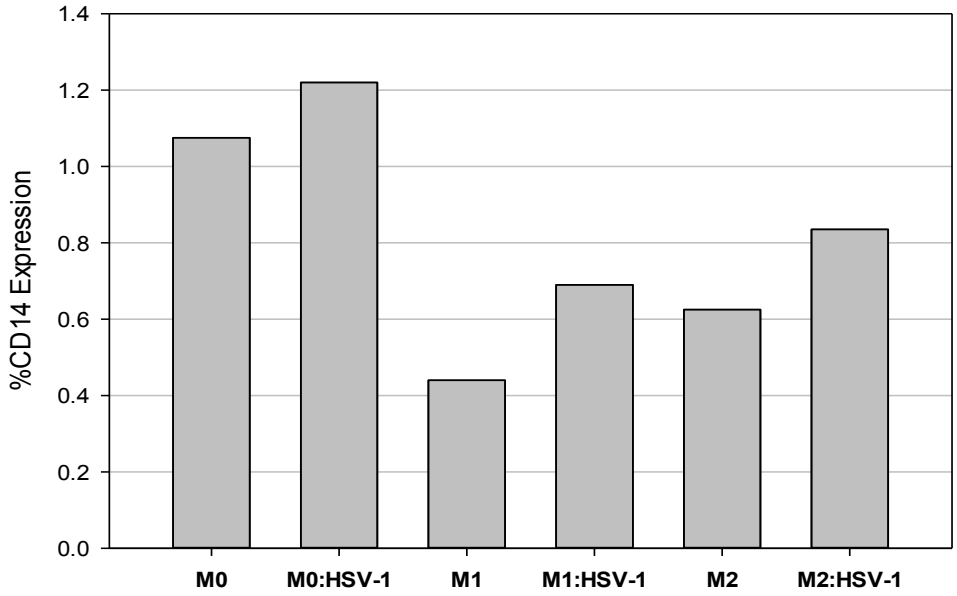


Figure 17. Expression Levels of CD14 and CD86 in Polarized Infected and Uninfected Macrophages. Figures represent flow cytometric analysis of CD14-CD86 expression levels in M0, M1 and M2 from a single experiment. M0 showed an increase in CD14 and CD86 expression levels after HSV-1 infection ($p<0.05$). Infected M1 cells showed a sligh increase in CD14. Infected-M2 macrophages showed the highest level of CD86 expression. Negative Isotope used to set quadrants.

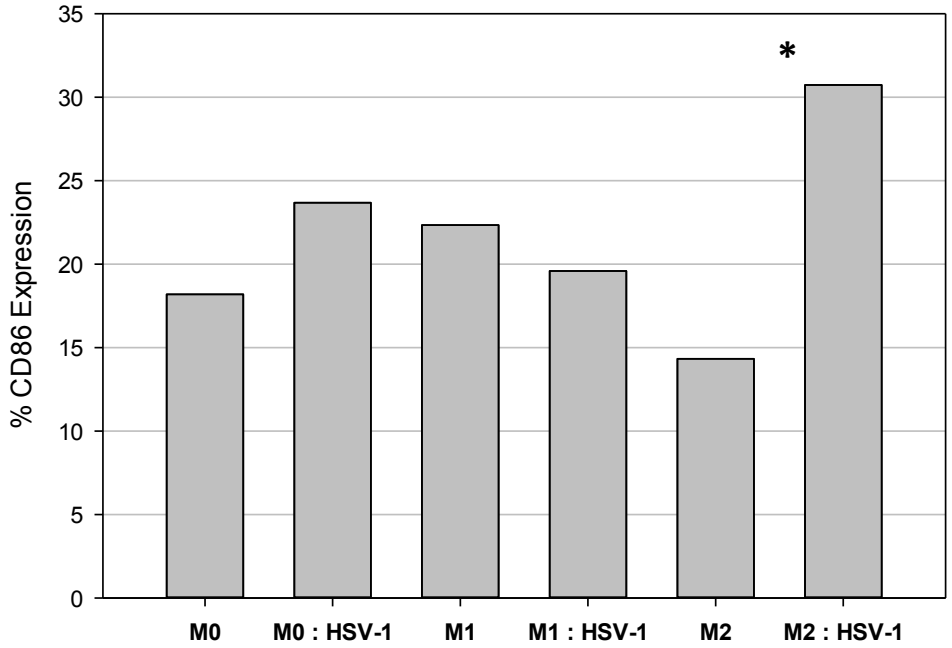
A)

Expression Level of CD14 in Differentiated and Polarized Macrophages Infected vs. Uninfected



B)

Expression Level of CD86 in Differentiated and Polarized Macrophages Infected vs. Uninfected



C)

Table.5) Significant Differences between Polarized Infected and Uninfected cells.

HSV-1	Marker	M0 CM/ SD	P-Value M0 - M1	M1 CM/ SD	P-value M1- M2	M2 CM /SD	P-value M2- M0
-	**CD14**	1.08 0.09	0.008	0.44 0.07	**NS**	0.63 0.03	0.015
	CD86	18.53 2.01	0.015	23.5 0.53	**NS**	22.43 1.9	0.020
+	**CD14**	1.22 0.11	0.015	0.69 0.01	**NS**	0.84 0.049	0.025
	CD86	23.68 0.5	0.014	19.59 1.0	**0.002**	30.73 0.67	0.006

Figure18. Comparison of Average percentage of CD14 $^{+}$ cells and CD86 $^{+}$ cells between Polarized Infected and Polarized uninfected Macrophages.

A) Expression of CD14 in polarized infected and uninfected macrophages. Infected M0, M1 and M2 cells showed insignificant changes in their expression compare to uninfected cells. B) Expression of CD86 in polarized infected and uninfected cells. Infected-M2 cells had a significant increase in CD86 expression compare to infected M0 ($p<0.006$) Infected M1 ($p< 0.002$) and uninfected M2 cells ($p< 0.001$). C) Table, Significant differences between polarized infected and uninfected cells. Data are represented as ± SEM.

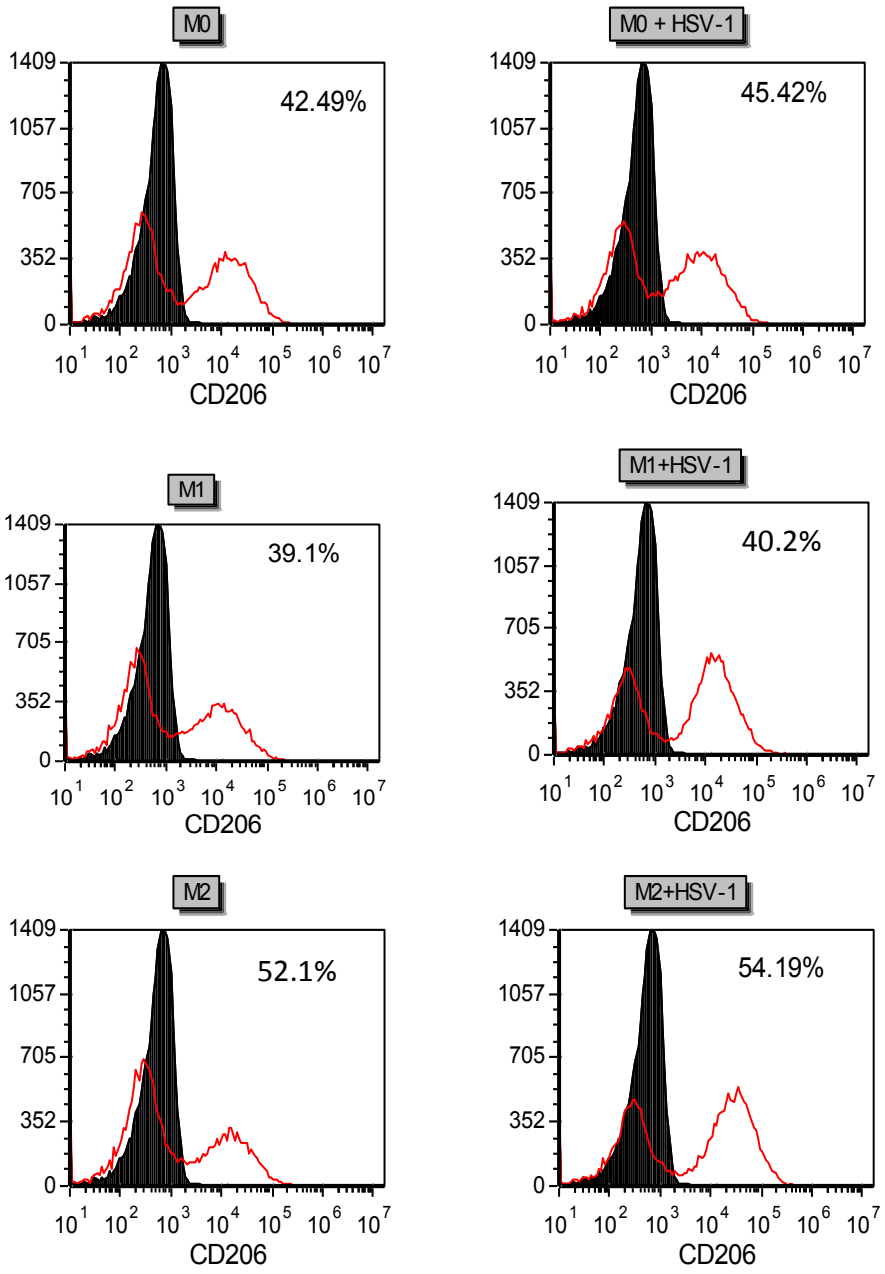


Figure 19. Expression of CD206 in Polarized Macrophages. Flow cytometric analysis of CD206 expression levels in polarized Macrophages (M0, M1 and M2) before (Left) and post HSV-1infection (right). M0, M1 and M2 showed insignificant differences in their CD206 expressions before and after HSV-1 infection. Figures above represent a single experiment. Negative Isotope control was used to set experiment quadrants.

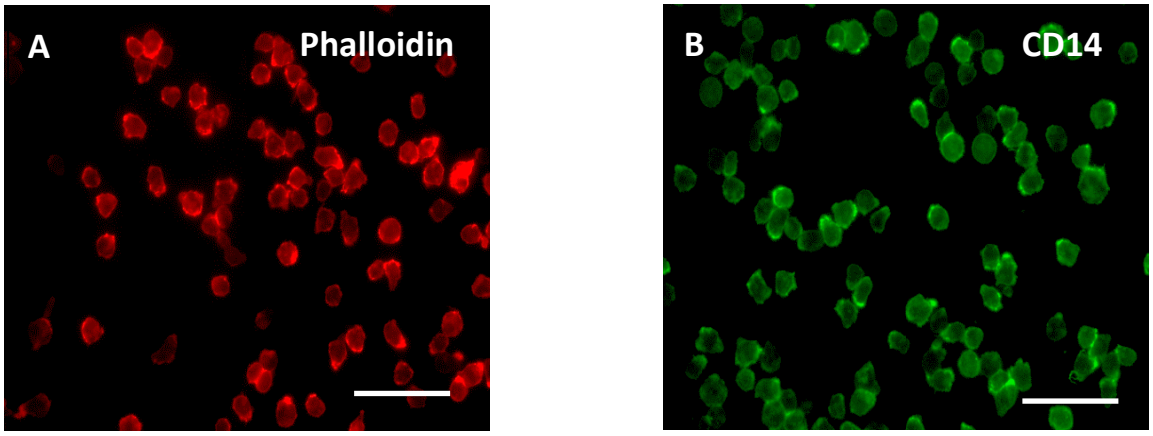


Figure20. U937 Cells. A) U937 cells stained with Texas-Red Phalloidin for Actin.Cells were un-adherent with irregular shape. B) U937 cells stained with FITC conjugated antihuman-CD14 antibodies. Untreated U937 cells expressed CD14. (Scale bar = 50μm).

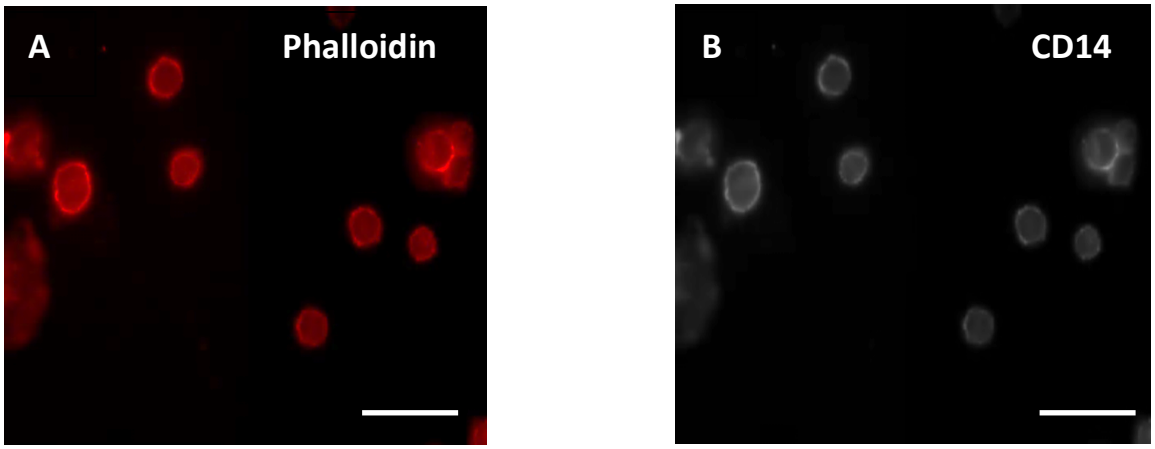


Figure 21. HSV-1 Infected U937 Cells. A) Infected U937 cells stained with Texas-Red Phalloidin for Actin. Cells showed irregular shape. B) Stained with FITC conjugated antihuman-CD14 antibodies. HSV-Infected U937 untreated macrophages express CD14 as well as CD86. (Scale bar = 50μm).

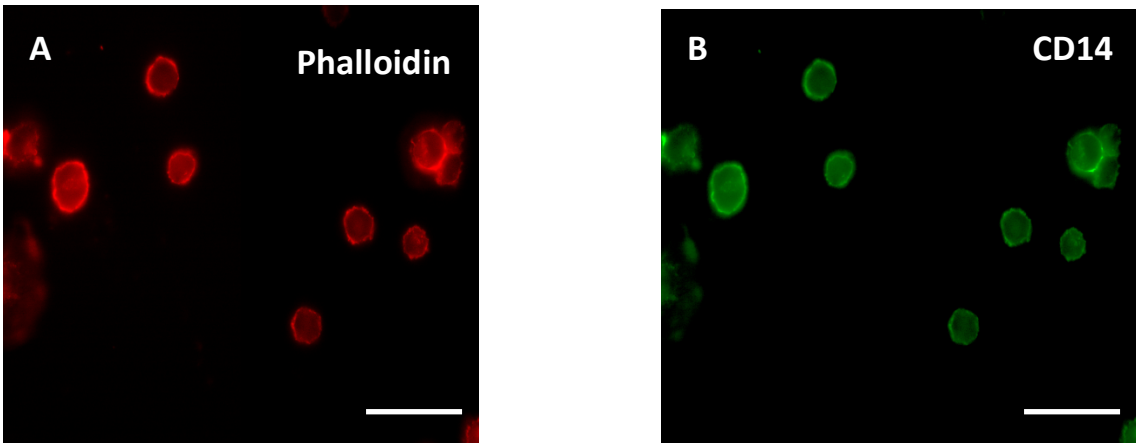


Figure 22. M0 Macrophages. U937 cells treated with PMA for 24 hours. A) Actin stained with Phalloidin Texes-Red . Cells were rounded and adherent. B) M0 cells stained with FITC conjugated antihuman-CD14 antibodies. Cells expressed CD14. (Scale bar = 50μm).

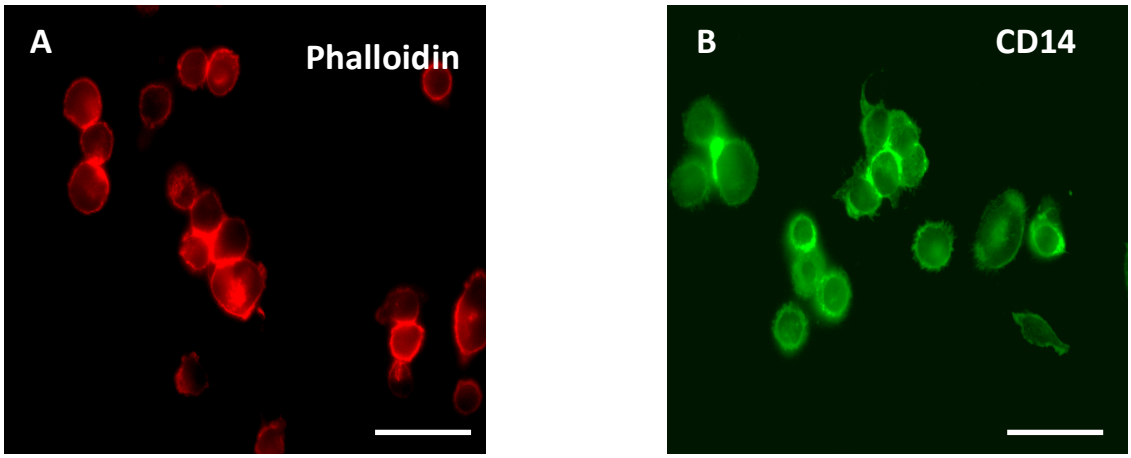


Figure 23. HSV-1 infected M0 macrophages. U937 cells treated with PMA and infected with HSV-1for 24 hours. A) Actin stained with Texes-Red Phalloidin .Infected cells were slightly irregular with rounded shape compared to uninfected cells and appeared in clusters. B) M0 cells stained with FITC conjugated antihuman-CD14 antibodies. Infected-M0 cells expressed CD14 as uninfected cells. (Scale bar = 50μm).

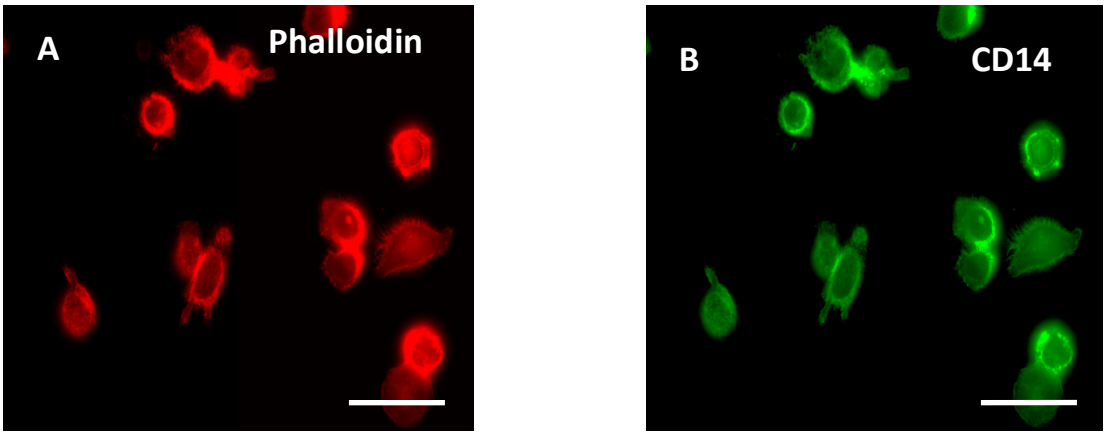


Figure 24. M1macrophages. U937 cells treated with LPS and IFN-γ for 24 hours. A) Actin stained with Phalloidin Texes-Red. Cells were elongated with defined shape and developed some pseudopodia. B) M1 cells stained with FITC conjugated antihuman-CD14 antibodies. M1 cells expressed CD14. (Scale bar = 50μm).

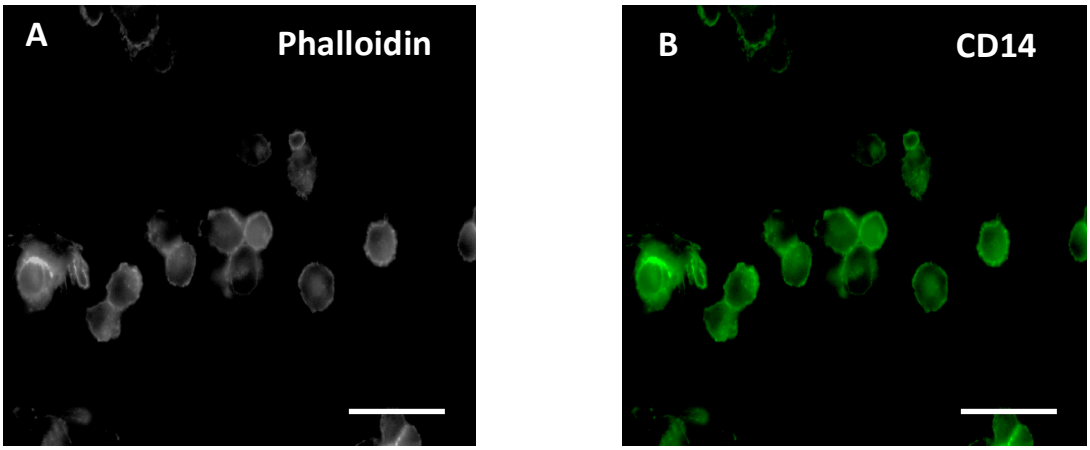


Figure 25. HSV-1 infected M1 macrophages. M0 cells treated with LPS/ IFN-γ and infected with HSV-1for 24 hours. A) Actin stained with Phalloidin Texes-Red. Infected M1 cells became irregular, clustered and slightly enlarged compared to uninfected cells. B) M1 cells stained with FITC conjugated antihuman-CD14 antibodies. M1 cells expressed CD14 as uninfected M1 cells with no significant variations. (Scale bar = 50μm).

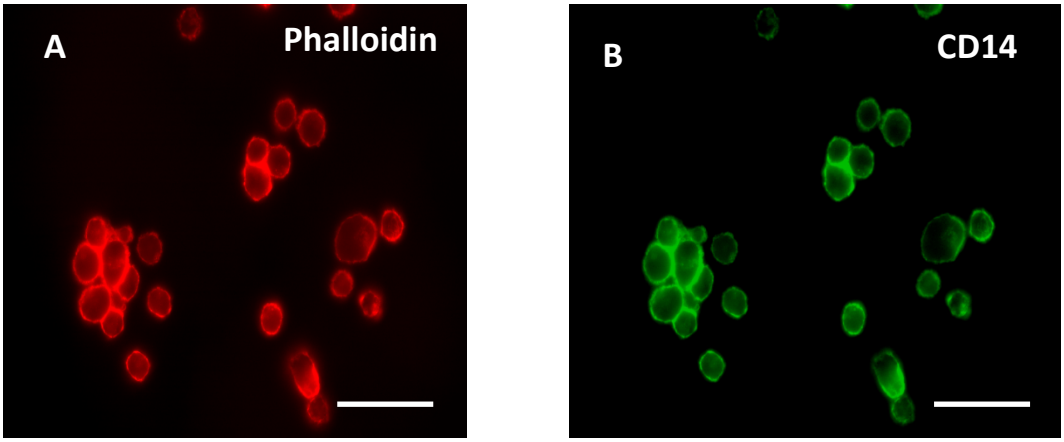


Figure 26. M2 Macrophages. M0 cells treated with IL-4 for 24 hours. A) Actin stained with Phalloidin Texes-Red. M2 cells were rounded and slightly clustered B) M2 cells stained with FITC conjugated antihuman-CD14 antibodies. M2 Cells expressed CD14 as M1cells. (Scale bar = 50μm).

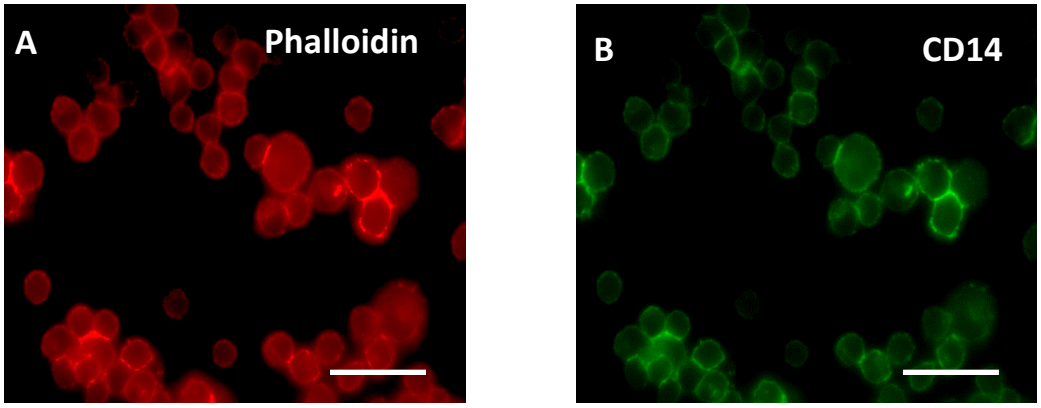


Figure 27. HSV-1 Infected M2 macrophages. M0 cells treated with IL-4 and infected with HSV-1 for 24 hours. A) Actin stained with Phalloidin Texes-Red. M2 infected cells were slightly flattened compared to uninfected M2 cells. B) M2 cells stained with FITC conjugated antihuman-CD14 antibodies. Infected M2 cells expressed CD14 as control M2 cells with no significant difference. (Scale bar = 50μm).

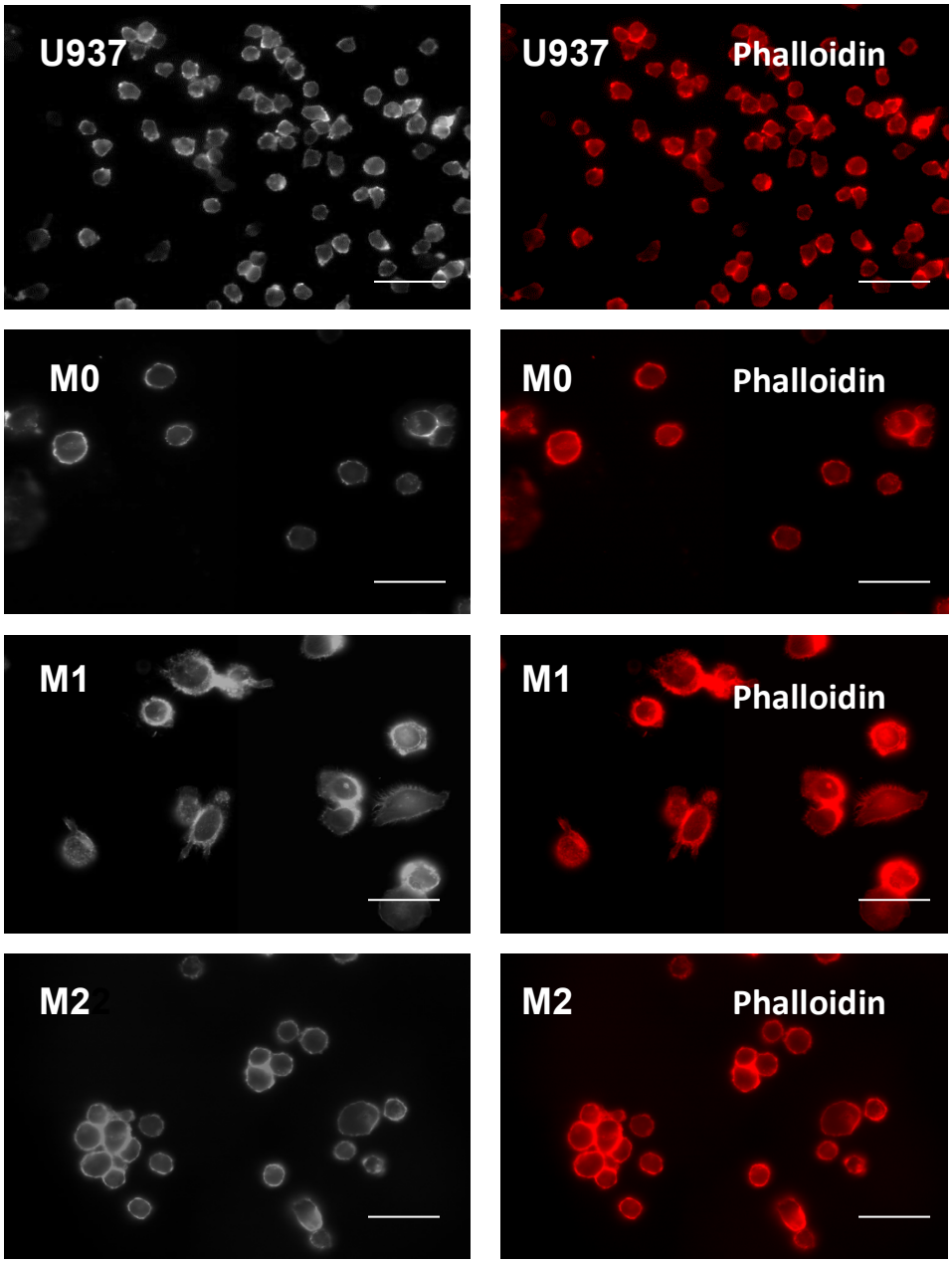


Figure28. U937 Cells Differentiated and Polarized. U937 cells undifferentiated stained with Texas-Red Phalloidin were un-adherent irregular cells. **M0** cells differentiated with PMA for 24 hours stained with Texas-Red Phalloidin were rounded and adherent. **M1**cells treated with LPS/IFN-γ for 24 hours, stained with Texas-Red Phalloidin were flattened. **M2** cells treated with IL-4 for 24 hours, stained with Texas-Red Phalloidin were rounded and cells appeared in groups (Scale bar = 50μm).

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