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The Anti-Apoptotic Effect of HSV-1 on Murine Macrophages: RAW 246.7 Murine Macrophage Cell Line

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The Anti-Apoptotic Effect of HSV-1 ON Murine Macrophages: RAW 246.7 Murine Macrophage Cell Line

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY MOFEDA ALHANGHARI ENTITLED The Anti-Apoptotic Effect Of HSV-1 On Murine Macrophages: RAW 246.7 Murine Macrophage Cell Line. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Herpes simplex virus type 1 (HSV-1) is a worldwide pathogen that affects humans and has the ability to establish a latent state of infection in the sensory nerve ganglia after primary infection of epithelial cells (Boutell and Everett, 2003). HSV-1 is a very contagious virus, which can be transmitted from person to person and cause cold sores in the infected person. Rarely, infection can lead to more serious complications, such as encephalitis. Most HSV-1 infections usually occur in childhood with lifelong potential for symptomatic or asymptomatic viral shedding episodes (Looker et al., 2015). HSV- 1 infects 60%–80% of people throughout the world (Cunningham et al., 2006). The purpose of this study was to examine the anti-apoptotic effect of HSV-1 on polarized and un-polarized RAW 246.7 murine at 4, 12, and 24 hours. We found that viability of M1 macrophages was significantly decreased compared to control cells at 4 hours (p-value<0.016), 12 hours (p-value<0.001), and 24 hours (p-value<0.001). Virus-infected M1 macrophages showed a significant increase in cell viability compare to uninfected M1 macrophage at 24 hours ($P = 0.025$). The percentage of late apoptotic cells in all cell groups (M0, M1, and M2) exhibited a significant decrease after infection with HSV-1 at 4 and 24 hours.

List of abbreviation

HSV-1	Herpes Simplex Virus type 1
HHV-1	Human Herpes Virus type 1
TLRs	Toll Like Receptors
INF- γ	Interferon Gamma
LPS	lipopolysaccharide
IL	Interleukin
NO	Nitric Oxide
TNF-α	Tumor Necrosis Factor Alpha
HVEM	Herpes Virus Entry Mediators
NPC	Nuclear Pore Core
MHC	major histocompatibility complex
TGF-β	Transforming Growth Factor Beta
TRAIL	TNF-related apoptosis-inducing ligand
APaf-1	Apoptotic protease activating factor 1
PRRs	pattern recognition receptors
PAMPs	pathogen-associated molecular patterns
RIG-I	retinoid acid inducible gene-I
DAI	DNA-dependent activator of interferon regulatory factors
CD	Clusters of Differentiation
hTERT	Human telomerase reverse transcriptase
PI	Propidium iodide
Av	Annexin v

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Acknowledgement

Firstly, I would like to express my sincere gratitude to my advisor Dr. Bigley for the continuous support of my study and related research. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my master study. Also, I would like to thank the program director Dr. Hull for her suggestions and guidance on my project. I would like also express my thanks to Dr. Wooley for serving as member on my thesis committee.

Dedication

- *To my parents*
- *To my husband and my beautiful daughter Maria*

1. Introduction

HSV-1, also known as human herpes virus -1 (HHV-1), is a large enveloped virus with a double stranded DNA genome from 120 to 230 kbp which encodes about 84 proteins (Zolini, et al., 2014). HSV-1 belongs to *herpesviridae* family that can be divided into three subfamilies (alpha, beta, and gamma); all herpviruses share common characteristics in their structure, replication cycle, and gene expression (Nishiyama, 1996). HSV-1 is a neurotropic herpes virus that can initiate infections in the stratified squamous epithelial tissue in the oral mucosa, cornea, conjunctiva, and skin. It can cause cutaneous disease on the mouth, face, or genitalia, and, rarely meningitis or encephalitis (Fan et al., 2014). Upon primary infection, the virus infects sensory neurons and establishes a latent infection. HSV-1 can be reactivated and migrates from the sensory neuron body to site of infection (Krug, 2004).

Host immune responses toward HSV-1 include both innate and adaptive immune responses. Innate immune response is mediated by white blood cells such as macrophages, dendritic cells, and neutrophils. These cells function in phagocytosis as well as produce a variety of inflammatory mediators and cytokines (Zolini et al., 2014). Adaptive immune response toward HSV involves the cellular response and is mediated by CD4+ and CD8+ T cells while the humoral response mediated by B cells and antibodies (Egan et al., 2013). Macrophages are a critical component of the innate immune system and have an important role in the host defense mechanism and in inflammation. Additionally, these cells have other functions beside host defense; they play a role in tissue remodeling (Sica and Mantovani, 2012). Macrophages are involved in defense against virus infection by two ways. First, they destroy the cells that are infected by the virus and this is called extrinsic resistance. The second mechanism involves

macrophages inhibiting virus replication within phagocytic vacuoles and this mechanism is called intrinsic resistance (Morahan et al., 1980). Macrophages respond to different signals and, depending on these signals, they differentiate into classical M1 activation, or alternative M2 activation. M1 macrophages are activated by Toll like Receptor (TLR) ligands, interferon (IFN-γ) (Sridharan et al., 2015), or lipopolysaccharide (LPS) **(**Ebtekar et al., 2006), whereas alternative M2 macrophages are activated by interleukin (IL-4), IL-13, or IL-10. /Interleukin-13 (Sridharan et al., 2015). However, M2 macrophages can be divided into M2a, M2b, or M2c that can be activated by different signals (Sridharan et al., 2015). Macrophages play a key role in the pathogenesis of endotoxic shock by producing NO and TNF-α, molecules, which can induce apoptosis in different cell types, including macrophages themselves (Soler et al., 2001). Apoptosis is distinguished by DNA fragmentation, chromatin condensation, and cell shrinkage (Aramaki et al., 1999). Most of the time, apoptosis is accomplished by activating a set of proteolytic enzymes called caspases. Apoptosis is utilized as a defense mechanism by eliminating the infected cells, which may be harmful if they survived (Lawen, 2003). This process can be induced in susceptible cells by different and normal physiological stimuli as well as harmful ecological conditions (Ramamoorthy and Tizard, 1998). Apoptosis acts as an extremely efficient defense mechanism against virus infection. Apoptosis helps to remove virus proteins and nucleic acid from the infected host (An et al., 1999). However, HSV-1, as other huge DNA-containing viruses, utilizes various viral resistance systems that collaborate to avert unexpected death of the host cell prompted by an assortment of intracellular and extracellular signals. The infection alonge increases the yield of viral offspring after lytic contamination of target cells. HSV-1 proteins can induce apoptosis as an immediate reaction of the host cell to infection (cell- autonomous apoptosis) (Raftery et al., 1999).

2. Literature background

2.1 Structure of HSV-1

HSV-1 is large complex virus (Lau & Crump, 2015). HSV-1 virion composed of four components: (1) dense core containing viral DNA contains at least 152 Kilo base pairs (kbp) organized into two exceptional groupings assigned as UL (unique long) and US (unique short), both of which are flanked by repeated components, (2) an icosahedral capsid which consist of 162 capsomeres (Whitley et al., 1998), (3) tegument which surrounds the capsid, consists of more than twenty different proteins that have an important function after the virus enters the cell (Kelly et al., 2009), and (4) envelope which is surrounded by capsid-tegument structure and consists of at least 10 glycosylated and many non-glycosylated viral proteins, lipids, and polyamines (Whitley et al., 1998). The assembly of HSV-1 particles occurs at the intracellular membrane to produce infectious virions, which can be released from the cell by secretion (Lau and Crump, 2015).

2.2 HSV-1 Entry and replication

HSV-1 infects several types of cell, but the major target cells during the primary infection are epithelial and nerve cells. HSV-1 can enter into the cell by one of two mechanisms fusion or endocytosis (Rahn et al., 2011). Five viral glycoproteins are important for HSV-1 to enter the cell: gC, gB, gD, gH, and gL. First, glycoprotein C (gC) and glycoprotein B (gB) initiate virus attachment by binding to cell surface proteoglycans which facilitate subsequent binding to co-receptor, then gD glycoprotein binds specifically to one of the primary entry receptors: nectin-1 and/or herpes virus entry mediator (HVEM) (Giovine et al., 2011); (Peterman et al., 2015). The gD-receptor interaction initiates the process that ultimately leads to gB-mediated membrane

fusion. gH and gL form a heterodimer and are important for entry, but their function in entry is not clear. They probably act as a bridge between gD and gB and/or bind to cellular receptors (Fan et al., 2014). Following attachment, the virus enters into the cell and releases a DNA-containing capsid with part of the tegument layer into the cytoplasm, which is after that transferred to the nuclear pore complex. At this point the genome is injected through nuclear pore complex into the nucleus (Mettenleiter et al., 2009). Immediate early genes are the primary set of gene expressed, which work as a trans-activator for a second set of virus transcript genes called early genes. For the most part proteins these genes encode required in viral genome replication. Lastly, the late genes mainly encode the virus structural components. Before the beginning of DNA replication there is a small set of late genes that is transcribed. Yet the really late genes are transcribed after the beginning of DNA synthesis (Boehmer and Lehman, 1997).

HSV-1 DNA replication takes place in the nucleus of the host cell. The linear genome in the nucleus replicates and circularizes by a rolling-circle mechanism. As a result, concatemers are split into monomers through the assembly of nucleocapsids. The fully developed DNA consisting of capsids bud through the inner lamella of the nuclear membrane. Throughout this process, the tegument, and a first form of the envelope are obtained. Then, the enveloped virions assemble in endoplasmic reticulum, and the mature virion is transported through the trans-Golgi network, where the envelope acquires its last composition and posttranslational adjustments of the glycoprotein (Nishiyama, 1996).

Herpes simplex virus type 1 (HSV-1) can enter cells via endocytic pathways or direct fusion at the plasma membrane depending on the cell line and receptor (Nishiyama, 1996). During the primary infection, HSV-1 uses the epithelial cells as the initial gate of entry and spreads through the epithelium. Then, the virion can infect the terminal ends of the sensory

neurons. HSV migrates to the neuronal cell body by retrograde process at that point the virus stop replication process to establish a latent infection. Following reactivation, HSV-1 can travel back to the first site of infection by anterograde axonal flow, and from there it spreads to infect the epithelial cells which often leads to lesion, and in some cases the HSV-1 travels to the central nerves system to cause encephalitis. Reactivation is normally related with stress factors such as immunosuppression, light exposure, hormonal alterations, and ultra-violate (Zolini et al., 2014).

2.3 Immune response to HSV-1

The host immune response toward HSV-1 includes both innate and adaptive immune response (Zolini at el., 2014).

2.3.1 Innate immunity to HSV-1

Host defense against any microbial pathogen is started by the innate immune system through pattern recognition receptors (PRRs). Pattern recognition receptors (PRRs) distinguish pathogen-associated molecular patterns (PAMPs) and activating host defense through the production of pro-inflammatory cytokines, tumor necrosis factor, and antiviral INF-α/β. There are various pattern recognition receptors (PRRs) included in the detection of HSV-1 which promote the activation of the immune response including Toll like receptors, retinoid acid inducible gene-I (RIG-I)-like receptors (RLRs), and DNA receptor. Even though viral RNA and glycoproteins can serve as pathogen-associated molecular patterns (PAMPs), the most common PAMP recognized by these PRRs is the HSV-1 genomic DNA (Egan et al., 2013). The identification of the HSV-1 involves: viral glycoproteins which are recognized by TLR2, HSV viral DNA recognized by TLR9 in the endosomes or by RNA polymerase III or potentially DAI in the cytosol, and finally the identification of virally-derived double-stranded (ds)RNA by RLRs (figure 1). However, to increase the antiviral immune response the cells have to identify the virus

and stimulate a cascade of signaling pathways involving: mitogen-activated protein kinase (MAPK) pathways, IFN regulatory factors (IRFs), and nuclear factor Kappa B.

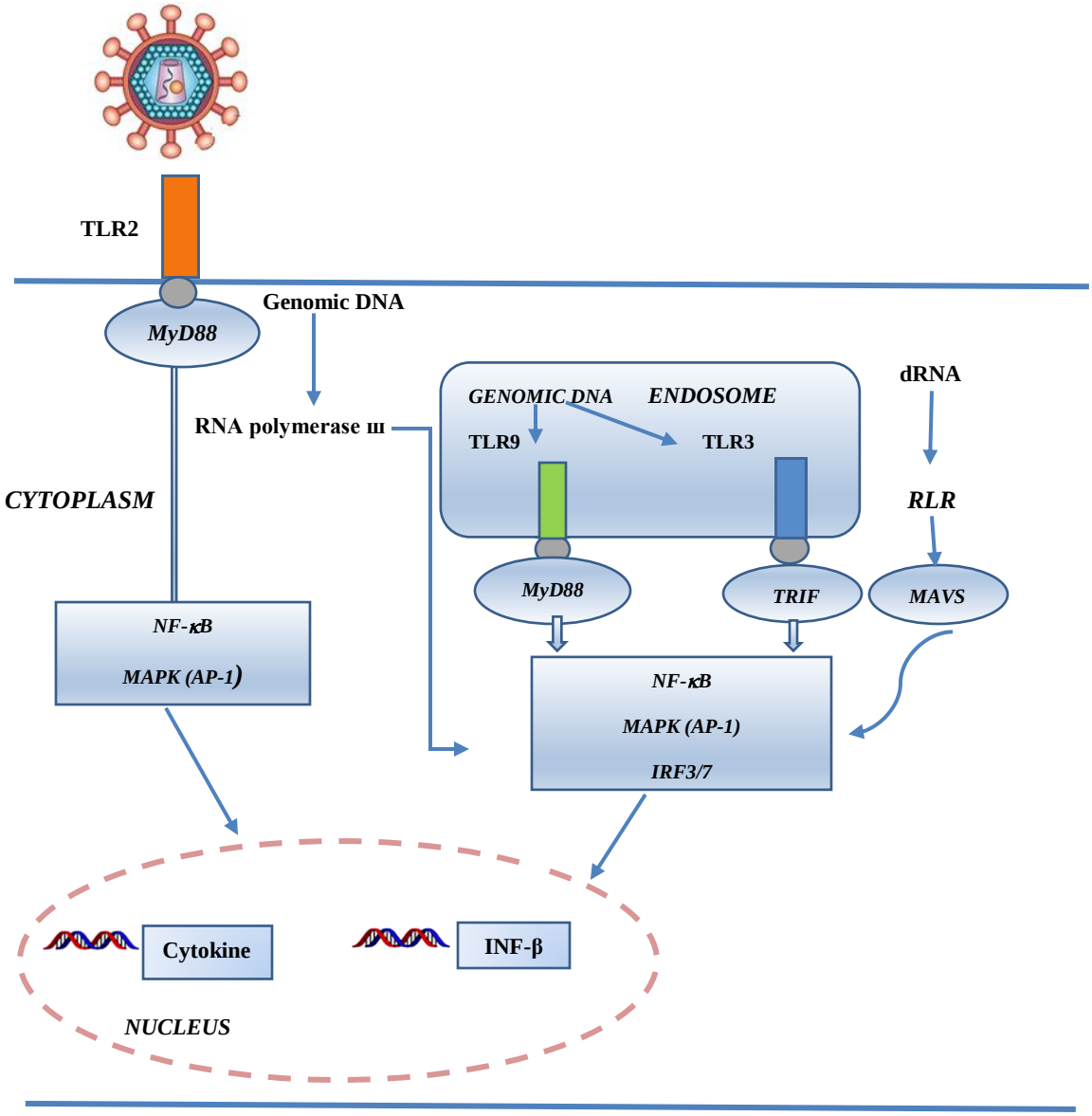


Figure 1: Recognition of HSV-1 and stimulation of antiviral and pro-inflammatory responses

The production of antiviral substances including nitric oxide and defensins, and in particular the secretion of IFNs and chemokines, is the primary response in HSV-1 infected cells. The point of innate immune response initiation is to restrict the virus proliferation, and to destroy the pathogens. The produced substances stimulate and activate immune cells and as a result assist the organization of antiviral response successfully. Innate immune response is mediated by white blood cells such as natural killer cells, dendritic cells, and macrophages. These cells function in phagocytosis as well as produce different inflammatory mediators and cytokines (Zolini et al., 2014). In HSV infection, NK cells that release IFN-γ have an anti-viral effect. This effect inhibits the viral replication (Lucin et al., 1994). Virus-infected cells can be distinguished and effectively killed by NK cells through two immune processes; the first one is opsonization of specific virus antibodies followed by interaction with Fc receptors, and the other one involves a recognition of viral antigen by MHC (Fitzgerald-Bocarsly et al., 1991). Additionally, during Infection with HSV can lead to a downregulation of MHC class I expression (Hill et al., 1995), as a result infected cells are more susceptible to be killed by NK. Dendritic cells (DCs) produce pro-inflammatory cytokines, engulf antigen, and show viral peptides to adaptive immune system cells. To activate the adaptive immune response, the innate immune response limits viral replication in the periphery and presents antigen to the naive lymphocytes. Monocytes travel to the site of HSV-1 infection and differentiate into tissue macrophages that phagocytose released virions and apoptotic epithelial cells. Macrophages also function as professional antigen presenting cells APCs that present viral proteins to the cells of the adaptive immune system (Egan et al., 2013). At the terminals of sensory neurons (Kodukula et al., 1999), activate macrophages decrease viral replication until the immune response of the adaptive system. Macrophages can be activated by infected cells such as epithelial cells that produce type I IFNs

and other chemotactic components. After the macrophages are activated, they produce inflammatory molecules such as nitric oxide (NO), IFNs type I, tumor necrosis factor-a (TNF-a), C-C motif chemokine 5 (CCL5; RANTES), and IL-6. Nitric Oxide (NO) production occurs through the action of the enzyme called inducible nitric oxide synthase. This enzyme is activated by IFN-γ produced by NK cells in the initiation of the infection, and by T cells (CD8+ T, CD4+ T, and γδ T cells) later in infection (Egan et al., 2013).

2.3.2 Adaptive immunity to HSV-1

Adaptive immune response toward HSV involves the cellular responses mediated by CD8+ and CD4+ T cells and the humoral response mediated by B cells and antibodies. In HSV-1 infection, CD8+T cells are activated in the lymph node and then migrate to the infection site to destroy virus-infected cells (Coles et al., 2002); (van Lint et al., 2004). CD8+T cells play an important role in both antiviral responses at the primary site of infection and in latent infection of the sensory neurons. CD8+ T cells produce and release IFN-γ as a response to viral infection. IFN-γ enhances the Th1 immune response, prevents virus replication, stops the cell cycle as well as enhances virus peptide presentation. IFN-γ stimulates expression of immune proteosomal subunits, and promotes viral peptides loading onto MHC class 1 molecules in the endosome. Additionally, IFN-γ promotes the MHC II pathway for antigen presentation by increasing expression of MHC class II molecules by professional and non-professional antigen-presenting cells (Egan et al., 2013). CD4+ T cells participate in the development of a specific humoral immune response. Together CD4+ and CD8+ T cells play a major role in antiviral immunity directly by their cytolytic characteristics or indirectly by their production of immunologically active cytokines (Schmid and Mawle, 1991). Antibody activity for a number of viral proteins includes antibodies specific for tegument proteins, glycoproteins, and capsid proteins (Ashley et

al., 1985); (Ashley et al., 1994). Antibodies have two ways to control the virus replication: through neutralizing free viral particles, and through killing virus-infected cells by complement-mediated cytotoxicity or antibody-dependent cell- mediated cytotoxicity (ADCC). The ADCC mechanism has been demonstrated as an important component of antiviral defense versus HSV infections, particularly in immunocompromised individuals and neonates (Kohl, 1991).

2.4 Macrophages functions and polarization

In addition to the resident tissue macrophage, the precursor of blood-borne macrophages are the circulating monocyte, which can pass the endothelium of the blood vascular to differentiate into macrophages in the peripheral tissue to be activated in different ways by exogenous or endogenous factors (Vogel et al., 2014). Macrophages are professional phagocytes that internalize large particles like dead cells or micro-organisms (Petermann et al., 2015). Macrophages are activated and their killing ability enhanced by soluble mediators produced by T cells such as IFN-γ. Macrophages accomplish two main functions in immunity, an innate and an adaptive function. Through effective ingestion and destruction, the pathogens are eliminated from the blood, and second, the present antigens on the cell surface which initiate an adaptive immune response. These events involve the production of cytokines, chemokines or reactive oxides (Mercer and Greber, 2013). These cytokines and chemokines activate other cells in the innate and adaptive immune system (Tripathi et al., 2008) as well as regulate T cell activity and inflammation in response to the pathogen (Mercer and Greber, 2013).

Macrophages play an important role in the innate immune system in the absence and presence of antigens by secreting a number of cytokines and chemokines. These cytokines and chemokines activate other cells in the innate and adaptive immune system (Tripathi et al., 2008). Macrophages play an important role in the immune system as a first line defense against a

variety of bacteria, viruses, and other micro-organisms (Bell et al., 2013). Macrophages respond to various signals and, depending on the signals, macrophages differentiate into classical M1 cells (activated by TLR ligands and IFN- γ), or alternative M2 cells (activated by IL-4/IL-13).

Classically activated or M1 phenotype cells (Sridharan et al., 2015), develop when macrophages interact with microbial products such as lipopolysaccharide (LPS) and pro-inflammatory signals such as Interferon- γ, that induce the M1 phenotype (Ebtekar et al., 2006). M1 macrophages have a high capacity to present antigens, as well as activate Th1 cells to produce pro-inflammatory cytokines (INF- γ and IL-2) in response to intracellular pathogens. M1 cells play a role of preventing the growth of infections by maintaining high level of iron to limit the availability of micro-environmental iron that require bacteria for growth (Raftery et al., 1999). However, these cells have harmful effects on the neighboring cells in the micro-environment as a result of increasing pro-inflammatory response and producing toxic oxygen intermediates (Sica and Mantovani, 2012).

Alternatively activated or M2 phenotype macrophages, develop when the macrophage receives signals such as Interleukin-4(IL-4) or Interleukin-13 from mast cells, basophils, and other granulocytes (Sridharan et al., 2015). M1 macrophages express scavenger, and mannose receptors (CD206), and release anti-inflammatory cytokines such as IL-10. In contrast to M1, M2 cells function in tissue remodeling by maintaining a high level of iron export. M2 macrophages can be divided into M2a, M2b, or M2c that can be activated by different signals. M2a is activated by IL-4 and IL-13, whereas M2b activated by toll like receptor (TLR) and immune complexes. However, both M2a and M2b initiate Th2 lymphocyte anti-inflammatory responses by producing IL-10, IL-1ra, and IL-6. M2c is activated by IL-10 and functions in

tissue remolding as well as suppression of inflammatory immune reaction by producing transforming growth factor-β (TGF-β) and IL-10 (Sridharan et al., 2015).

2.5 Apoptosis

Macrophages exert a significant function in the pathogenesis of endotoxin shock by producing NO and TNF-α, which can induce apoptosis in different cell types, including macrophages themselves (Soler et al., 2001). Unlike necrosis, which is uncontrolled cell death caused by a pathogen, apoptosis is a physiologically controlled mechanism. Necrotic cell products induce inflammation and stimulate signals whereas the rest of apoptotic cells are removed by phagocytosis without stimulating additional inflammatory signals (Lawen, 2003). Apoptosis is distinguished by DNA fragmentation, chromatin condensation, and cell shrinkage (Aramaki et al., 1999). Most of the time, apoptosis is accomplished by activation of set of proteolytic enzymes called caspases. The caspases are synthesized as inactive precursor molecules called procaspases. When the N-terminal pro-domain is cleaved from the precursor molecule, the active caspases are ready to cleave their substrates. Caspases are involved in cascades that are released in response to pro-apoptotic signals and cleave a set of protein, leading to cell death. Apoptosis can be triggered by extracellular signals (extrinsic pathway) or intercellular signals (Intrinsic pathway). Extrinsic pathway is initiated by sub group of tumor necrosis factor superfamily receptors (TNFRs) such as TNFR, Fas, and TRAIL. Activating these so-called death receptors by binding to cognate ligands recruits adaptor molecules that enable the binding, self-cleavage, and activation of procaspase 8, which subsequently leads to the cascade of events culminating in the activation of the effector caspases, typically caspase 3. The active caspase 3 functions to cleave a number of death proteases that lead to the characteristic apoptosis including DNA fragmentation, nuclear fragmentation, and other morphological and biochemical

changes. The intrinsic pathway is regulated by mitochondria, and can be activated by different stress events such as virus infection, cytotoxic drugs, or oxidative stress. Apoptotic signals are transmitted to the mitochondria and subsequent release of cytochrome c. releasing cytochrome c from the mitochondria resulting in a form called apoptosome by binding cytochrome c to apoptosis activating factor-1 protein (APaf-1). The apoptosome activates pro-caspase 9, which subsequently activates caspase 3 (Schultz et al., 2003) (Figure 2).

Apoptosis or programmed cell death is a critical process in the homeostatic maintenance of multicellular organisms. Apoptosis eliminates the infected cells, which may be harmful if they survived (Lawen, 2003). This process can be induced in susceptible cells by a variety of physiological stimuli as well as harmful ecological conditions (Ramamoorthy and Tizard, 1998). Apoptosis acts as an extremely efficient defense mechanism against virus infection. Apoptosis helps to remove virus proteins and nucleic acids from the infected host. There are two types of apoptotic stimuli that induce apoptosis in the virus-infected cells (An et al., 1999). Firstly, virus-infected cells go through apoptosis by presenting viral peptides on the cell surface with major histocompatibility antigens (O'Brien, 1998), which can be recognized by cytotoxic cells such as natural killer cells (NKs), and cytotoxic T cells. Secondly, virus-infected cells go through a cell autonomous apoptosis without attack by the immune cells (An et al., 1999). However, HSV-1, like other huge DNA-containing viruses, utilizes various viral resistance systems that collaborate to avert unexpected death of the host cell prompted by an assortment of intracellular and extracellular signals. The infection alonge increases the yield of viral offspring after lytic contamination of target cells. HSV-1 proteins can induce apoptosis as an immediate reaction of the host cell to infection (cell- autonomous apoptosis) (Raftery et al., 1999).

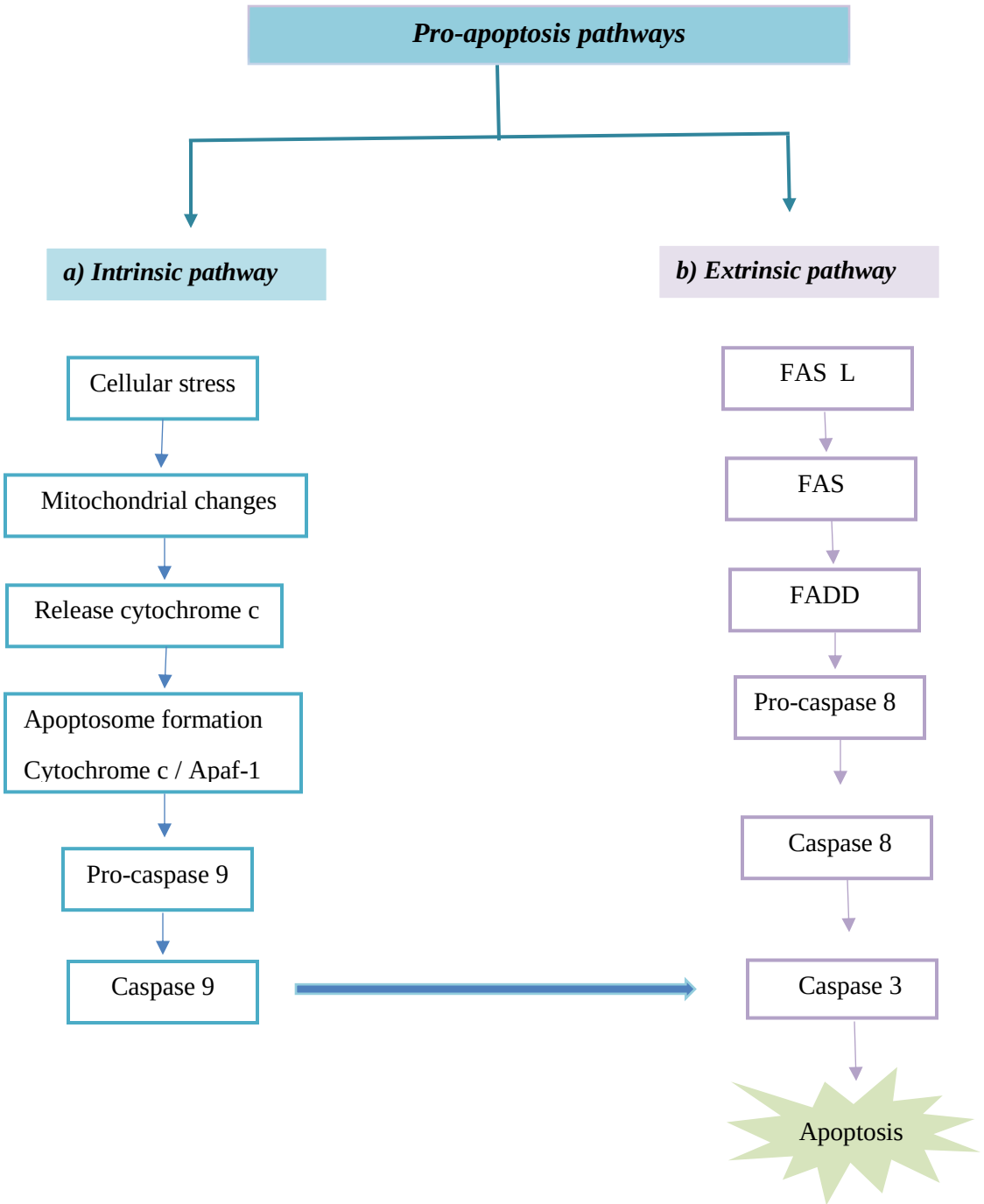


Figure 2: Intrinsic and extrinsic pathways of apoptosis. The intrinsic pathway is regulated by mitochondria and can be activated by different stress events. such as virus infection, cytotoxic drugs, or oxidative stress. Apoptotic signals are transmitted to the mitochondria and subsequent release of cytochrome c. releasing cytochrome c from the mitochondria resulting in a form called apoptosome by binding cytochrome c to APaf-1 protein. The apoptosome activates pro-caspase 9, which subsequently activates caspase 3. The extrinsic pathway is induced by binding death ligand such as Fas to death receptor and recruit adaptor molecule FADD which activate procaspase 8 and subsequently activate caspase 3 which lead to apoptosis.

2.6 Apoptosis detection

A number of methods have been expanded to detect apoptosis; each method depends on assessment of specific features related with cell death (Fink et al., 2005). The activation of caspases can be assessed by using fluorescent, colorimetric and luminescent substrates that are added to cells. Cleavage of caspases can be detected by western blots and ELISAs. Changes of the mitochondrial membrane permeability is indicated by using fluorescent dyes such as JC-1, rhodamine 123, and TMRE, which accumulate in healthy mitochondria. Releasing cytochrome c from mitochondria (early stage of apoptosis) is assessed by immunoblotting, and flow cytometry. DNA fragmentation can be detected by gel electrophoresis, ELISA, and TUNEL {terminal UTP nick end labeling, which marks the DNA cleavage by using terminal deoxynucleotidyl transferase (TdT)}. Loss of membrane integrity during early phase of apoptosis is associated with exposure of phosphatidylserine to the outer side of the cell. FITC conjugated Annexin V is a phospholipid and calcium binding protein that combined with Pi, and bind to phosphatidylserine. This is assessed by using flow cytometry to detect apoptotic cells in early stages from the apoptosis (Cobb, 2013).

Annexin V (AV) was first known as a vascular protein with a strong effect against coagulant. It belongs to a multigene family of proteins recognized by a sequence motif named the endonexin loop. Annexin V, a calcium-dependent, phospholipid-binding protein, characteristically attaches to phospholipids in a Ca2+ dependent manner. Annexin V binds especially to phospholipid species such as phosphatidylserine (PS). In normal cells PS, is located in the inner surface of the plasma membrane. During cell death, PS is translocated to the external layer of the membrane. This happens in the early periods of apoptotic cell death during which the cell layer itself stays intact (Vermes et al., 1995).

Propidium iodide (PI) is widely used in conjunction with Annexin V to figure out whether cells are viable, apoptotic, or necrotic through changes in plasma membrane integrity and permeability. The Annexin V /PI assay is a usual method to detect apoptotic cells. PI is utilized more regularly than other nuclear stains since it is efficient, stable and a good detector of cell viability. PI does not stain live or early apoptotic cells because of the existence of an intact plasma membrane. In late apoptotic and necrotic cells, reduction on the integrity of the plasma and nuclear membranes permit PI to go through the layers, enter into nucleic acids, and show red fluorescence (Rieger et al., 2011).

3. Specific aims and hypothesis

M1 activated macrophages produce cytotoxic molecules such as TNFα, ROS, and NO which can cause apoptosis and cell death.

I hypothesized that HSV-1 has an anti-apoptotic effect on murine RAW 246.7 macrophages.

The goals of this study were:

- To examine cell viability of un-polarized and polarized murine RAW 246.7 macrophages for 4, 12, 24 hours (trypan blue).
- To analyze cells for apoptosis using annexin V staining of un-polarized and polarized murine RAW 246.7 macrophages for 4, 12, 24 hours (Flow cytometry).

4. Materials and Methods:

4.1 Cell Line and cell culture conditions

RAW 264.7 is Abelson murine leukemia virus-induced tumor derived from an adult male BALB/C mouse, and was obtained from the American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin at 37 °C in a 5% CO2 humidified incubator.

4.2 Polarization treatment

RAW 264.7 macrophages were grown to approximately 80% confluency, at which time the polarization treatment was administered. To induce the M1 phenotype RAW 264.7 macrophages were treated with IFN-γ (20 ng/mL) and LPS (100 ng/mL) for 4, 12, and 24 hours; IL-4 (20 ng/mL) was used to induce the M2 phenotype. After 4, 12, and 24 hours cells were removed by pipette from the cell culture flasks for further analyzed. The murine cytokines (IFN-γ and IL-4) were purchased from (Rocky Hill, NJ); LPS was purchased from (Redmond, WA).

4.3 Cell viability

RAW 264.7 were grown to approximately 80% confluences. They were treated with IFN-γ (M1), and LPS or IL 4 (M2) for 4, 12, and 24 hours with and without infection. Untreated cells used as a control. After 4, 12, 24 hours, cells were transferred to centrifuge tubes and pelleted in a table tope centrifuge at 1500 rpm (4˚C) for 5 minutes. The supernatants were discarded and pellets were re-suspended in 1 ml of fresh medium. Trypan blue dye (Fisher Sciences, Pittsburgh,

PA) was used to perform the trypan blue exclusion test and viable cells were counted using hemocytometer. The following equation was used to estimate the cell viability.

Cell Viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) ×100

4.4 Plaque assay

HSV-1 (Syn 17+ strain) (obtained initially from Dr. Nancy Sawtell,Children's Hospital Medical Center, Cincinnati, OH) was propagated in Vero cells. The Vero cells were grown in 100 mm culture dishes as a monolayer and, at 90-100% confluency, cells were infected with HSV-1 at 0.1 MOI. Cytopathic effect (cells rounding and detaching from the bottom of the dish) appeared by microscopic examination 3-5 days post-infection. Then, the cells were harvested and the medium was stored as virus stock in 200 µl aliquots at 80°C.

5.5 Flow cytometry and Annexin v FITC/PI double staining

Cells were grown to approximately 80% confluency in cell culture flasks, at which point M1 or M2 treatment was administered with or without virus. After 4, 12, 24 hours of treatment, cells were removed and centrifuged at 1500 revolutions per minute (4˚C) for 5 minutes. After centrifugation the supernatant was aspirated and the cell pellet was resuspended using 1 mL of complete growth medium. A hemocytomter was used to obtain viable cell counts; one million cells were utilized for each sample.

Annexin V staining to detect apoptosis: one million cells were placed in 1.5 ml centrifuge tubes and washed two times with cold cell staining buffer (Biolegend) and then resuspended in Annexin V binding buffer. After that, 100 µL of cell suspension was transferred to a 5 ml test tube. Then 5 µL flourochrome conjugated annexin V and 10 µL of PI solution were added. The cells were gently mixed and incubated for 15 min in room temperature in the dark.

Then 400 μL of annexin V binding buffer was added to each tube. The samples were analyzed using an Accuri C6 flow cytometry. The flow cytometry data was gated by using cytospec software from Purdue University (http://www.cyto.purdue.edu/Purdue_software) .

Table: Stains and buffer concentrations used to show apoptotic cells using flow cytometry

Stain/Buffer	Concentration/Dilution	Company
FITC-Annexin v	5 μL/million cells	Biolegend
Propidium iodide (PI)	10 μL/million cells	Biolegend
Cell staining buffer	0.5ml/million cells	Biolegend
Annexin v binding buffer	500 μL/million cells	Biolegend

Statistical Significance

All experiments were repeated three times. The statistical significance between testing and control experimental groups was analyzed by one way ANOVA (Sigma Plot 12.0, YSTAT).

5. Results

5.1 RAW 246.7 Cell viability prior to and after HSV-1 infection

RAW 642.7 macrophages were treated with IFN-γ/LPS (M1) or IL-4 (M2) for 4, 12, and 24 hours, and the cells stained with trypan blue to determine the number of live cells versus dead cells. Cell viabilities for M1 and M2 were compared with untreated cells (control). At 4 hours following polarization, M1 macrophages showed a slight decrease of 13.65% (p-value<0.016) in cell viability following IFN-γ/LPS treatment. M2 macrophages experienced a slight, non-significant, decrease of 3.35% (p-value =0.33) in cell viability following treatment with IL-4 compared to control. M2 showed a decrease of 10.3% (p-value<0.037) in cell viability compared to that seen for M1 cells (Figure 3A). At 12 hours following polarization, M1 macrophages showed also a significant decrease of 14.6% (p-value<0.001) in cell viability following treatment IFN-γ/LPS treatment. M2 macrophages had a slight non-significant decrease of 3.06% (p-value=0.18) in cell viability following treatment with IL-4 (Figure 4 A). At 24 hours following polarization, M1 macrophages showed a significant decrease in viability of 71.4 %(p-value<0.001) while M2 macrophages showed a small, non-significant reduction of 4.46% (p-value=1.1) (Figure 5A).

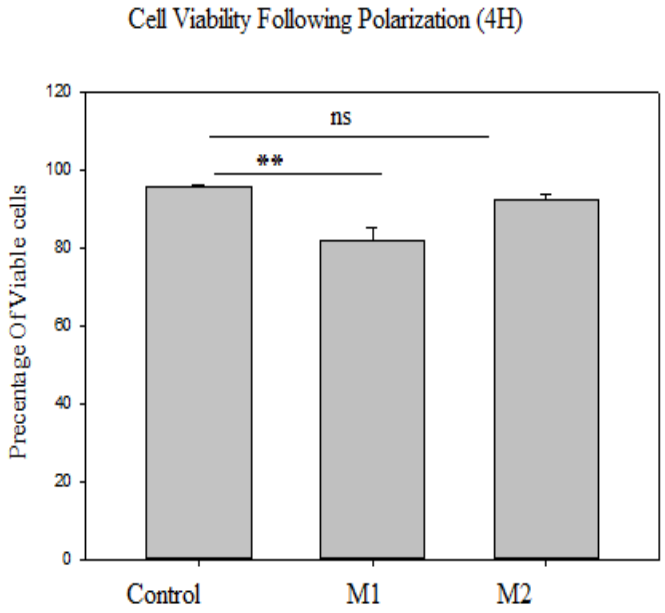
At 4 hours following polarization and HSV-1 infection, M1 macrophages exhibited a significant decrease of 12.84% (p-value<0.001) in cell viability compared to virus infected control cells. In contrast, M2 macrophages did not show a significant decrease in cell viability (1.36 % p= 0.084) (Figure 3B). At 12 hours following polarization and HSV-1 infection, M1 macrophages showed a significant reduction of 15.01 % (p-value<0.001) in cell viability compared to virus infected control cells. M2 macrophages did not display a significant difference

compared to infected control cells (Figure 4B). At 24 hours following polarization and HSV-1 infection, M1 macrophages displayed a significant decrease in cell viability of 53.12 %(p-value<0.001) when compared virus-infected control cells. M2 macrophages showed a non-significant slight reduction in viability of 5.9% (p= 0.354) (Figure 5B).

5.2 Detection of apoptosis with annexin V in 246.7 RAW cell prior to and after HSV-1 infection

RAW 642.7 macrophages were treated with IFN-γ/LPS (M1) or IL-4 (M2) for 4, 12, and 24 hours, and the cells were stained with FITC-annexin V and PI double staining to detect apoptosis stages. At 4 hours after polarization and HSV-1 infection. There were significant decreases in AV+/PI+ cell populations (late apoptosis) after infection with HSV-1 in all cell groups (M0, M1, and M2). There were no statistically significant differences in necrotic cells in all macrophage cell groups. In early apoptotic cells there were significant decreases after HSV-1 infection in M2 and M0 macrophages while in M1 there was no statistical significantly difference between infected and uninfected M1 macrophages (Figure 6). At 12 hours following polarization/infection with HSV-1, there was a significant increase in late apoptosis percentage in M0 and M1 infected cells compare to uninfected M0 and M1, while in M2 there were no significant differences in late apoptosis percentage between infected and uninfected M2 macrophages. There were significant decreases in the percentage of necrotic cells in virus infected M1 and M2 macrophages while there was not a significant difference between infected and uninfected M0 cells. In early apoptotic cells there were no significant differences between infected and uninfected M0 and M1 macrophages whereas M2 macrophages showed a significant decrease in necrotic cell percentage after infection with HSV-1 (Figure 7). At 24 hours following polarization/ and HSV-1 infection, there were statistically significant differences

in late apoptosis after and before infection with HSV-1; all cell groups (M0, M1, and M2) experienced significant decreases percentage of cells in late apoptosis after infection with HSV-1. There were not statistically significant differences in necrotic cells in any macrophages groups. M0 and M1 macrophages displayed a decrease in early apoptotic cell percentage after HSV-1 infection, while there was no result showed in infected and uninfected M1 macrophages (Figure 8).



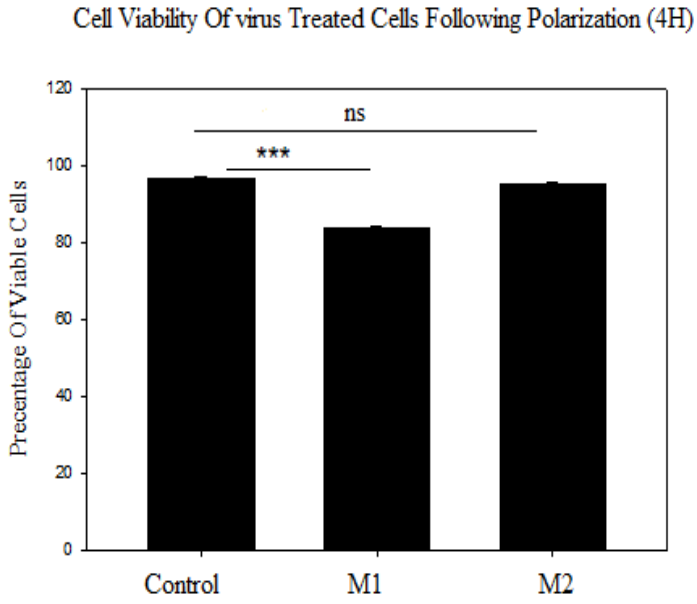
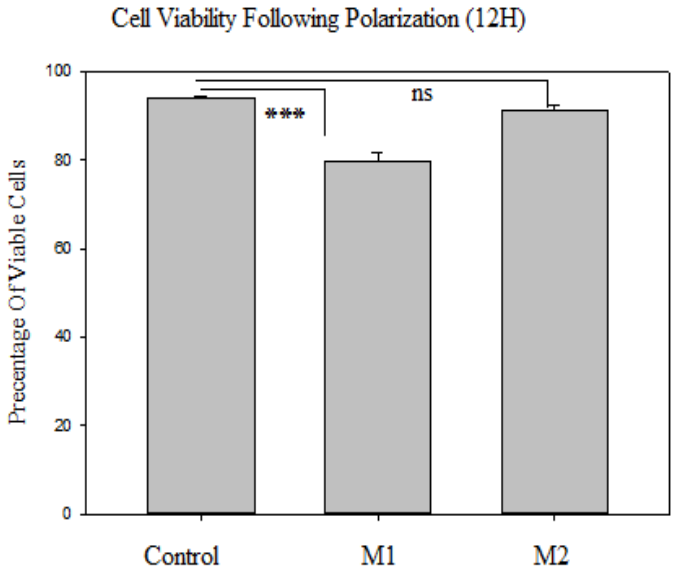


Figure 3: Percentage of viable cells following polarization treatment 4H. **A)** M1 cells experienced a decrease (13.65% (p-value<0.016)) in cell viability following INFγ/LPS treatment. M2 macrophages had a slight decrease (3.35% p-value =0.33) in cell viability following treatment with IL-4. **B)** Percentage of viable cells following polarization and HSV-1infection at 4H, M1 macrophage exhibited a significant decrease with 12.84% (value<0.001) in cell viability compared to virus infected control cells. M2 macrophage showed an inconsiderable decrease with 1.36 %(p= 0.084). (ns= not significant, **=p-value<0.01, ***= p-value<0.001)



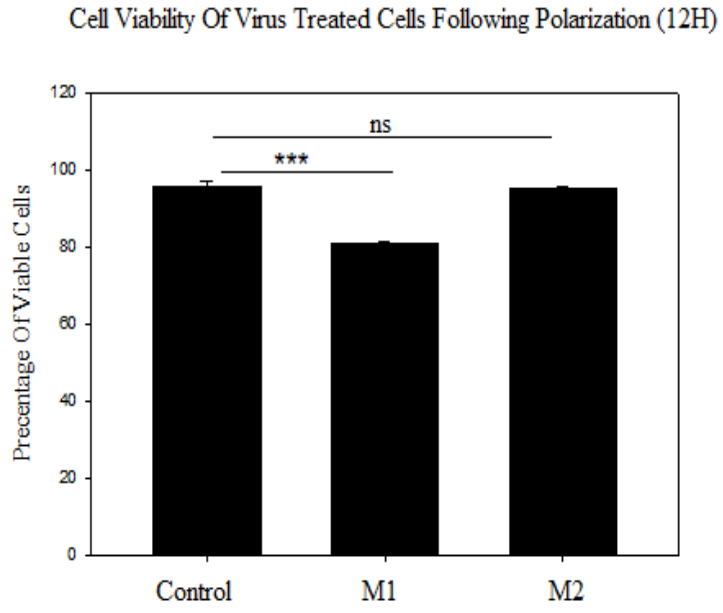


Figure 4: Percentage of viable cells following polarization treatment 12H. **A)** M1cells experienced a decrease (14.6% p-value<0.001) in cell viability following INFγ/LPS treatment. M2 macrophages had a slight decrease (3.06% p-value=0.18) in cell viability following treatment with IL-4. **B)** percentage of viable cells following polarization and HSV-1 infection at 12H, M1 macrophage showed a significant reduction with 15.01 %(p-value<0.001) compared to virus infected control cells. (ns= not significant, **=p-value<0.01,*** = p-value<0.001)

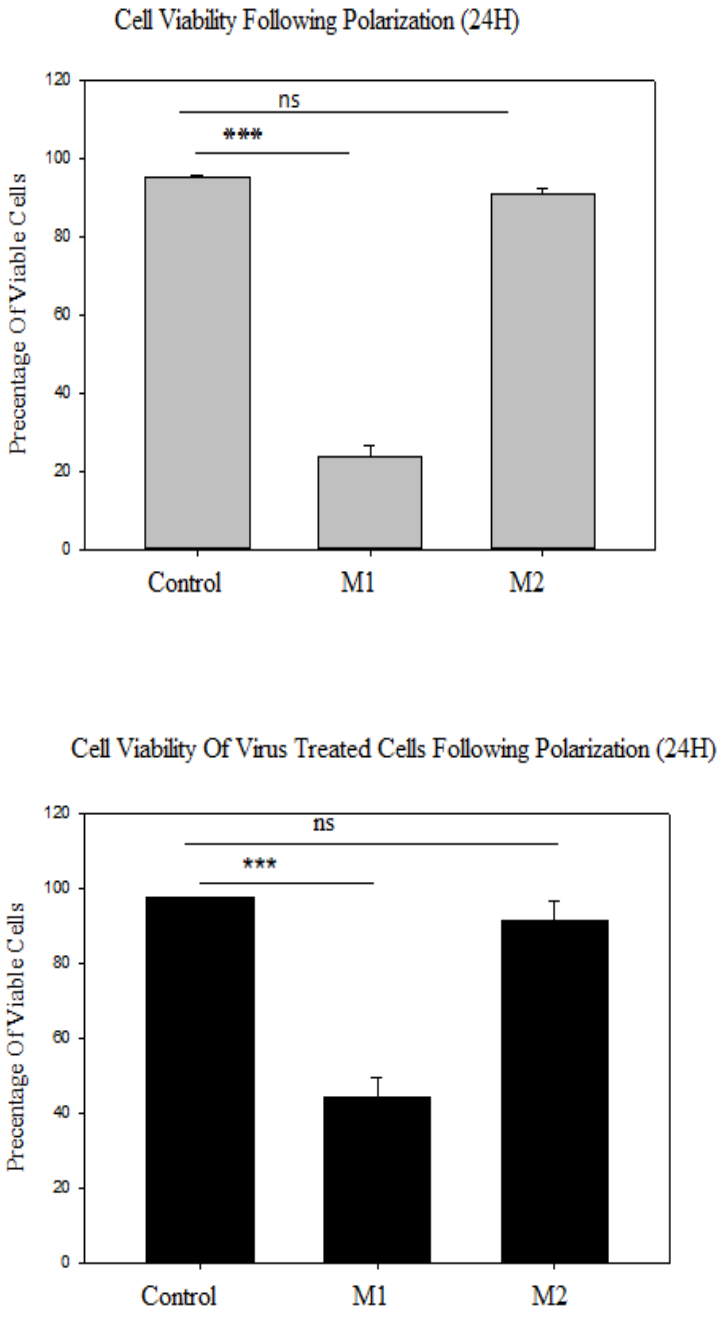


Figure 5: Percentage of viable cells following polarization treatment 24H **A)** M1 cells experienced a decrease (71.4 %p-value<0.001) in cell viability following INFγ/LPS treatment. M2 macrophages had a slight decrease (4.46% p-value=0.1) in cell viability following treatment with IL-4. **B)** percentage of viable cells following polarization and HSV-1 infection at 24 H, M1 macrophage displayed a significant result with 53.12 %(p-value<0.001) when it compared to viability of virus-infected control cells. M2 macrophage showed a slight reduction with 5.9% (p= 0.354). (ns= not significant, **=p-value<0.01, = p-value<0.001)

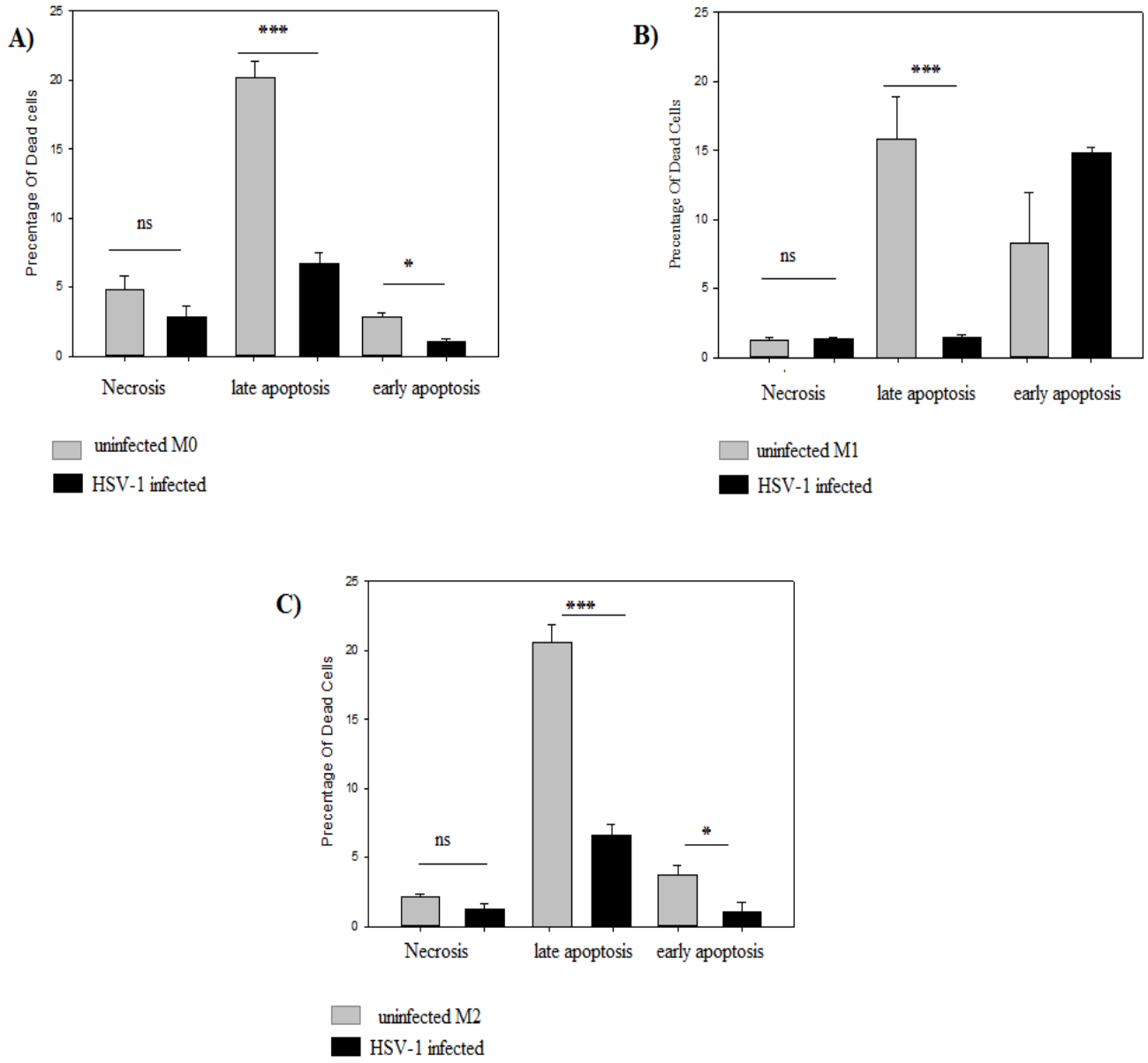


Figure 6: Annexin v staining of polarized and non-polarized after 4H without HSV-1 infection and with h infection. **A)** shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior and after infection with HSV-1 on M0 macrophages(untreated cells).**B)** shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior and after infection with HSV-1 on M1 macrophages(treated with INFγ/LPS).**C)** shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior and after infection with HSV-1 on M2 macrophages (treated with IL-4). (ns= not significant, * =p-value<0.05,**=p-value<0.01, = p-value<0.001)

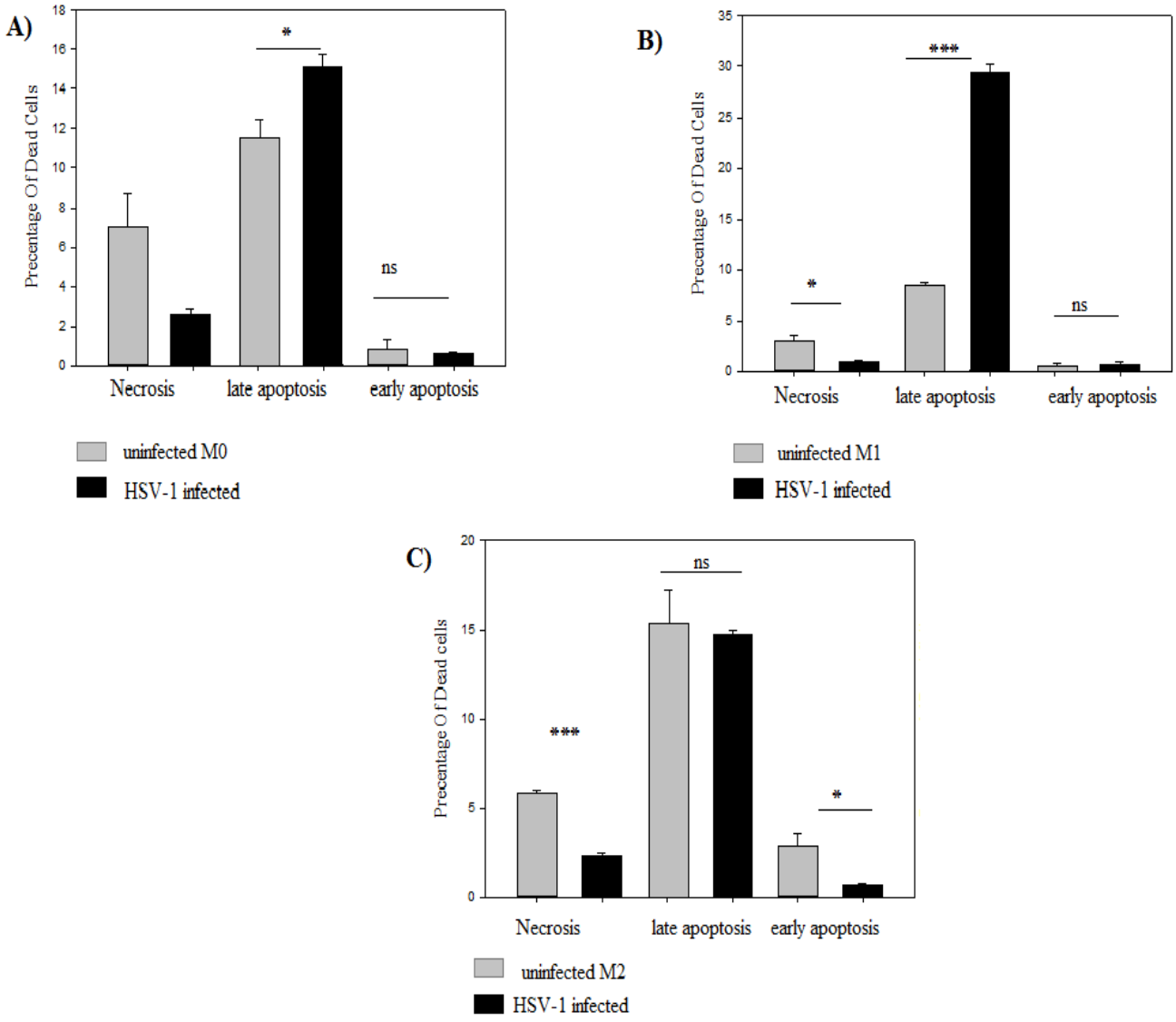


Figure 7: Annexin v staining of polarized and non-polarized after 12 H without HSV-1 infection and with HSV-1 infection. **A)** Shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior and after infection with HSV-1 on M0 macrophages. **B)** Shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior and after infection with HSV-1 on M1 macrophages. **C)** Shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior and after infection with HSV-1 on M2 macrophages. (ns= not significant, * =p-value<0.05,**=p-value<0.01, = p-value<0.001)

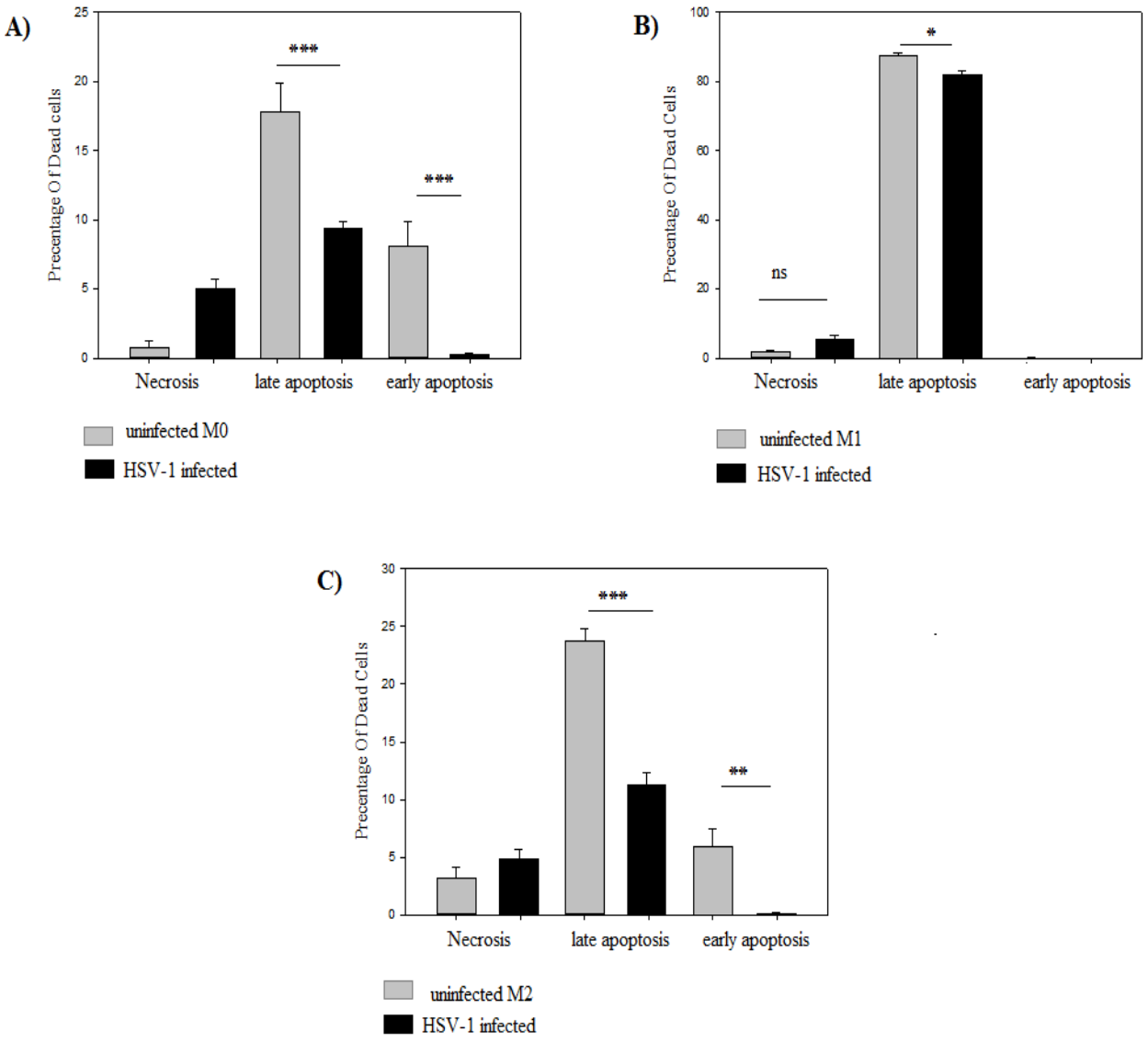


Figure 8: Annexin v staining of polarized and non-polarized after 24H without HSV-1 infection and with HSV-1 infection. **A)** Shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior and after infection with HSV-1 on M0 macrophages. **B)** Shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior to and after infection with HSV-1 on M1 macrophages.**C)** shows the percentage of necrotic cells, late apoptotic cells, and early apoptotic cells prior to and after infection with HSV-1 on M2 macrophages. (ns= not significant, =p-value<0.05, **=p-value<0.01, = p-value<0.001)

6. Discussion and Future Directions

In the current study we found that HSV-1 has anti-apoptotic effect on RAW 246.7 macrophages. RAW 246.7 were polarized to either the M1 or M2 phenotype. By using trypan blue exclusion to detect cell viability, the results revealed that uninfected and infected M1 macrophages showed a significant decrease in cell viability compared to uninfected and infected M2 and M0 (control) macrophages after 4 hours, 12 hours, and 24 hours of treatment. Whereas uninfected and infected M2 macrophages did not display a significant reduction in cell viability compare to uninfected and infected M0 (control) macrophages after 4, 12, and 24 hours of treatment. Infected M1 macrophages displayed an increase in cell viability compare to uninfected M1 macrophages after 24 hours of treatment. The reduction of cell viability in M1 macrophages may result from the activation and stimulation of the inducible nitric oxide synthase (iNOS) transcription, which can lead to produce high concentration of NO. High level of NO can make macrophages receptive to apoptosis (Seminara et al., 2007). Additionally, M1 macrophages regulate the transcription of many genes involving nitric oxide synthase-2 and phagocyte oxidase that are related with reactive oxygen species (ROS) production that can lead to cell death in macrophages (Bell et al., 2013). For the flow cytometric analysis by using Annexin V and propidium iodide, we observed that the percentage of late apoptotic cells decreased in polarized and un-polarized macrophages after infection with HSV-1 at 4 hours and 24 hours. At 12 hours, M2 macrophages showed a slight increase in late apoptotic cell percentage after HSV-1 infection. The anti-apoptotic effect of HSV-1 on RAW 246.7 macrophages may result from the pro-inflammatory cytokines (IFN) suppression. Viral Immediate early genes for ICP4, and ICP27 that express on Vp16 decrease the stability of mRNA to express pro-inflammatory cytokines (Mogensen et al., 2004). During HSV-1 primary

infection mitogen-activated protein kinase (MAPK), IFN regulatory factors (IRFs), and nuclear factor Kappa B signaling are activated to produce pro-inflammatory and cytokines. ICP27 inhibits the induction of IFN and cytokines through inhibition of IRFs and NF-κB activation (Melchjorsen et al., 2009). ICP27 inhibits apoptosis indirectly through inducing early and late anti-apoptotic gene products. The early gene products that suppress apoptosis are glycoprotein D (gD), Us3 protein kinase, and ribonucleotide reductase 1 (R1). gD binds to its receptor, which is a member of TNF family receptor, and is able to activate NF-κB signaling pathway. gD-mediated inhibits Fas-induced apoptosis and enhance the expression of anti-apoptotic genes. Us3 inhibits apoptosis through its action to phosphorylate pro-apoptotic proteins (Bid and Bad) and prevents their function to induce apoptosis. Also, Us3 can prevent intrinsic apoptosis process by inhibiting the release of cytochrome c from mitochondria. R1 inhibits apoptosis induced by TNFα and Fas ligand through its binding to caspase 8 effector death domain and blocking the activation of caspase 8 (Xiaoliang and Sudan, 2016).

Our observations (figure 6, 7, and 8) show that after HSV-1 infection there was a decrease in the percentage of apoptotic cells at 4 and 24 hours, while the percentage of apoptotic cells increased at 12 hours. The increase and the decrease in the percentage of apoptotic cells may result from an apoptotic balance between viral and cellular factors at early stages from HSV-1 infection, early immediate gene expression induces apoptosis. The cellular caspases and hTERT help to induce apoptotic process. At late stages from HSV-1 infection, early and late anti-apoptotic viral genes inhibit apoptosis process. The cellular factors p53, Bcl2 family are blocking apoptosis during viral infection (Marie and John, 2009).

As we mentioned previously, the anti-apoptotic effect of HSV-1 may result from the suppression of IFN- γ so, in the future studies it would be very helpful to study the changes of

IFN signaling pathway prior to and during HSV-1 infection on 246.7 RAW macrophages. The DNA microarray method would be useful to study the effect of viral infections on the regulation of IFN gene expression. Using the DNA microarray method would allow us to study the cellular gene expression such as the MAPK, IRF, and nuclear factor kappa B gene expression that are responsible for producing cytokines and IFN as an innate immune response of viral infections. For example, to study the changes in IFN signaling pathway in RAW 246.7 macrophages prior to HSV-1 infection, we would treat the cells by IFN-α and IFN-β at different time points (4, 8,18, and 24 hours) to differentiate early and late gene expression changes. Then to examine the effect of individual viral gene expression on IFN-regulated gene expression, we would add the HSV-1 (MOI=0.1) at the time of adding IFN treatment (at 4, 8, 18, and 24 hours). Following this step, DNA microarray analysis would be used on RAW 246.7 macrophages in two different conditions prior to and after virus infection. After that, we could compare the cellular gene expression patterns before and after HSV-1 infection to determine which viral genes have an effect on the IFN signaling pathway. Also, it would be beneficial to compare the anti-apoptotic effect of HSV-1 on 246.7 RAW macrophages with other immune cells, such as natural killer cells and dendritic cells or other type of macrophages such as J777, to see if the anti-apoptotic effect of HSV-1 cell type is dependent or has the same effect on other cells. by determining cell viabilities directly by using trypan blue exclusion and by flow cytometry by using Annexin V and propidium iodide to detect apoptotic and necrotic cells.

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