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## The Impact of Cytokines and HSV-1 on Rab5 Protein Expression, F-actin Cytoskeleton Rearrangement, and Cell Viability of Uninfected and Virus-Infected M0, M1, and M2 RAW264.7 Murine Macrophages

Muhannad Falah Alruwaili  
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THE IMPACT OF CYTOKINES AND HSV-1 ON RAB5 PROTEIN EXPRESSION, F-  
ACTIN CYTOSKELETON REARRANGEMENT, AND CELL VIABILITY OF  
UNINFECTED AND VIRUS-INFECTED M0, M1, AND M2 RAW 264.7 MURINE  
MACROPHAGES

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

By

MUHANNAD FALAH ALRUWAILI  
B.S., Northern Border University, 2012

2018

Wright State University

**WRIGHT STATE UNIVERSITY**

**GRADUATE SCHOOL**

April 20, 2018

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Muhannad Falah Alruwaili ENTITLED The Impact of Cytokines and HSV-1 on Rab5 Protein Expression, F-actin Cytoskeleton Rearrangement, and Cell Viability of Uninfected and Virus-Infected M0, M1, and M2 RAW264.7 Murine Macrophages BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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## ABSTRACT

Alruwaili, Muhannad Falah M.S. Microbiology and Immunology Graduate Program, Wright State University, 2018. The Impact of Cytokines and HSV-1 on Rab5 Protein Expression, F-actin Cytoskeleton Rearrangement, and Cell Viability of Uninfected and Virus- Infected M0, M1, and M2 RAW264.7 Murine Macrophages.

The endocytic pathway in all eukaryotic cells is necessary to maintain cellular functions, such as initiating transport of intracellular cargos and ingesting pathogens. The main regulator of this process is a member of small GTPase family, Rab5 protein. Rab5 protein plays a key role in the endocytic dynamic delivery of molecules, receptors, and pathogens from the cell membrane to cytoplasmic vesicles.as well as in the exocytic delivery of cellular products to the cell's exterior (Bonifacino & Glick, 2004). Many pathogens have exploited this protein to enter cells. Herpes Simplex Virus Type 1 (HSV-1) enters most cells by fusion or utilizes the endocytic pathway using Rab5 protein (Spearman, 2017). Also, HSV-1 depends on Rab5 in the enveloping process to produce mature viral particles (Hollinshead, 2012). F-actin is a major microfilament of the cell's cytoskeleton, aiding migration to the site of infection, muscles contraction, and cell division (Khadijeh, Amir, & Maryam, 2015). As a barrier, F-actin also protects the cellular organelles within the cytoplasm (Vitale, Seward, & Trifaró, 1995). The goals of this study were to determine the effect of the HSV-1 infection on Rab5 expression in RAW 264.7 murine macrophages and on F-actin distribution at 2, 4, 6, and 24 hours or late time at 24 hours after polarization of the macrophages to M1, M2a, and M2c phenotypes. M1 macrophages were polarized by interferon gamma (IFN- $\gamma$ ) and lipopolysaccharides (LPS). Unpolarized cells (M0) were converted to M2a phenotype by treating them with interleukin 4 (IL-4). M2c phenotype was polarized with interleukin 10 (IL-10). HSV-1 infection upregulated Rab5 protein expression at 2-6 hours in both M2a and M2c phenotypes but not in M1 polarized macrophages. No

increase in Rab5 expression was seen at 24 hours after virus infection in any of the polarized macrophages but was seen in HSV-1 infected unpolarized M0 cells. Both M1 and M2 polarizing agents caused an upregulation in Rab5 expression from 2 to 6 hours after polarization. HSV-1 infection caused a decrease F-actin distribution (staining intensity) levels among test groups at most time points. Polarization caused a decrease in cell viability of M1 macrophage; HSV-1 infection did not enhance this decrease in cell viability after 24 hours. M2 phenotypes, uninfected or HSV-1-infected, did not exhibit any decrease in cell viability at 24 hours. Treatment of HSV-1 phenotypes with M2 polarizing anti-inflammatory cytokines, Il-4 and Il-10, as well as with SOCS3, an inducer of IL-10 expression, enhanced expression of Rab5 and F-actin distribution.

## **HYPOTHESIS**

HSV-1 and macrophage polarizing cytokines upregulate expression of Rab5 GTPases by 2- 6 hours following binding to their respective cell surface receptors in unpolarized M0 cells as well as M1 and M2 macrophage phenotypes. Cell viabilities are more diminished in uninfected and HSV-1-infected M1 phenotypes. Consequently 24 hours after HSV-1 infection, only the M0 and M2 phenotypes show an upregulation of Rab5 expression. Following virus entry, F-actin cellular distribution is diminished in uninfected and virus-infected M0, M1, and M2 phenotypes. Treatment of HSV-1 phenotypes with M2 polarizing anti-inflammatory cytokines, Il-4 and Il-10, as well as with SOCS3, an inducer of IL-10 expression, will enhance expression of Rab5 and F-actin cellular distribution.

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## ACKNOWLEDGMENT

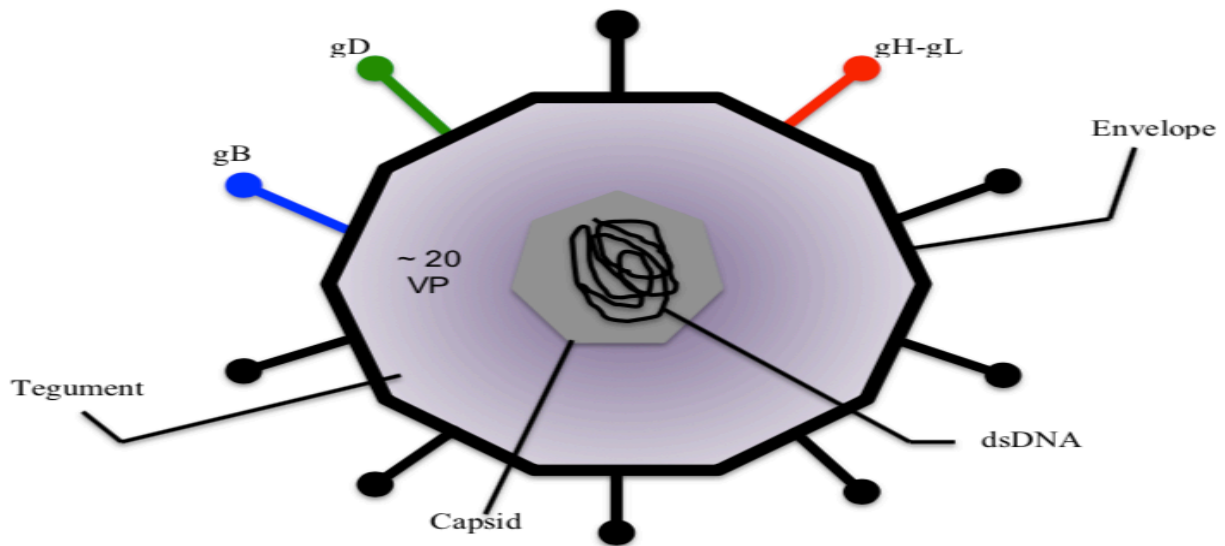
I would like to express my sincere gratitude to my advisor, Dr. Nancy J. Bigley for her support and guidance during the time I was working on this project. I could not get this work done without her experience that mainly contributed to my work. I would like to thank Dr. Hull where she was always allowing me to talk to her even though I did not schedule any appointments. Her ideas were helpful for me to complete this work. I would like to sincerely thank Dr. Wooley for all the knowledge I have learned from her classes. They have opened a new world to me in the research field where I will use this knowledge to pursue a Ph.D degree.

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I cannot achieve this without showing my gratitude to my family, especially my mother and father. They have been always taking care of me since I was a child. They had spent their power and time just to build a successful future for me. I cannot be what I am without your effort.

## Introduction

The Herpesviridae family consists of a number of DNA viruses. The most common viruses in this family that contribute to human diseases are HSV-1, HSV-2, Cytomegalovirus, Epstein-Barr virus and Varicella Zoster virus. HSV-1 is a double stranded DNA (dsDNA) virus that contains eighty conserved genes in a 154-kilobase long genome (Roizman, Knipe, Whitley, 2007). HSV-1 glycoprotein D (gD) on HSV-1 particle initiates the infection by binding to two major receptors on the target cell, the herpesvirus entry mediator (HVEM) and nectin-1. This attachment facilitates the entry of the virus particle using two different mechanisms: fusion or endocytosis. Then, the HSV-1 genome is released into the cytoplasm by the acidification of endosome containing the virus in order to enter the nucleus and replicate (Knebel-Moersdorf, 2016). This early stage of infection is the lytic phase and will be encountered by the immune system.



**Figure 1.** Virion Structure of HSV-1.

Macrophages are one of the first immune cells responding to an infection. It has been shown that the macrophage is the most abundant cell type infiltrate at site of infection (Ghiasi et al., 2000). This observation confirms the importance of macrophage during lytic infection as the depletion of macrophage enhances the replication of HSV-1 in trigeminal ganglia (Mott, Brick, van Rooijen, Ghiasi, 2007). During HSV-1 infection, virus replication is suppressed by macrophage polarized into M1 macrophage (classically activated) or maintained by M2 macrophage (alternatively activated) (Lee & Ghiasi, 2017).

Macrophage cells polarized to M1 or M2 are essential in maintaining the immune system. M1 macrophage cells are polarized when they encounter microenvironmental stimuli such as interferon-  $\gamma$  (IFN-  $\gamma$ ) that are produced by infected cells or bacterial components such as lipopolysaccharides (LPS). The M1 macrophage is able to produce pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- $\alpha$ ), IL-1 $\beta$ , and interleukin six (IL-6). These soluble molecules are able to fight the infection as they contribute to an increase in body temperature, which decreases the pathogen replication (Nan, Hongwei, & Ke, 2014). However, this process is highly regulated by the immune system, especially M2 macrophage.

M2 macrophage cells are categorized into three subtypes: M2a, M2b, and M2c. Macrophage cells are polarized to M2a by IL-4 and IL-13, while M2c is polarized by IL-10. The main function of M2 macrophage is to heal tissue wounds after the clearance of the pathogen by M1 macrophage. M2 macrophage cells are able to repair the damaged tissue and control the inflammatory status by producing ant-inflammatory cytokines, like IL-10 (Nan, Hongwei, & Ke, 2014). Moreover, not only the secretion of IL-10 but also by the production of suppressor of cytokines signaling (SOCS) proteins can control and suppress the inflammation status. SOCS1

maintains the inflammatory response while SOCS3 suppress it by inhibiting the cytokine signaling pathway of JAK and STAT (Alsharif, 2015).

Eukaryotic cells are complex in their structure and function compared to prokaryotic cells. Eukaryotic cells have membranous organelles composed of lipids and proteins. The transport of cellular cargo between membranous organelles is essential in the cell function, as this helps recycling or degrading cellular components (Bonifacino & Glick, 2004). Rab GTPases proteins are most abundant in the small GTPase family (Stenmark, 2009). In humans, seventy Rab family members belong to the Rab GTPase family with specific functions and locations inside the cell for each protein (Zerial & McBride, 2001). The main function of Rab proteins is in trafficking intracellular components, the uptake of antigens and pathogens, and the exocytosis of signaling molecules (Stenmark, 2009).

F-actin is another cellular factor that can determine the entry route of HSV-1 (Marsh, & Bron, 1997). F-actin is an integral part in the cells and is made of protein that forms a unique structural microfilaments. Filamentous structure of this protein (F-actin) is composed of many monomer globular actins called (G-actin). These monomer globular actins are held together after the hydrolysis of Adenine triphosphate molecule (ATP) forming intertwined structure of F-actin. F-actin is crucial for the cells to maintain the proper function inside the body. It helps cells migrate to infection site and cell signaling (Khadijeh, Amir, & Maryam, 2015).

## Review of Literature

### Herpes Simplex Virus-1 (HSV-1)

HSV-1 is an enveloped virus composed of double strand DNA and considered to be a large virus. The genome material is conserved in the capsid in the icosahedral shell. HSV-1 has a unique structure between the envelope and the capsid called tegument (Kelly, Cunningham, & Diefenbach, 2009). The tegument contains twenty-four viral proteins designated from VP1 to VP24 (Heine, Honess, Cassai, & Roizman, 1974). The main function of tegument proteins is to evade the immune system and aid viral replication (Kelly, Fraefel, Cunningham, & Diefenbach, 2009). This virus has the capability to infect two different types of cells: neuronal and epithelial cells. It enters neuronal cells by a pH-independent process that facilitates the fusion of the viral envelope while in non-neuronal cells HSV-1 infects and enters the cell by both pH-dependent or pH-independent endocytic processes (Nicola et al., 2005). The type of glycoprotein interacting with the host receptors determines these pathways. gB, gD, and gH-gL heterodimers are required for the fusion process in cultured cells, like Vero cells. In contrast, it has been determined that the HSV-1 particle can enter the cell by endocytosis in some cultured cells, such as Chinese hamster ovary (CHO) cells, by attaching the gD glycoprotein to the HVEM or nectin receptors. In endocytosis, the acidification of a vesicle-containing the virus particle is important in order to release the capsid, but not in the fusion process where the virus fuses and releases its contents upon binding (Milne, Nicola, Whitbeck, Eisenberg, & Cohen, 2005).



## **Endocytosis and Rab5 protein**

Endocytosis is an integral part of the cell biology. This process plays an important role in sorting molecules or proteins, recycling them to the plasma membrane, uptaking virus particles, and internalizing receptors. The Rab5 protein is a small GTPase protein acting as an early endosome near to the plasma membrane and can be found in the cytoplasm (Stenmark, 2009). The endocytosis process is highly regulated in the cell via GTP bound “on” or GDP bound “off” switch. The “on” switch is initiated when Guanine nucleotide exchange factor (GEF) catalyzes the addition of one molecule of guanosin-5' - triphosphate (GTP) to GDP-bound Rab, which activates the Rab protein. In contrast, the GTP-ase activating protein deactivates Rab5 protein by replacing GTP molecule by GDP. It serves as the “off” switch by hydrolyzing GTP molecule to GDP (Delprato, Merithew, & Lambright, 2004). As previously described, Rab5 dynamic in the cell depends on the creation of vesicles, helping many process, such as the internalization of viruses. This dynamic results in four different types of endocytic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae, and phagocytosis (Hansen, & Nichols, 2009).

## **The effect of cellular processes of macrophage and HSV-1 on Rab5**

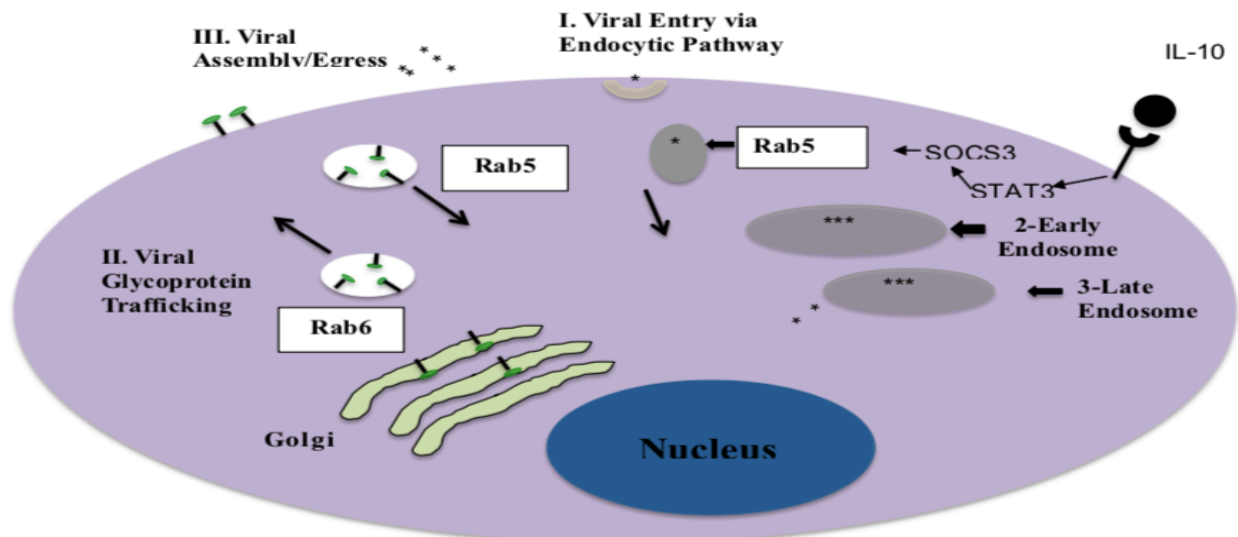
Macrophage cells are known for their function in immune defense against an infection and the production of different cytokines. A set of cytokines regulates the expression of Rab5 protein and other Rab proteins upon binding to their receptors. The binding of soluble cytokines causes an activation of Rab5 protein in order to complete the process. Receptors bound to a cytokine undergo recycling or degradation process. This is important for the cell as this renew the surface receptor for new ligand binding (Cendrowski, Mamińska, & Miaczynska, 2016). This

activation is essential for this process in order to IFN-  $\gamma$  is an important cytokine during an infection course and is mainly produced by immune cells, such as T lymphocytes, Natural killer cells (NK), and antigen presenting cells (APC). It is critical as an antiviral during the activation of innate and adaptive immune system as well as presenting antigens (Schroder, Hertzog, Ravasi, & Hume, 2004). Treating M0 macrophage in vivo with IFN-  $\gamma$  that is produced by other cells or in vitro results in an M1 macrophage phenotype (Nan, Hongwei, & Ke, 2014). Upon binding of IFN-  $\gamma$  to its receptor, it initiates a cascade of downstream signals leading to the production of various antimicrobial proteins and the stimulation of phagosome maturation (Flannagan, Cosío, & Grinstein, 2009). IFN-  $\gamma$  utilizes the Rab5 protein by regulating its dynamic in order to increase phagosome maturation. Priming macrophages with IFN-  $\gamma$  increased the expression of Rab5 protein in the macrophage cells but not fibroblast cells expressing IFN-  $\gamma$  receptors. Both Rab5 newly synthesized protein and mRNA increase upon IFN-  $\gamma$  activation at early hours. However, Rab5 expression decreases after eighteen hours to the basal level. The relation between the increase or the decrease in the expression is highly regulated by the addition or the hydrolysis of GTP molecules. Alvarez-Dominguez & Stahl showed that priming the macrophage cells with IFN-  $\gamma$  increased the GTP:GDP bound Rab5 ratio twofold (Alvarez-Dominguez, & Stahl, 1998).

IL-4 is a cytokine that plays an important role in healing damaged tissue and suppressing the inflammatory state (Nan, Hongwei, & Ke, 2014). IL-4 polarized M2a macrophage showed more enlarged endosome vesicles with or without prostaglandin E2 (PGE2). However, the combination of IL-4/PGE2 increased the number of mannose receptors (MR), which function as pattern recognition receptors, accumulated in the enlarged endosome with Rab5 protein. The

existing Rab5 in the enlarged endosome is important in order to form large endosomes while a dominant negative mutant version of Rab5 did not. IL-4 cytokines, in contrast to IFN- $\gamma$ , enhanced the activity of (GEF) as it catalyzes the addition of GTP molecules to Rab5 proteins (Wainszelbaum, Proctor, Pontow, Stahl, & Barbieri, 2006).

The HSV-1 virus also utilizes intracellular membrane trafficking to enter the cell via endocytic pathways. Rab5 has been shown to be the main regulator of virus trafficking inside the cell and the maturation of viruses. Influenza, HIV-1, and other enveloped viruses rely on the Rab GTPase proteins **Figure 2 (I)** (Spearman, 2017). Little is known about the interaction between HSV-1 and the Rab5 protein. Hollinshead and his colleagues conducted one study where they found that Rab5 is a critical step in HSV-1 maturation. Transfection of cells with siRNA



**Figure 2.** The involvement of Rab5 protein in Viral entry. (I) Viral entry in early time post infection. (II) Newly synthesized viral glycoprotein transferred to plasma membrane. (III) Viral glycoprotein are redirected inside the cell by Rab5. Prediction of effect of IL-10 on STAT3, SOCS3, and increase in Rab5 is indicated.

depleted Rab5 protein, and cells were infected with HSV-1 expressing GFP-gD. The GFP-gD fluorescence intensity were assessed at eight hours and found to be high and located in the plasma membrane. Later at eleven hours, the intensity increased further. After the depletion of Rab5 protein, the virus yield was quantified and showed a significant decrease in virus production **Figure 2 (III)** (Hollinshead et al., 2012). Both M2 are known for producing IL-10, but IL-10 treated macrophage cells produce more IL-10 compared to IL-4 treated M2c. Binding of IL-10 to its receptor activates STATA3 leading to the production of more of SOCS3 (Bode, Ehlting, & Häussinger, 2012).

### **HSV-1 virus and Host Cytoskeleton Interactions**

The entry route of viruses and their ability to infect neighboring cells are also determined by F-actin distribution (Marsh, & Bron, 1997; Herman W. Favoreel et al., 2005). Dense cortical cytoskeleton can serve as a barrier that protects organelles by separating them from the plasma membrane (Vitale, Seward, & Trifaró, 1995). HSV-1 has the ability to enter cells by fusion or endocytosis (Milne, Nicola, Whitbeck, Eisenberg, & Cohen, 2005). The fusion of a virus through the plasma membrane of some cultured cells can be hindered by dense cortical cytoskeleton. Marsh & Bron (1997) showed that even some viruses prefer to enter baby hamster kidney (BHK) and (CHO) cells by endocytic pathway; they can also enter a cell by fusion. However, the induction of an acid environment, which facilitates the process of pH-dependent fusion, helped the fusion of the virus but no productive infection was seen. They suggested that the virus could undergo fusion but could not deliver the nucleocapsid into the cytoplasm. They attributed this observation to the dense cortical cytoskeleton that prevented the virus from delivering its contents into the cytoplasm. Therefore, HSV-1 and other viruses take advantages of the

endocytic pathway (Marsh, & Bron, 1997). Viruses such as HIV-1 have evolved a unique way to overcome this biological barrier. HIV uses its own protease to break down actin protein in order to fuse into cells (Yoder, 2008).

## **Materials and Methods**

### **Cell Lines**

The 264.7 RAW murine macrophage cell line was purchased from American Type Culture Collection (ATCC). This cell line was obtained from adult male BALB/c mice. This cell line was transformed by Abelson murine leukemia virus-induced tumors. The cells were grown on vented BioLite flask (Fisher Scientific, Pittsburgh, PA). The cells were supplemented with Dulbecco's Modified Eagle's Medium (DMEM; HyClone), 10% Fetal Bovine Serum (FBS) (Fisher Scientific), and the pH was maintained by 10 mM HEPES buffer. The cells were split when they are 70% confluent using a cell scraper (Fisher Scientific) to detach the cells from the flask surface since they are adherent cells. Flasks containing cells were kept in 100% humid incubator and 5% carbon dioxide CO<sub>2</sub> at 37° C degree.

CCL-81 Vero cells were purchased from American Type Culture Collection (ATCC). This cell line was obtained from kidney tissue of normal African green monkey. The cells were grown on vented BioLite flask (Fisher Scientific, Pittsburgh, PA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; HyClone), supplemented with 10% Fetal Bovine Serum (FBS) (Fisher Scientific), and the pH was maintained by 10 mM HEPES buffer. The cells were split when they were 70% confluent using Trypsin (Fisher Scientific). The cells were grown in 100% humid incubator and 5% carbon dioxide Co<sub>2</sub> at 37° C degree.

### **Plaque Assay**

Plaque assay technique was used to determine HSV-1 (syn17+) (originally supplied from Dr. Nancy Sawtell, Children's Hospital Center, Cincinnati, OH) virus stock in order to infect cells with 0.1 MOI. Vero cells were grown on six wells plates (Fisher Scientific) until they reached around 90% confluence. Then, the media in each well were aspirated, and a different

serial dilution of virus stock was incubated on each well for two hours in 37° C degree incubator with one well without virus as a control. After two hours of incubation, the virus was discarded from each well and 2% of methylcellulose gel was applied on all wells including the control. The plate was incubated between three to five days until the plaques can be visualized. The gel was removed, and the wells were washed with PBS to remove the remaining gel. Cells were fixed with 4% paraformaldehyde for 30 minutes and stained with 1% crystal violet. To choose the best well to count the plaques, a well that has a number of plaques between 30-100. The following equation was used to determine the virus titer:

$$\text{PFU/ml (of original stock)} = \frac{\text{Number of Plaques}}{\text{Dilution factor} \times (\text{ml of inoculum/well})}$$

### **Polarization Treatment**

Cells were treated for two, four, six, and 24 hours to see the changes in M1, M2a, and M2c at these time points. M1 phenotype was achieved by adding IFN-  $\gamma$  (20ng/mL) (Biolegends) and LPS (100ng/mL) for two, four, six, and 24 hours to see the changes in M1 at these time points. M2 phenotypes was polarized by two different treatments to convert cells to either M2a or M2c. M2a cells were polarized from naïve macrophage cells by adding murine IL-4 cytokine (20ng/mL) (Biolegends). M0 macrophage cells were converted to M2c subtype by IL-10 cytokine (20ng/mL) (Biolegends). SOCS1 and SOCS3 were used in this study, as they are known for suppressing cytokines signaling, production, and the ability to interfere with STAT signaling pathway. SOCS1 and SOCS3 treated M1, M2a, and M2c with (35mM/mL) **Table 1.**

**Table 1:** Table showing the treatments for each experimental group

<b>Treatment</b>	<b>Concentration</b>
Negative (Control)	No treatment
Lipopolysaccharides (LPS)	100ng/mL
Interferon gamma (IFN $\gamma$ )	20ng/mL
Interleukin-10 (IL-10)	20ng/mL
Interleukin-4 (IL-4)	20ng/mL
Suppressor of cytokines signaling Peptide Mimetic 1 & 3 (SOCS 1& 3)	35uM/mL

### **Immunofluorescence Staining**

In this study, F-actin staining shows a decrease of increase in intracellular actin. For example, increase staining indicates aggregation of F-actin where decrease staining shows a deaggregation of F-actin and an increase in aggregated G-actin.

5000 Cells were grown on a 12 well chambers slide and incubated in the incubator until cells attached to the bottom of chamber. Appropriate treatment and 0.1 MOI of virus were added and incubated for two, four, six, and 24 hours **Table 1**. After the incubation time, media containing treatments and virus were discarded, and chambers were washed twice with Phosphate Buffer Saline (PBS). Cells in chambers were fixed with 4% paraformaldehyde for 10 minutes at room temperature to stabilize the cellular structures and protein and then washed with PBS three times. Then, cells were permeabilized using 0.01% Saponin for 5 minutes at room temperature to form pores and allow antibodies to get in the cell. Cells were washed again with PBS three times to remove excess Triton. To prevent non-specific binding, cells were blocked



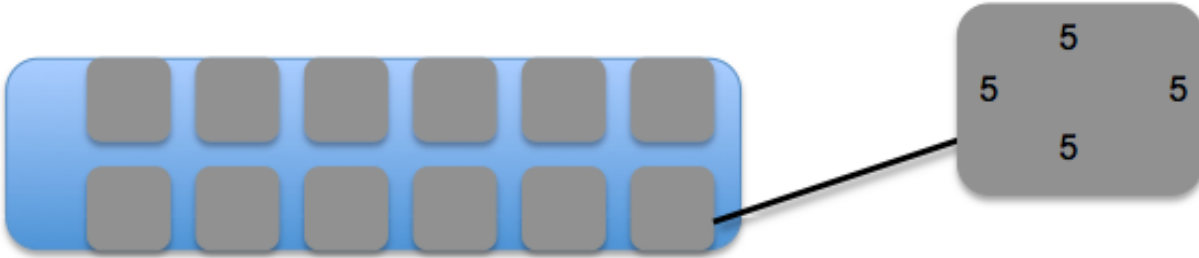
with 10% Goat serum for one hour at room temperature. After one hour incubation, blocking solution was removed, and cells were washed with PBS three times. Primary antibody to Rab5 was added and incubated at 4° C overnight **Table 2**. Next day, primary antibody was discarded, and 12 chambers slides were washed with PBS three times. Secondary antibody to Rab5 primary antibody was incubated with cells for one hour at room temperature. The secondary antibody then was discarded, and chambers were washed three times with PBS and F-actin stain was applied for 1 hour at room temperature **Table 2**. Then, chambers again were washed with PBS three times and left for 10 minutes until they were dry. Mounting medium was applied on chambers and cover slip on the top of the medium. Slides were visualized using ACCU-SCOPE EXC-350 microscope with scopeLED fluorescence illuminator at 500x magnification.

**Table 2:** Table showing the concentration of antibodies used for immunofluorescence staining

<b>Antibody</b>	<b>Concentration</b>	<b>Company</b>
Anti-Rab5 antibody [34A] 1° antibody	1:200	<u>Abcam</u> , San Francisco, <u>Ca</u>
Goat Anti-Mouse <u>IgG H&amp;L (Alexa Fluor488)</u> 2° antibody	1:500	<u>Abcam</u> , San Francisco, <u>Ca</u>
Texas Red- <u>Phalloidin</u> (F-actin stain)	1:40	<u>ThermoFisher Scientific</u> , Waltham, MA

### **Selection of fields for taking pictures**

Five pictures were taken from each edge **Figure 3**.



**Figure 3.** Pictures were selected systematically

### **Image Analysis**

ImageJ (from National Institute of Health NIH) was used to analyze the pictures fluorescence intensity by quantifying the region of interest (qROI). Initially, pictures were colored and, their color was changed to black and white. In order to get rid of all artifacts and non-specific binding of antibodies in the background, pictures were subtracted. Threshold was applied on the pictures to exclude black background, which range between zero to 20 pixels, and the bright artifacts, which range between 111 to 255 pixels. Then, pictures were analyzed using analyze particles option in the software. This gives the average size, which is the total area divided by count.

### **Cell Viability**

Cells were seeded on six wells plate with the appropriate treatments. After the desired time point was reached, cells were scraped and transferred into 1.5 mL centrifuge tube. The tubes were spun on 2000 Round Per Minute (RPM) for five minutes at 4 degrees. The pellet was suspended in one mL complete media. A 1:2 ratio of cells to Trypan blue was mixed together,

and 10 ul of the mixture were pipetted into the hemocytometer. Alive cells appeared colorless due to ability of the lipid bilayer of the cell to exclude the dye. On the other hand, dead cells appeared blue because the cells membrane is permeable to the dye and can take it up. Four squares were counted, and two edges in each square were counted but not the other two edges.

The percentage of cell viability were determined based on the following equation:

$$\text{Cell Viability} = \frac{\text{Alive cells}}{\text{Total Cells (Alive and Dead Cells)}} \times 100$$

### **Statistical analysis**

All experiments were done three times. All data were run on SigmaPolt 12.0 software using One-Way ANOVA to identify the significant values.

## Results

### **Polarized M1 macrophage with or without HSV-1 infection exhibited a significant differences compared to the control at early hours**

The level of Rab5 expression was evaluated at two, four, and six hours after the polarization with or without the HSV-1 infection. Uninfected cells were compared to unpolarized M0 cells, and HSV-1 infected cells were compared to HSV-1 infected unpolarized M0 cells. The Rab5 protein in uninfected and infected M1 cells localized in the plasma membrane. Uninfected and HSV-1 infected Polarized M1 macrophage with LPS and IFN- $\gamma$  and M1 with SOCS3 showed a dramatic increase of Rab5 expression compared to M0 unpolarized cells where SOCS3 challenged uninfected M1 expressed more Rab5 compared to HSV-1 infected M1 at two hours. The HSV-1 infection did not affect Rab5 activity in both uninfected and HSV-1 infected cells at two hours **Figure 4**.

At four hours, both uninfected and HSV-1 infected M1 cells showed a similar pattern as at two hours. However, SOCS1 and SOCS3 challenged HSV-1 infected M1 cells induced the intensity of Rab5 significantly compared to HSV-1 infected unpolarized cells. Virus infection sharply decreased the level of Rab5 in HSV-1 infected M1 cells but showed an increase expression of Rab5 in SOCS3 challenged HSV-1 infected M1 **Figure 5**.

After six hours of treatment and infection, all groups uninfected and HSV-1 infected cells did not show any significant changes compared to their controls. However, when comparing the effect of HSV-1 infection on both SOCS1 and SOCS3 challenged M1 cells, Rab5 expression in HSV-1 infected M1 was slightly lower than uninfected cells **Figure 6**.

**HSV-1 infection of IL-4 polarized M2a macrophage showed a rapid increase the level of Rab5 expression at two hours, but IL-4 treatment increased Rab5 in uninfected cells after four hours**

HSV-1 virus infection caused a sudden increase in Rab5 expression (staining intensity) at two hours in all infected groups compared to uninfected unpolarized and polarized phenotypes both treated and untreated with SOCS1 or SOCS3 peptides **Figure 7**. At four hours, the level of Rab5 expression in HSV-1 infected unpolarized M0 cells returned to the basal level compared to the uninfected control. IL-4 treatment enhanced Rab5 expression of uninfected cells with or without SOCS3 treatment compared M0 cells but not with the HSV-1 infection **Figure 8**.

After six hours, uninfected IL-4 polarized M2a cells Rab 5 expression remained significantly higher than in the M0 ( $p < 0.001$ ). HSV-1 infection did stimulate an increase in Rab5 expression in unpolarized M0 and IL-4 polarized M2a cells treated with SOCS3 compared to uninfected control groups **Figure 9**.

**IL-10 treatment stimulated a marked increase of Rab5 protein expression following HSV-1 infection**

At two hours, HSV-1 infected macrophages exhibited a significant increase in Rab5 protein expression in IL-10 polarized M2c cells; SOCS3 treatments did not alter this response. SOCS3. Infecting cells with the HSV-1 virus drastically enhanced the level of Rab5 expression in SOCS1 and SOCS3 challenged, IL-10 polarized M2 cells compared to uninfected groups **Figure 10**.

At four hours, IL-10 treatment of uninfected cells showed a higher Rab5 expression compared to the control; SOCS1 or SOCS3 treatment did not alter this response. Rab5

expression was enhanced in both HSV-1 infected unpolarized M0 and IL-10 polarized M2c cells. A decrease in the Rab5 expression was apparent following SOCS1 or SOCS3 treatment of HSV-1 infected M2c macrophages **Figure 11**.

After six hours of treatment and virus infection, no significant changes in Rab5 expression were seen for both uninfected and HSV-1-infected cells except in SOCS3 treated cells. Rab5 expression for both SOCS3 treated, uninfected and HSV-1 infected, M2c macrophages were higher than in their controls. When comparing each uninfected sample to HSV-1 infected sample, HSV-1 infected M2 cells with or without SOCS1 treatment showed significantly higher expression of Rab5 compared to uninfected M2c cells **Figure 12**.

#### **Rab5 expression in M1, IL-4 and IL-10 polarized M2 cells with or without HSV-1 infection at 24 hours (predicted time of initial viral egress)**

After 24 hours, Rab 5 expression in HSV-1-infected and uninfected, polarized and unpolarized, and treated or untreated with SOCS peptides was determined. This time was selected as a potential time of initial release of mature virus from the infected cell.

HSV-1-infected and uninfected M1 polarized cells showed an insignificant decrease in Rab5 expression; treatments of these cells with the SOCS peptides did not alter this observation.

#### **Figure 13.**

Rab5 protein expression in IL-4 polarized M2a cells was upregulated by HSV-1 infection at 24 hours. Uninfected IL-4 polarized M2a cells treated with either SOCS1 ( $p < 0.05$ ) or SOCS3 ( $p < 0.001$ ) peptide showed significant increases in Rab5 expression. In contrast, Rab5 expression was only upregulated following HSV-1 infection of unpolarized M0 cells and in IL-4 polarized M2a cells treated with SOCS1 peptide **Figure 14**.

HSV-1 infected M0 (unpolarized) cells showed an increase in Rab5 expression over uninfected M0 cells ( $p < 0.05$ ). IL-10 polarized M2c macrophages showed significant increases in Rab5 expression ( $p < 0.001$ ) in uninfected cells compared with HSV-1 infected cells. IL-10 polarized M2c cells exposed to either SOCS1 (NS) or SOCS3 ( $p < 0.05$ ) peptide, showed decreases in Rab5 expression following HSV-1 infection compared with uninfected counterparts.

**Figure 15.**

**F-actin distribution- M1 Macrophages**

In general, F-actin staining intensity indicative of distribution with the cell was diminished in HSV-1-infected M1 polarized at two, four, and six hours after polarization and infection. This observation was attributed to the polarizing agents, IFN- $\gamma$  and LPS, on F-actin expression (Hu, Hu, & Boumsell, 2008).

At two hours, only HSV-1-infected M1 polarized macrophages showed an increase in F-actin staining intensity ( $p < 0.05$ ). In contrast, only the uninfected M0 ( $p < 0.05$ ) and M1 cells treated with either SOCS1 ( $P < 0.001$ ) or SOCS3 ( $P < 0.001$ ) peptide exhibited increase in F-actin staining intensity over their infected counterparts **Figure 16.**

At four hours, uninfected M0 and M1 polarized macrophages showed increases in F-actin staining intensity ( $p < 0.001$ ) over HSV-1-infected counterparts. SOCS1 peptide treatment enhanced F-actin staining intensity in M1 cells ( $p < 0.001$ ) while SOCS3 treatment of M1 cells caused a decrease in F-actin staining intensity ( $p < 0.01$ ) **Figure 17.**

After six hours of incubation, uninfected M0 cells ( $p < 0.001$ ), M1 cells ( $p < 0.05$ ) and M1 cells treated with SOCS1 peptide ( $P < 0.001$ ) showed increases in F-actin staining intensity when compared with their HSV-1-infected counterparts. Although not significant, a slight increase in

F-actin staining occurred in virus-infected M1 cells treated with SOCS3 peptide over that seen in their uninfected counterpart **Figure 18**.

#### **F-actin distribution- IL-4 polarized M2a Macrophages**

After 2 hours of polarization and HSV-1 infection, F-actin staining intensities were increased in similar in M0, M2a, and M2a cells and M2a cells treated with SOCS1 peptide. A noticeable increase in F-actin staining occurred seen in the HSV-1-infected M2a cells treated with SOCS3 peptide ( $p < 0.01$ ) **Figure 19**.

At four hours after polarization and HSV-1 infection, increases in F-actin staining occurred in infected M0 ( $p < 0.05$ ) and M2a cells treated with SOCS3 peptide ( $p < 0.01$ ) over uninfected counterparts; increase in F-actin staining occurred in uninfected M2a macrophages ( $p < 0.001$ ) and M2a cells treated with SOCS1 peptide (NS) over their virus-infected counterparts **Figure 20**.

Six hours after polarization and HSV-1 infection, only uninfected M0 cells ( $p < 0.001$ ) and M2a macrophages treated with SOCS3 peptide ( $P < 0.001$ ) showed increases in F-actin staining over their virus-infected counterparts **Figure 21**.

#### **F-actin distribution- IL-10 polarized M2c Macrophages**

After 2 hours of polarization and HSV-1 infection, F-actin staining intensities were increased in uninfected M0 cells ( $p < 0.01$ ), M2c cells, M2c cells treated with SOCS 1 peptide, and M2c cells treated with SOCS3 peptide over their virus-infected infected counterparts **Figure 22**.



After 4 hours of polarization and HSV-1 infection, F-actin staining intensities were slightly increased (NS) in M0 and M2c cells treated with SOCS3 peptide in virus-infected cells compared with uninfected cell counterparts. Increases in F-actin staining intensities were seen in M2c cells (NS) and M2c cells treated with SOCS3 peptide ( $p < 0.001$ ) **Figure 23.**

After 6 hours of polarization and HSV-1 infection, F-actin staining intensities were increased only in HSV-1-infected the M2c cells treated with SOCS1 ( $P < 0.001$ ) or SOCS3 ( $P < 0.01$ ) peptides over uninfected cell counterparts. Interestingly, a significant increase in F actin staining occurred in uninfected M2c cells ( $p < 0.001$ ) over their virus-infected counterpart **Figure 24.**

#### **F-actin distributions-M1 Macrophages 24 hours After Polarizations and HSV-1 Infection**

At 24 hours after polarization and HSV-1 infection, F-actin staining intensities were generally lower in untreated and virus-infected M1 cells and M1 cells treated with SOCS peptides. M0 cells and M1 cell showed increases in F-actin staining in the uninfected cells over their infected counterparts ( $p < 0.001$ ). In contrast, the virus-infected M1 populations treated with SOCS1 ( $P < 0.01$ ) or SOCS3 ( $P < 0.05$ ) peptides showed an increase in F-actin staining over their uninfected counterparts **Figure 25.**

#### **F-actin distributions-M2a Macrophages 24 hours After Polarizations and HSV-1 Infection**

At 24 hours after polarization and HSV-1 infection, F-actin staining intensities were generally higher in the unaffected M0 cells ( $p < 0.05$ ), M2a cells ( $p < 0.001$ ), and M2a cells treated with SOCS1 peptide (NS) over their HSV-1-infected counterparts. **Figure 26.**

### **F-actin distributions-M2c Macrophages 24 hours After Polarizations and HSV-1 Infection**

At 24 hours after polarization and HSV-1 infection, F-actin staining intensities were higher in uninfected M0 cells over virus infect cm0 cells (NS), but significantly higher in virus-infected M2c cells ( $p < 0.05$ ) over their uninfected counterpart. Treatment with either SOCS1 ( $P < 0.001$ ) or SOCS3 peptide ( $p < 0.05$ ) significantly diminished F-actin staining of the M2c macrophages

**Figure 27.**

### **Cell Viabilities of Polarized and HSV-1 Infected Macrophages.**

M1 cells upon polarizing with LPS and IFN- $\gamma$  showed a significant decrease in cell viability ( $p < 0.001$ ) compared to the control. SOCS1 peptide mimetic decreased the viability percentage but was not significant. SOCS3 mimetic increased the viability (NS). Only M1 polarized cells or HSV-1-infected M1 polarized cells ( $p < 0.001$ ) cells showed significant decreases in cell viability **Figure 28**. On the other hand, all M2 phenotypes with or without the virus, and/or SOCS1, and SOCS3 treatments showed no significant differences in cell viabilities

**Figure 29 & 30.**

## Discussion

Many viruses rely on the endocytic pathway in order to enter and start replicating their viral genome (Spearman, 2017). Cytokines, such as those used in polarizing M1 and M2 macrophages, binding to their receptors also affect the dynamics of endocytosis (Flannagan, Cosío, & Grinstein, 2009; Wainszelbaum, Proctor, Pontow, Stahl, & Barbieri, 2006). Rab5 is the master regulator of this essential process during the activation of receptors or the recognition of an invading pathogen (Stenmark, 2009). The role of Rab5 in HSV-1 entry has not yet been investigated even though the virus is able to enter the cells via endocytosis. Several studies showed that HSV-1 entry by fusion or endocytosis depends on cell lines are being used. This observation suggests that HSV-1 can utilize Rab5 at entry (Milne, Nicola, Whitbeck, Eisenberg, & Cohen, 2005). Rab5 is referred to as an early endosome marker, acting early after cell entry. but Rab5 is also active later in vesicles during egress of molecules from the cell (Hollinshead et al., 2012). Based on previous literature, Rab5 protein was expected to increase eight hours after infection. Hollinshead et al. (2012) showed that the synthesized glycoprotein was transferred to the plasma membrane after eight hours post HSV-1 infection and then redirected by using the Rab5 protein for envelopment purpose.

This study demonstrated that uninfected M1 macrophages exhibit significant increases in Rab5 expression at 2 and 4 hours after polarization with LPS and IFN- $\gamma$  compared to unpolarized cells. This increase is not enhanced by HSV-1 infection of the M1 macrophages/. This was an expected result, as IFN-  $\gamma$  is known to induce maturation of phagosomes that uses the Rab5 protein (Alvarez-Dominguez, & Stahl, 1998). HSV-1 infected M0 and IL-4 polarized M2a cells showed increases in Rab5 expression at 2 hours compared to uninfected polarized M2a cells. One explanation for the observation is that the virus may be using the endocytic route at 2 hours

post infection. However, further study is needed to determine observation whether the upregulation of Rab5 was due to the HSV-1 infection. Interestingly, IL-4 polarized M2a cells gradually showed increased expression Rab5 endosomes at four and six hours after polarization (Wainszelbaum et al., 2006). Wainszelbaum, and colleagues (2006) showed that IL-4 induced endosome enlargement. Using a dominant negative mutant version of the Rab5, this group showed that the cells were not able to form any endocytic vesicles. These finding substantiate our findings that IL-4 upregulates expression of the Rab5 protein.

Both M2 polarizing agents, IL-4 and IL-10, induce secretion of IL-10 with IL-10 being an autocrine in M2c cells (Alsharif, 2015; Kim et al., 2015). IL-10 maintains the anti-inflammatory status in the M2 cell phenotypes. Because IL-10 stimulates production of SOCS3 protein through STAT3 (Bode, Ehling, & Häussinger, 2012), the effects of treatment with SOCS peptide mimetics was evaluated on Rab5 expression and F-actin cellular distribution in the present study (Figure 2). HSV-1 infection of M2 macrophage phenotypes treated with SOCS3 peptide enhanced expression of Rab5 and increased cellular distribution of F-actin. HSV-1 infection of M2 macrophage phenotypes treated with SOCS 3 peptide enhanced expression of Rab5 and increased cellular distribution of F-actin. Enhanced Rab5 expression was seen only in HSV-1 infected unpolarized M0 macrophages and in SOCS-1 treated M2a macrophages.

IFN-  $\gamma$  treatment of M1 cells was shown to decrease the F-actin expression gradually from 2 to 24 hours after polarization. Others have shown that IFN-  $\gamma$  negatively affects F-actin cellular distribution (Hu, Hu, & Bousmell, 2008). Interestingly, F-actin distribution was further decreased following HSV-1 infection of unpolarized M0 macrophages and M1-polarised cells. Also, HSV-1 infected Vero cells display a low level of F-actin after four hours of infection due to viral protein US3 (Bedows, Rao, & Welsh, 1983). This finding strongly agrees with our

finding that HSV-1 infected M1 cells showed decreased F-actin staining (distribution) compared to uninfected M1 cell. Cellular distribution of F-actin in IL-4 polarized M2a was higher than in the control cells at four and 24 hours but not at two and six hours. Frances and colleagues (2013) also note that IL-4 enhances F-actin staining in murine macrophages (Frances Y., Tingting, Phoebe, Thanh,c& Wendy ,2013).

This study supports the observation of Reichard et al (2015) that unpolarized and HSV-1 infected M1 macrophages showed marked decreases in cell viability at 24 hour after polarization; polarization treatment alone appears to be responsible for this decrease compared to This was an anticipated result, as the macrophage is known for its role in clearing viral infection. Wang et al. (2011) showed that the secretion of pro-inflammatory cytokines, such as TNF- $\alpha$ , by M1 macrophage contributes to cell death in vivo and in vitro.

## Future Studies

SOCS3 peptide treatment enhanced upregulation in Rab5 protein expression in some experimental groups. Because Rab5 expression varied among the test groups in this study, quantitation of these differences could be made using the more sensitive flow cytometry technique. This technique would permit identification of factors regulating Rab5 expression such as guanine exchange factor (GEF) and guanine activating proteins (GAP) to determine whether their fluctuations in expression correlated with those of Rab5. An increase in GEF:GAP after SOCS3 peptide treatment will simulate the activator protein GEF leading to an increase in Rab5 expression.

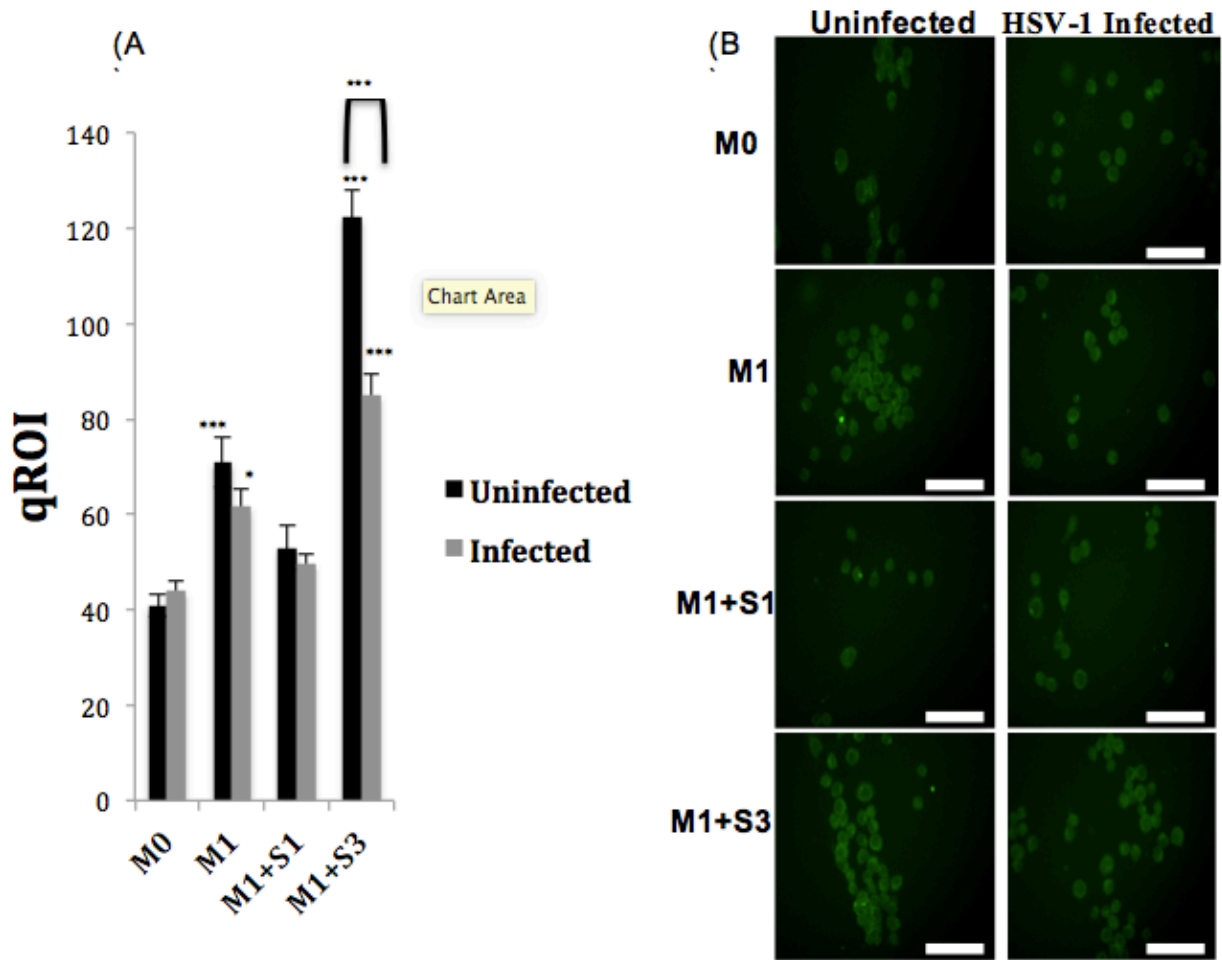
In his study, decreased viabilities of M1 phenotypes was attributed to the formation of inflammatory cytokines stimulated by the M1 polarizing agents,  $\text{IFN}\gamma$  and LPS. This supposition can be confirmed by intracellular staining of these cells for pro inflammatory cytokines -TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Also reactive oxygen species (ROS) and nitric oxide (NO) may also contribute this loss in cell viability in M1 phenotypes at 24 hours after polarization. In future experiments, the effects of inhibitors such as pyruvate kinase M2 (ROS inhibitory) and nitric oxide synthetase (NO inhibitor) could be evaluated in the M1 macrophage phenotypes.

To determine whether the virus co-localizes with Rab5 in endosomes, GFP-labeled HSV-1 gD or fluorescently labeled HSV-1 gD antibody can be used in immunolocalization and/or flow cytometry studies. If co-localization occurs, this would indicate that the virus used the endocytic pathway in entry. If not, this would indicate that the virus entered only by fusion.

Extending the observation time points to 48 and 72 hours, we will be able to do an evaluation of Rab5 changes that will happen during the complete viral egress. It is expected that

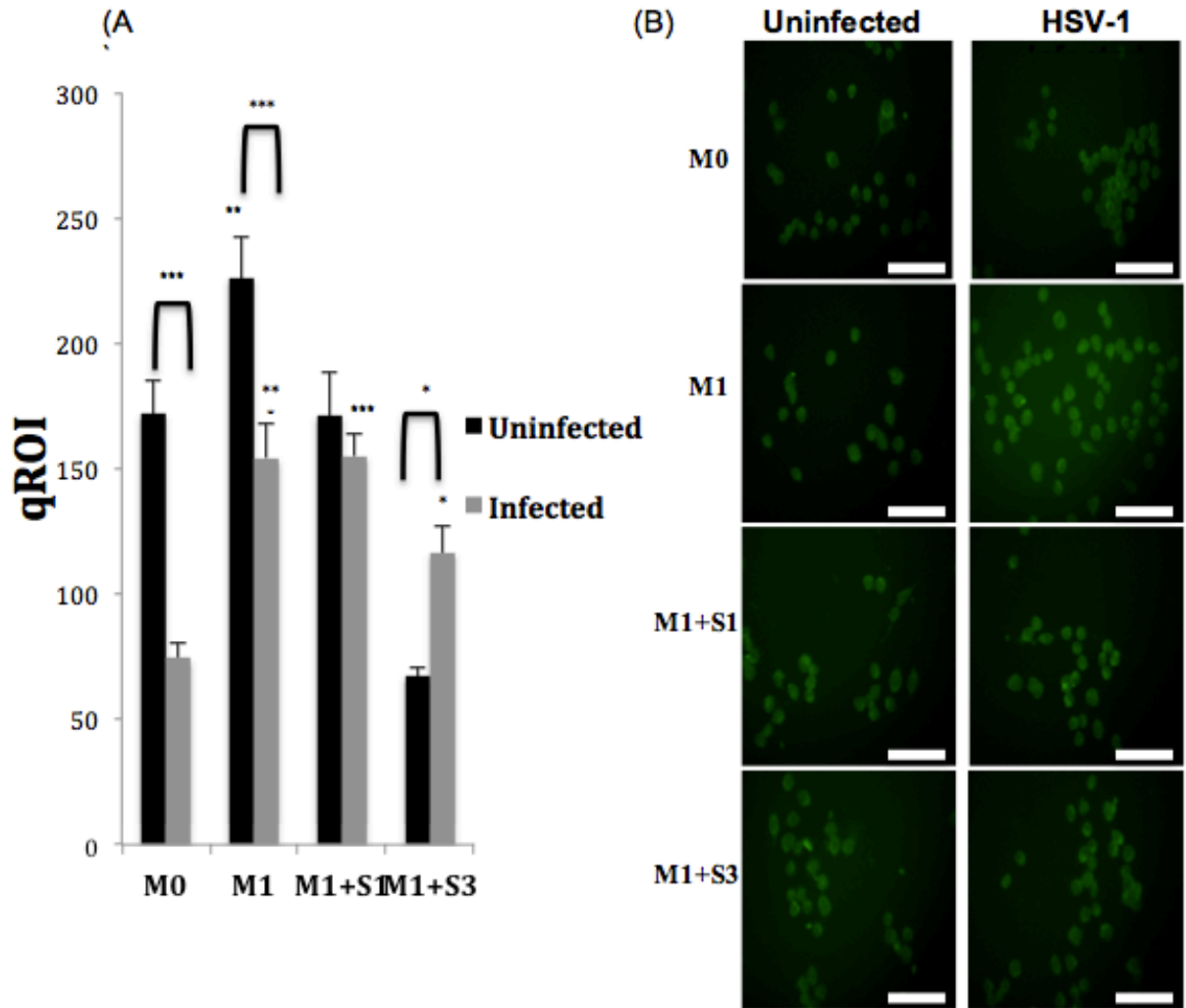
Rab5 protein expression will be enhanced in virus infected M0 and M2 phenotypes compared to that observed in M1 macrophage phenotype at 48 and 72 hours following HSV-1 infection.

## Figures

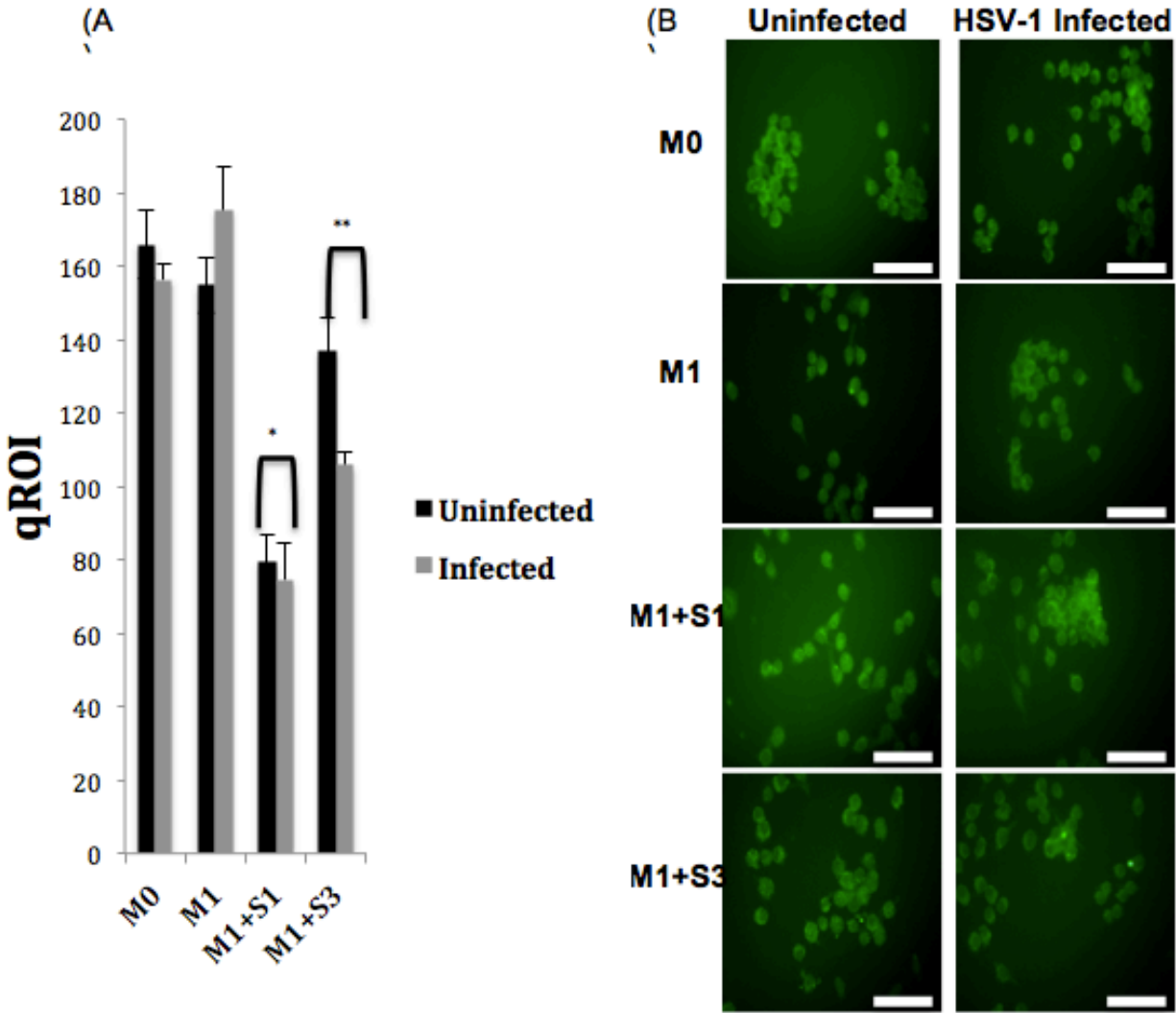


**Figure 4. Rab5 immunofluorescence intensity and morphological changes in polarized uninfected and HSV-1 infected M1 at 2h.** (A) Level of Rab5 expression. The stars on black bars shows the significant of uninfected M1 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M1 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups. (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) ( $P < 0.05$  \*,  $P < 0.01$  \*\*, and  $P < 0.001$  \*\*\*)

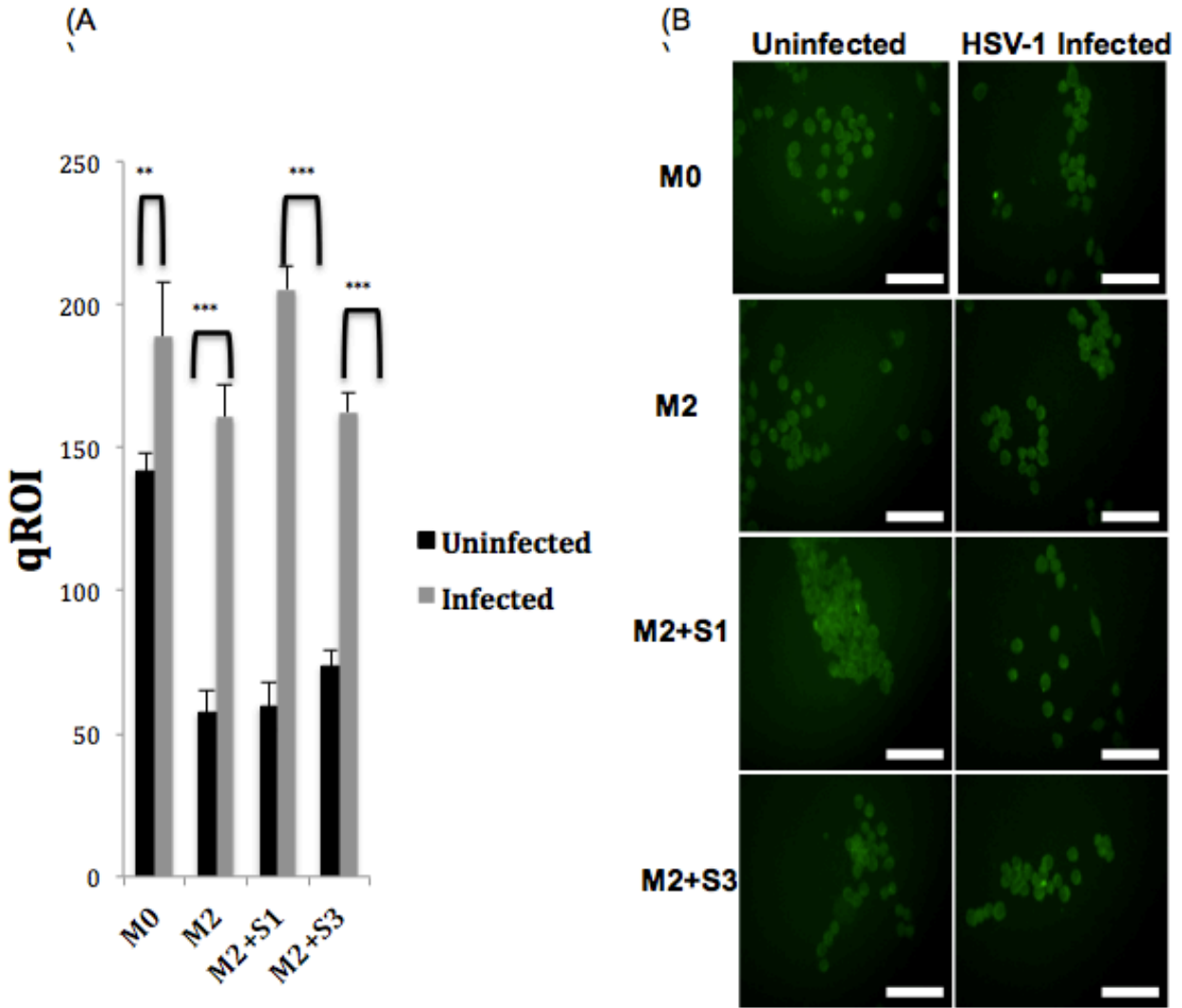




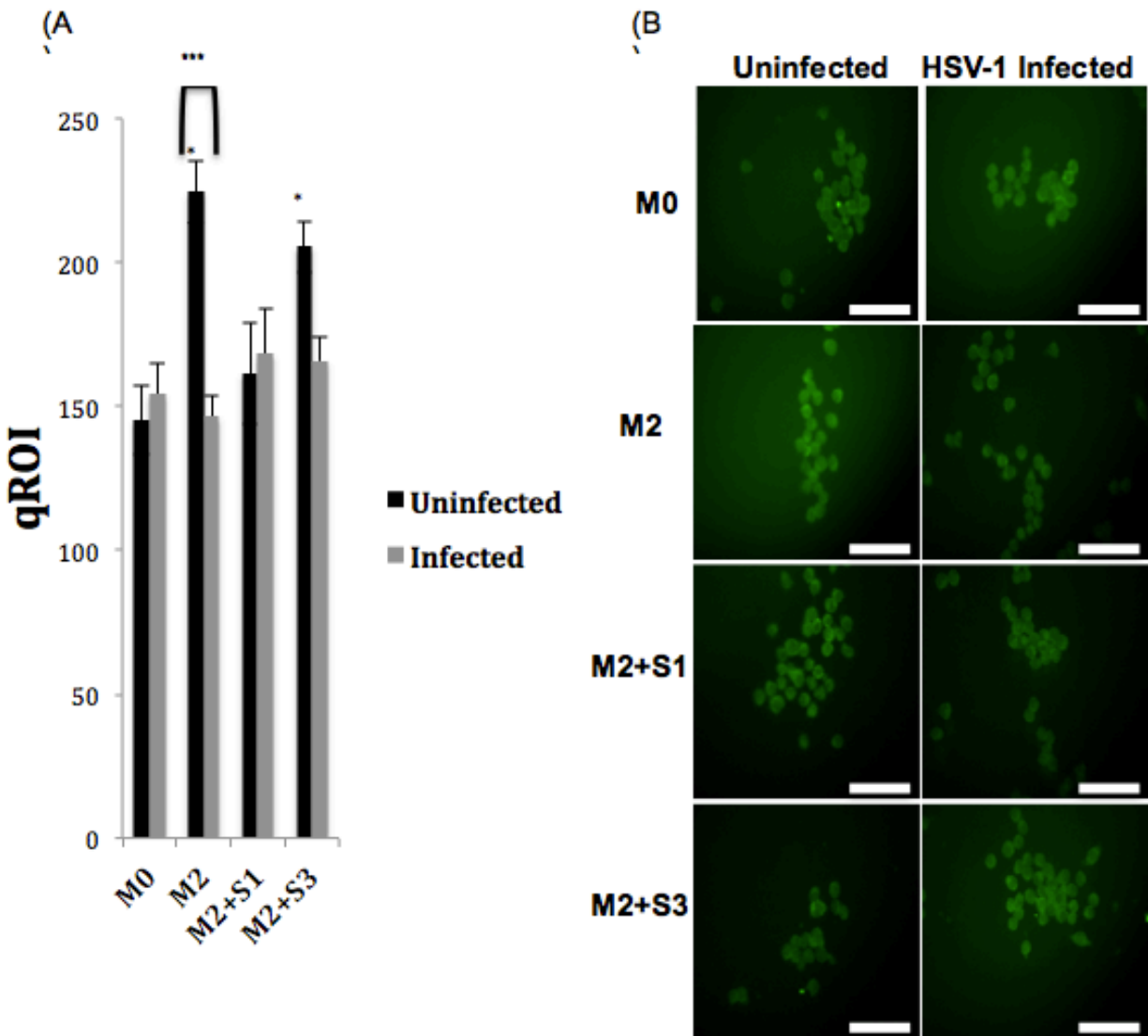
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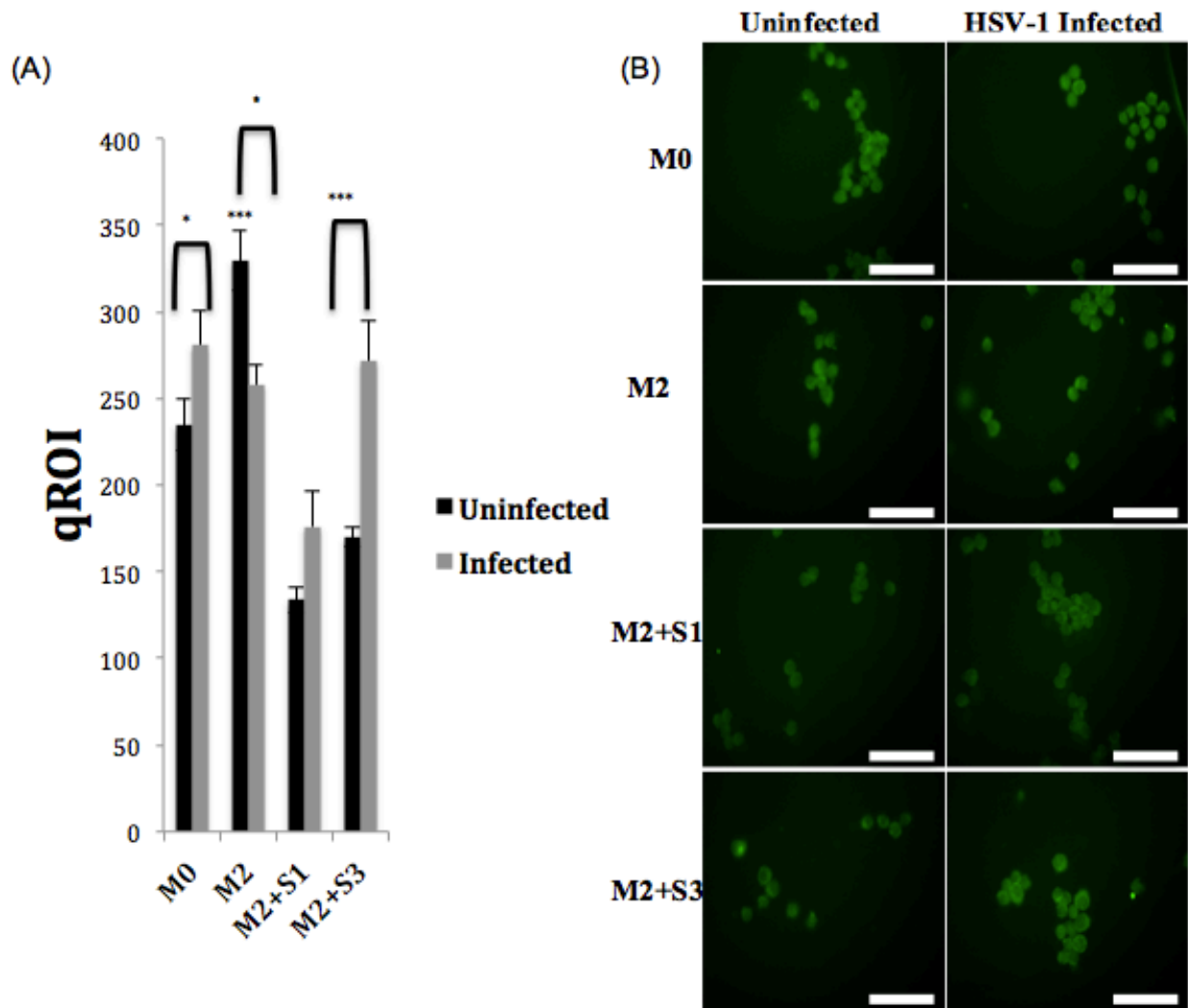
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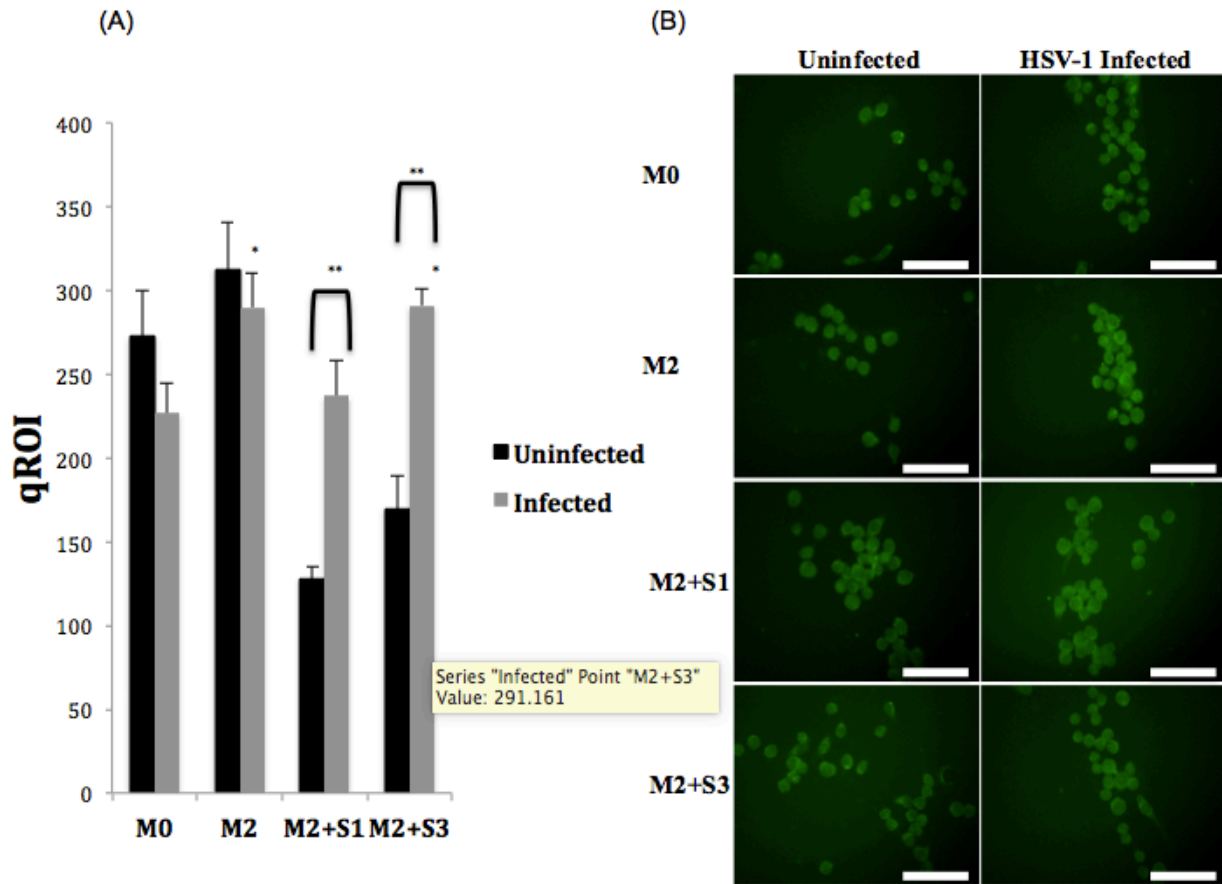
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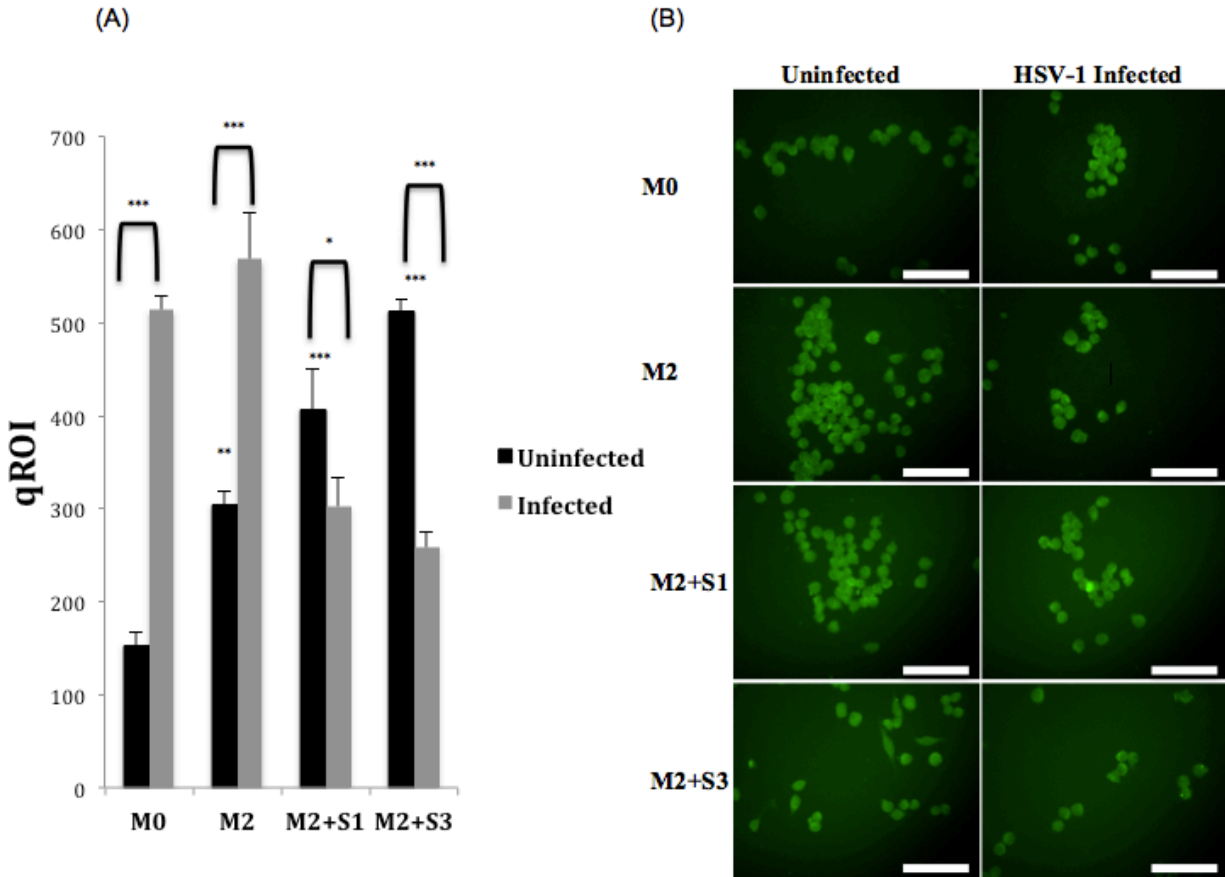
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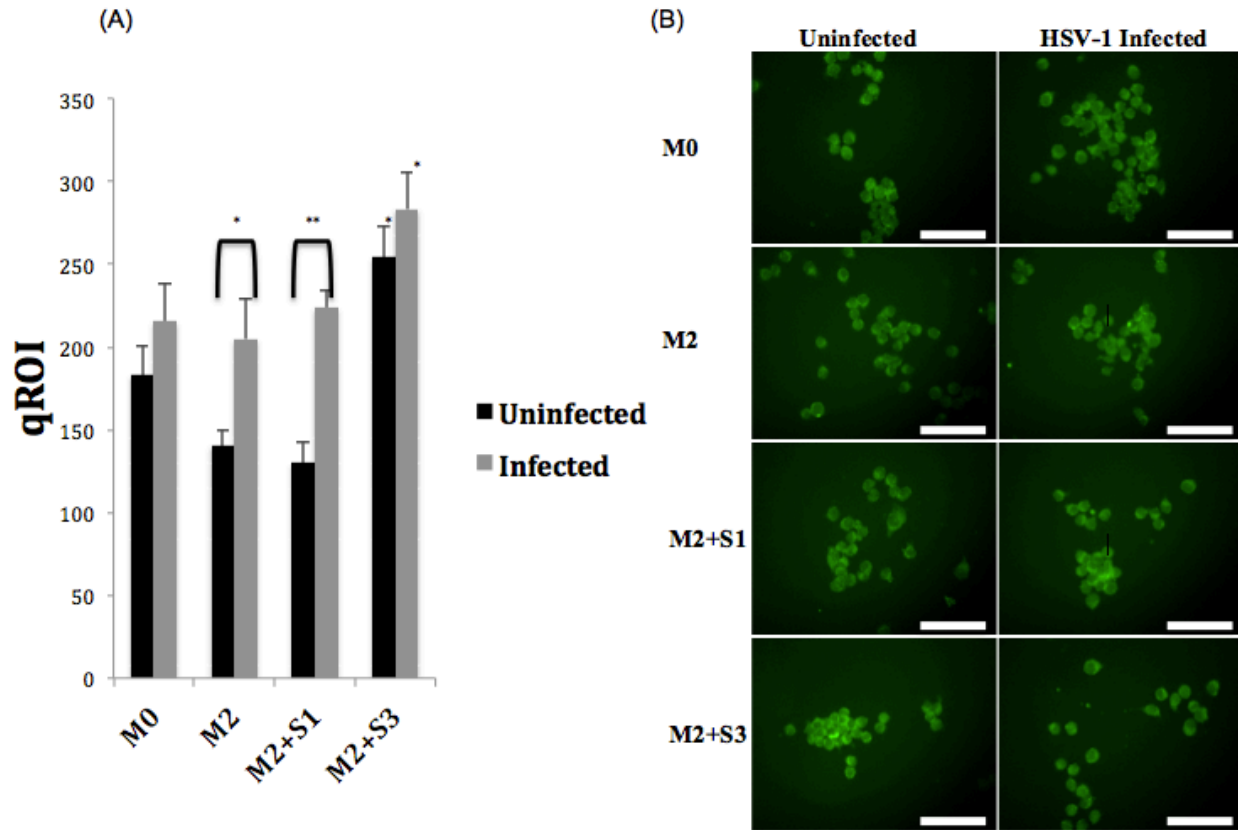
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**Figure 10. Rab5 immunofluorescence intensity and morphological changes in uninfected IL-10 polarized M2 and HSV-1 infected IL-10 Polarized M2c at 2h.** (A) Level of Rab5 expression. The stars on black bars shows the significant of uninfected M2c cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2c cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, and P<0.001 \*\*\*)

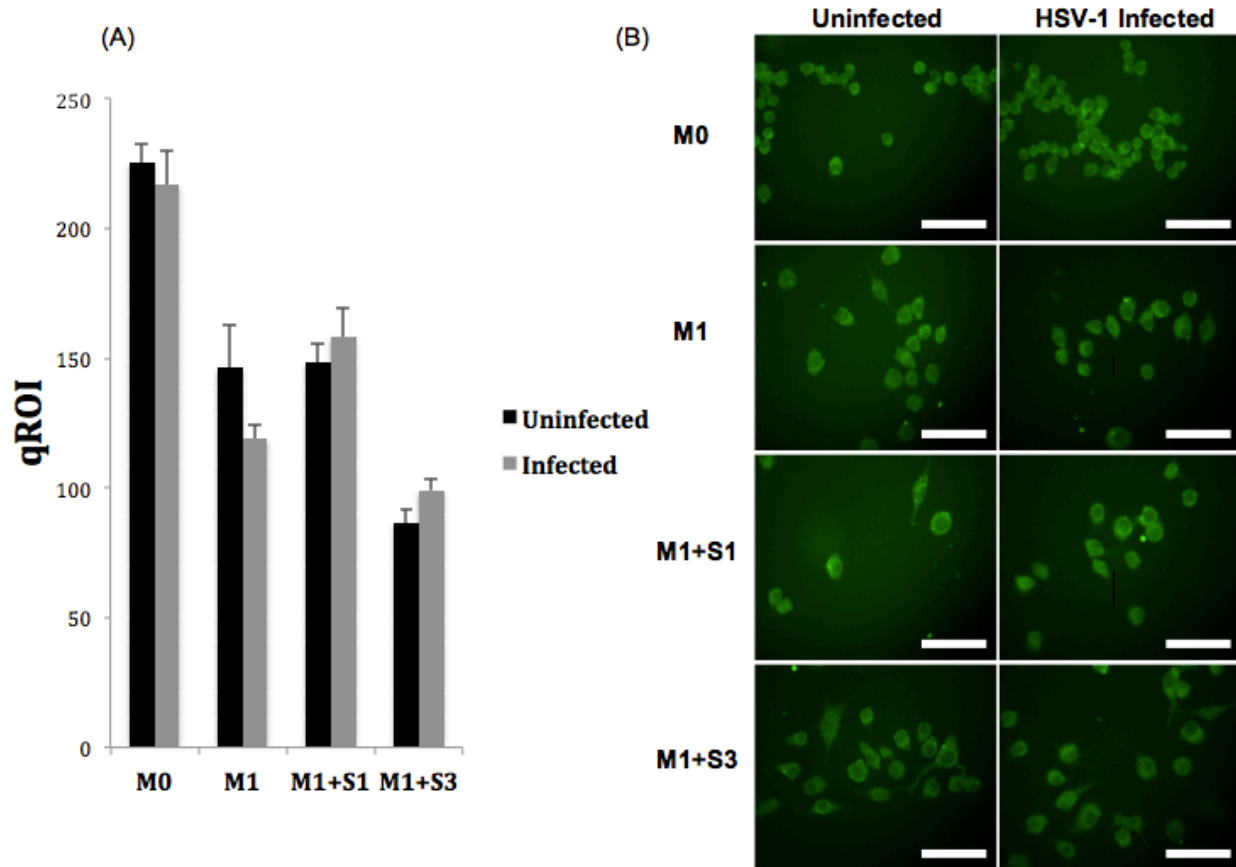


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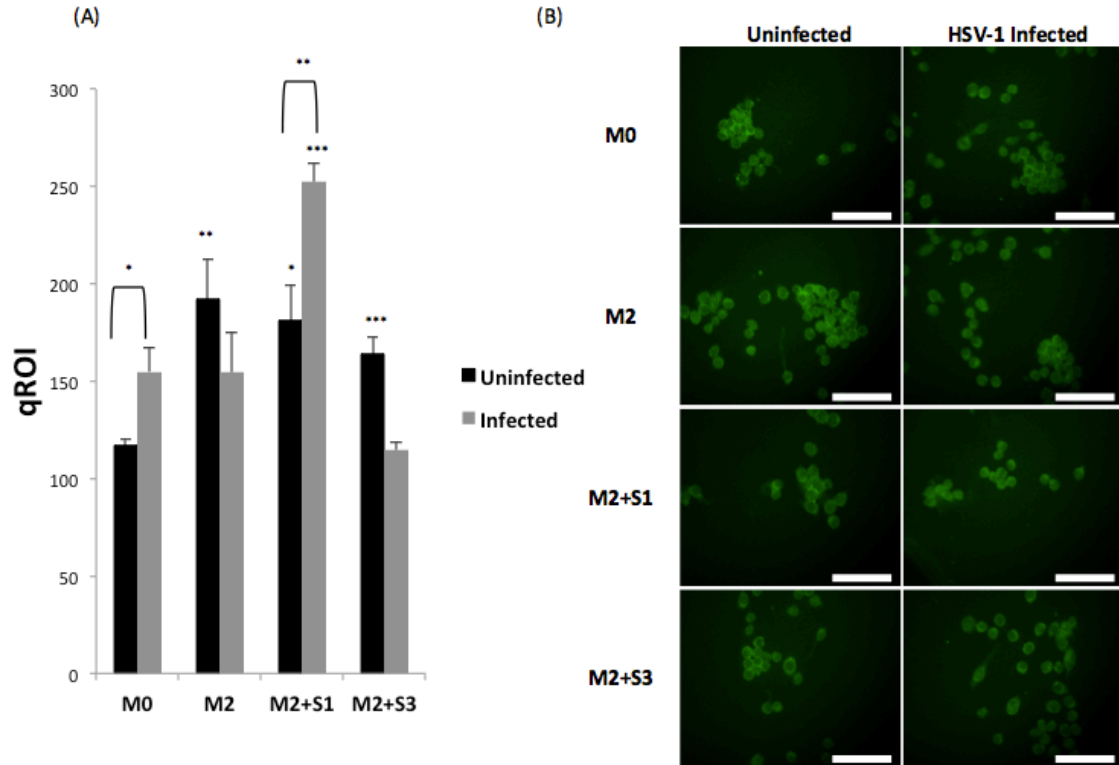


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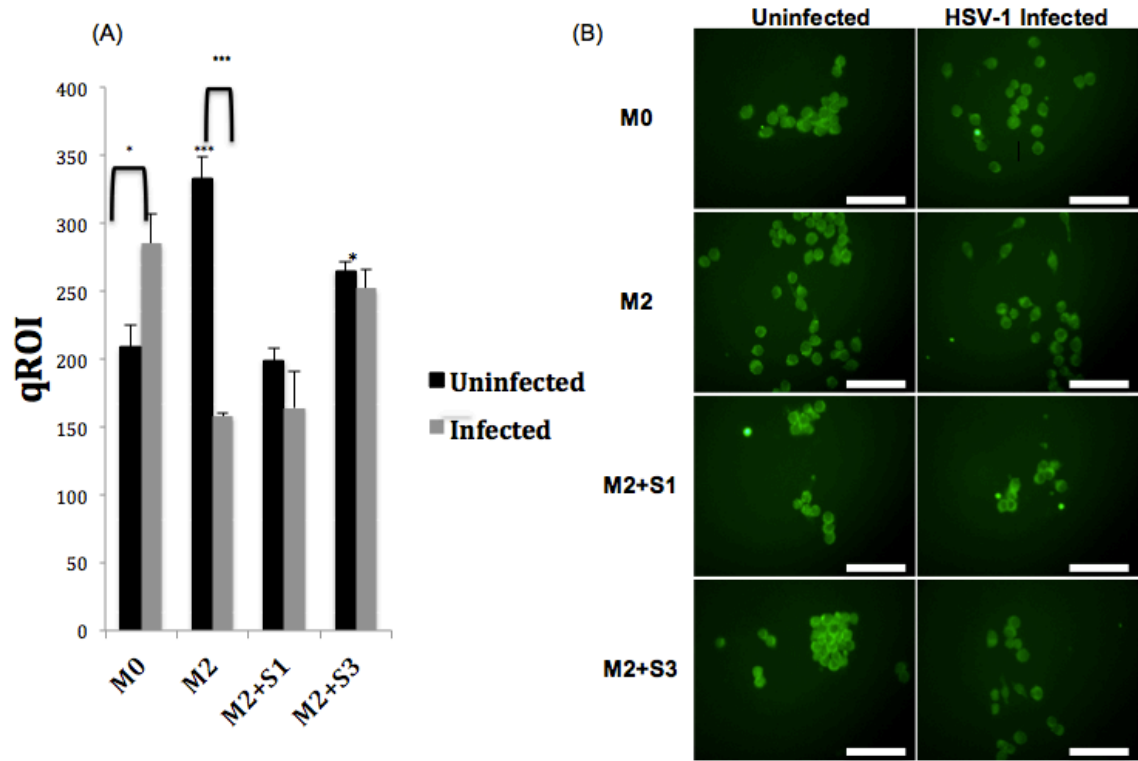




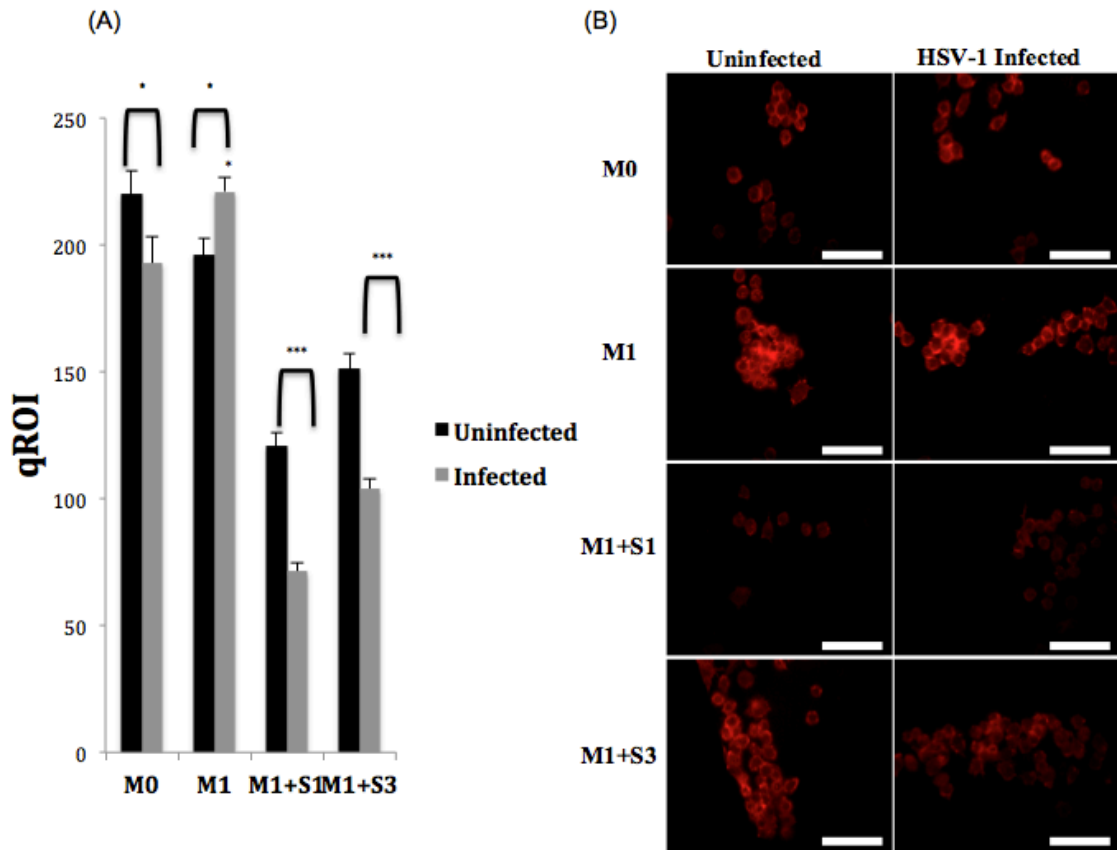
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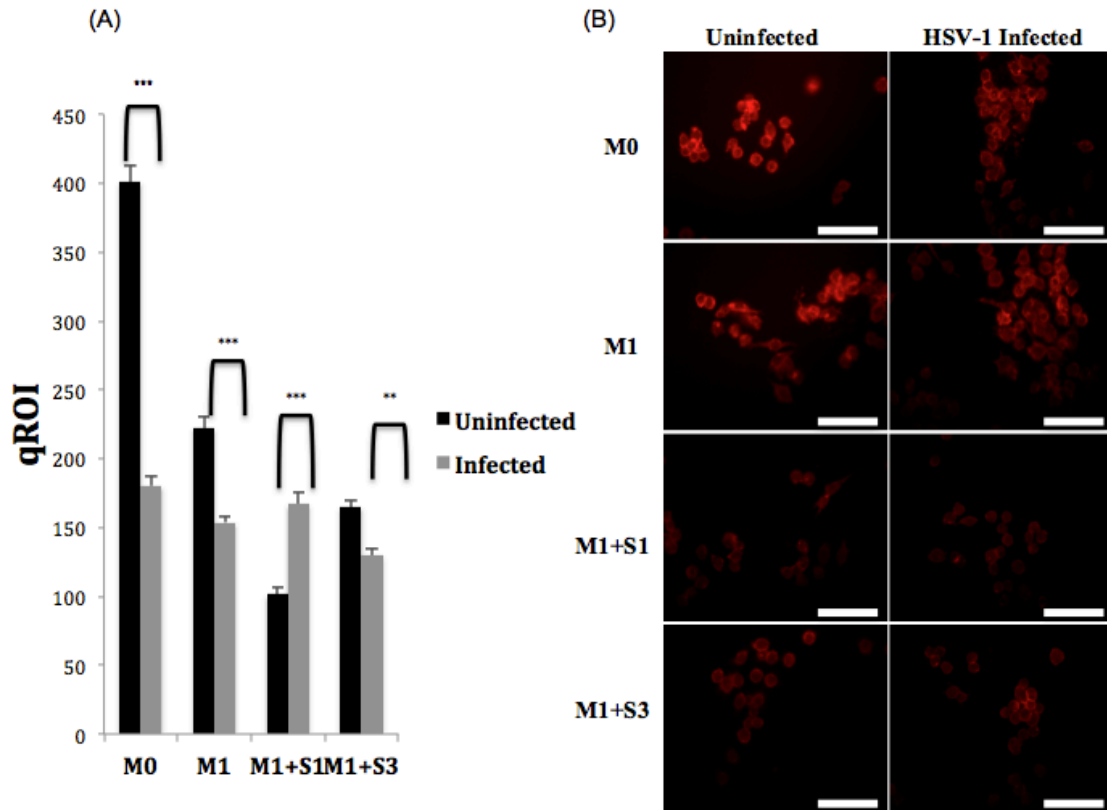
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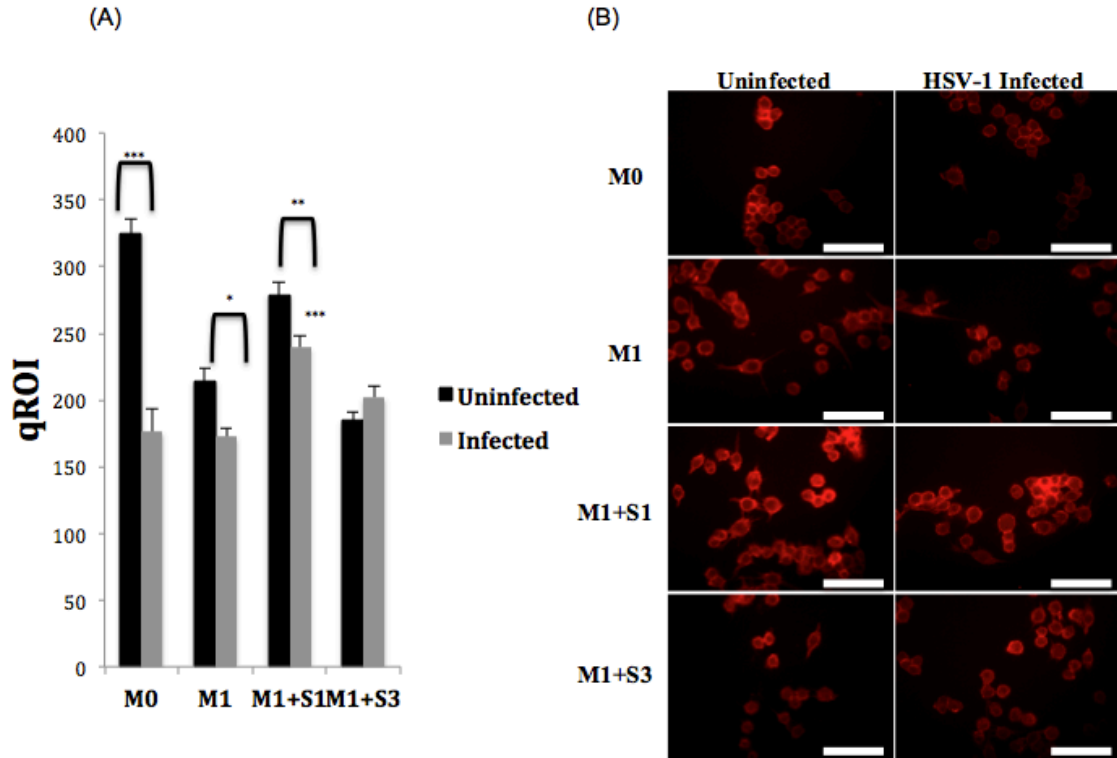
**Figure 15. Rab5 immunofluorescence intensity and morphological changes in uninfected IL-10 polarized M2 and HSV-1 infected IL-10 Polarized M2c at 24h.** (A) Level of Rab5 expression. The stars on black bars shows the significant of uninfected M2c cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2c cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50µm) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)



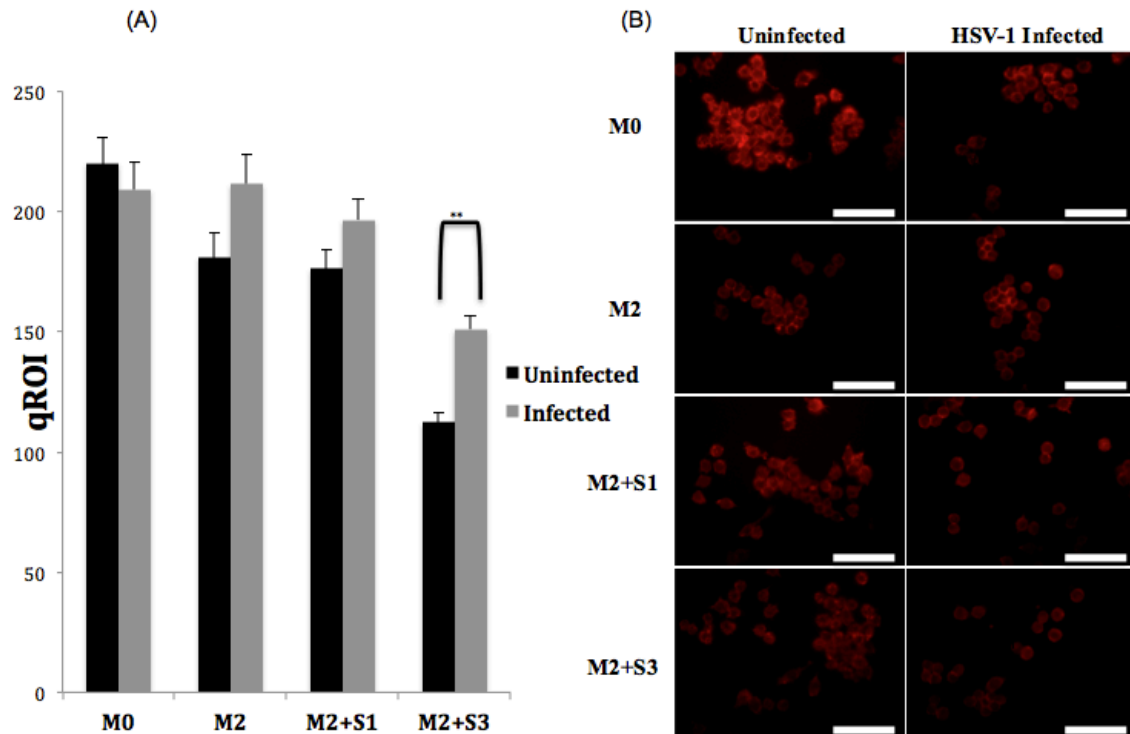
**Figure 16. F-actin immunofluorescence intensity and morphological changes in polarized uninfected and HSV-1 infected M1 at 2h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M1 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M1 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)



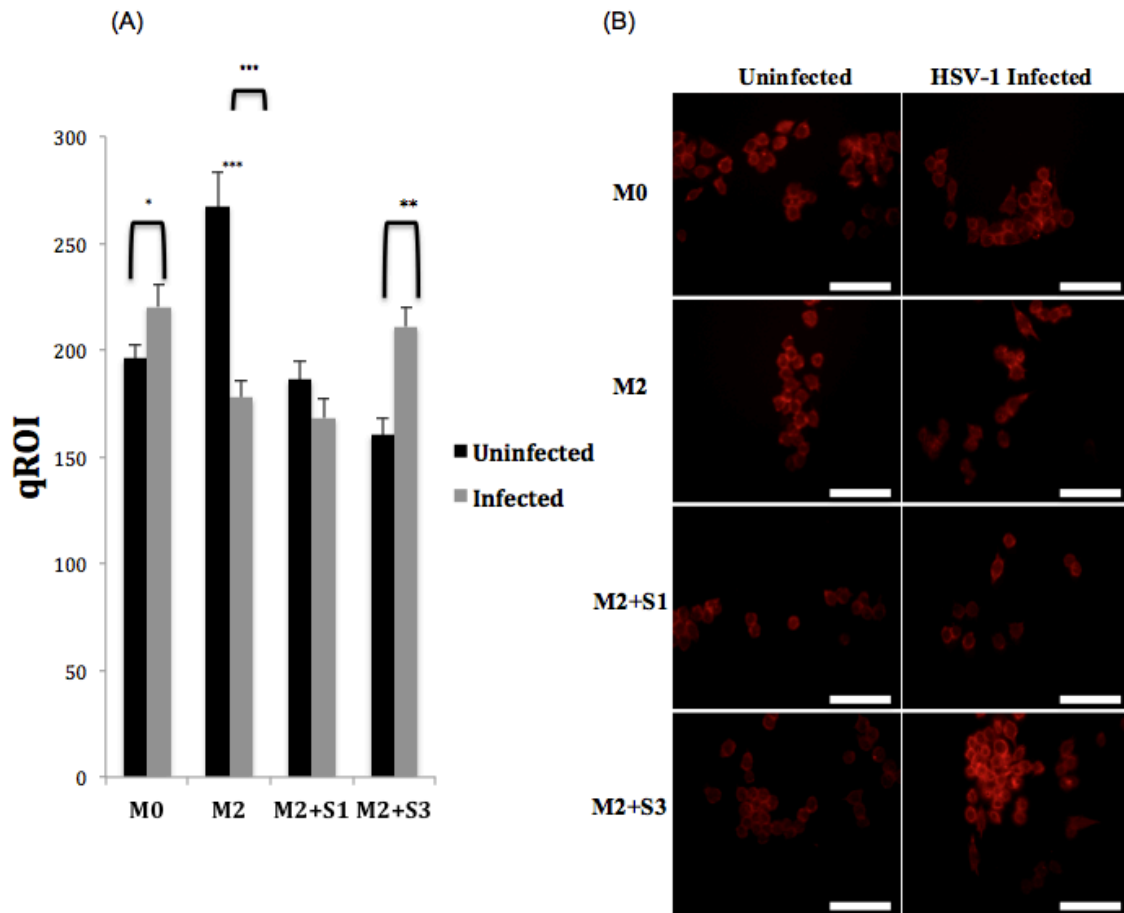
**Figure 17. F-actin immunofluorescence intensity and morphological changes in polarized uninfected and HSV-1 infected M1 at 4h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M1 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M1 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)



**Figure 18. F-actin immunofluorescence intensity and morphological changes in polarized uninfected and HSV-1 infected M1 at 6h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M1 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M1 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)

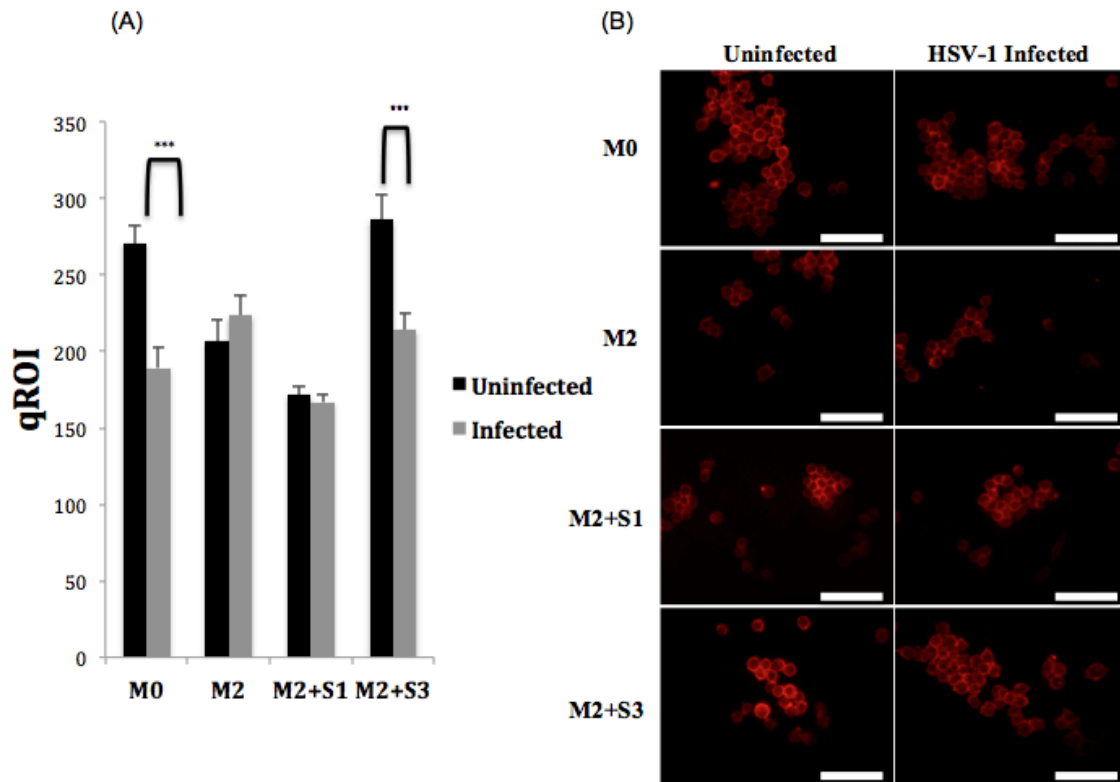


**Figure 19. F-actin immunofluorescence intensity and morphological changes in IL-4 polarized uninfected and HSV-1 infected IL-4 polarized M2a at 2h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2a cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2a cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)

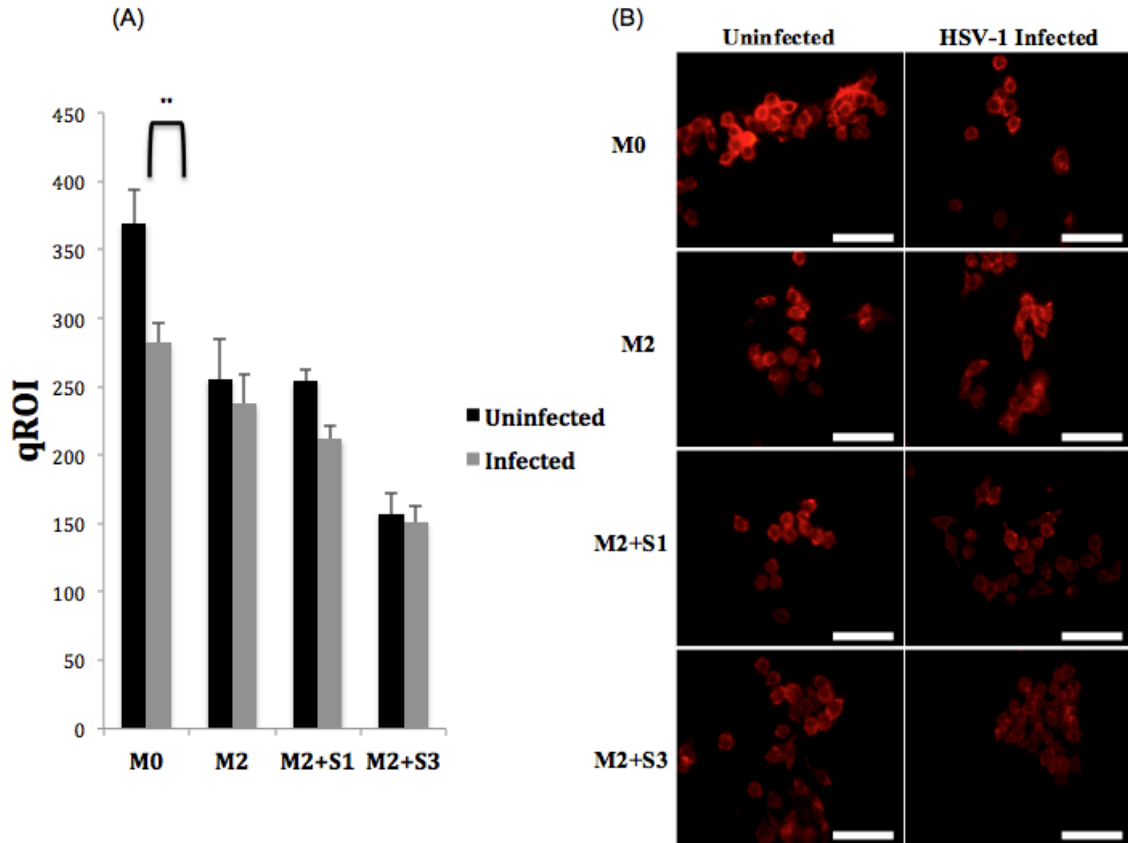


**Figure 20. F-actin immunofluorescence intensity and morphological changes in IL-4 polarized uninfected and HSV-1 infected IL-4 polarized M2a at 4h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2a cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2a cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)

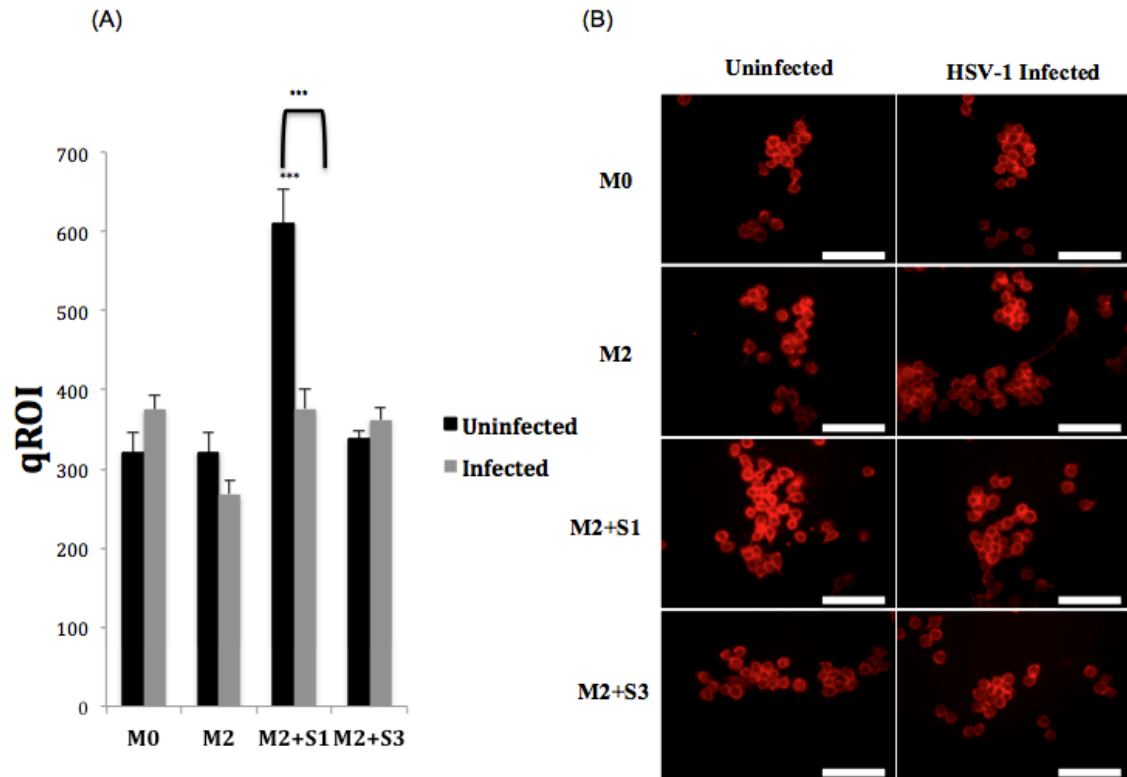




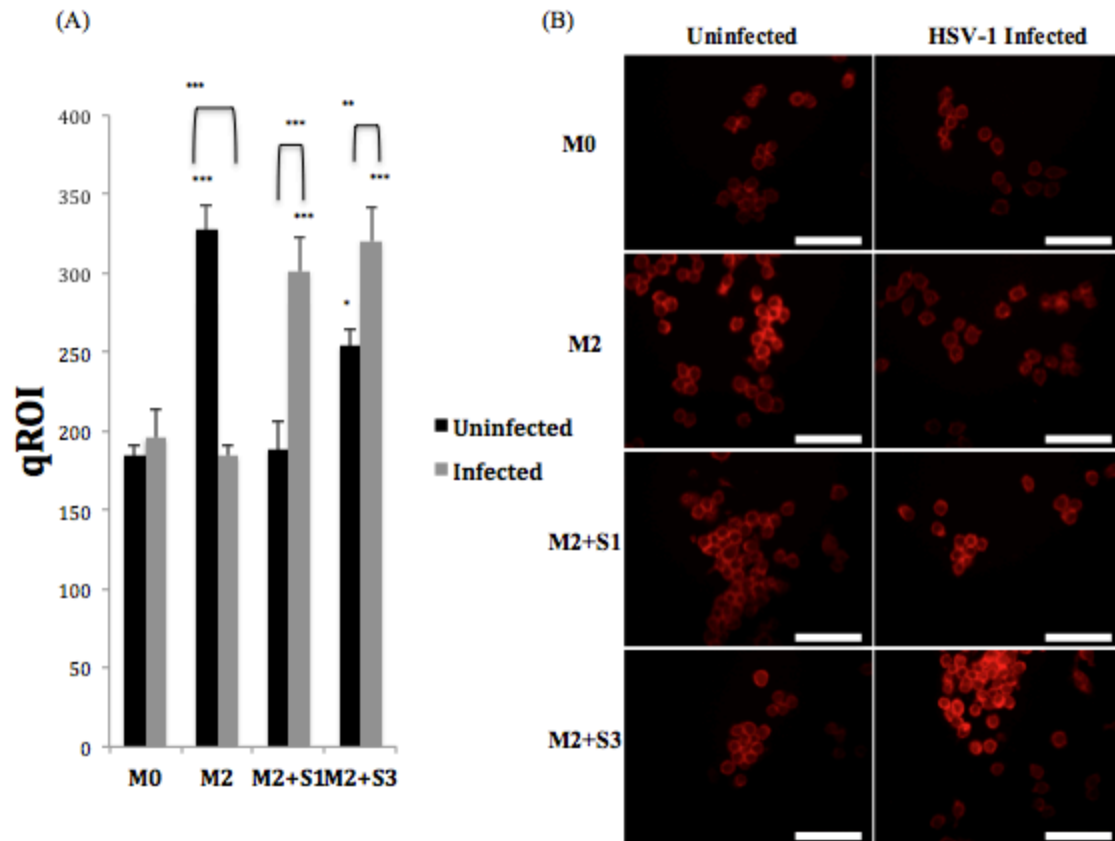
**Figure 21. F-actin immunofluorescence intensity and morphological changes in IL-4 polarized uninfected and HSV-1 infected IL-4 polarized M2a at 6h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2a cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2a cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)



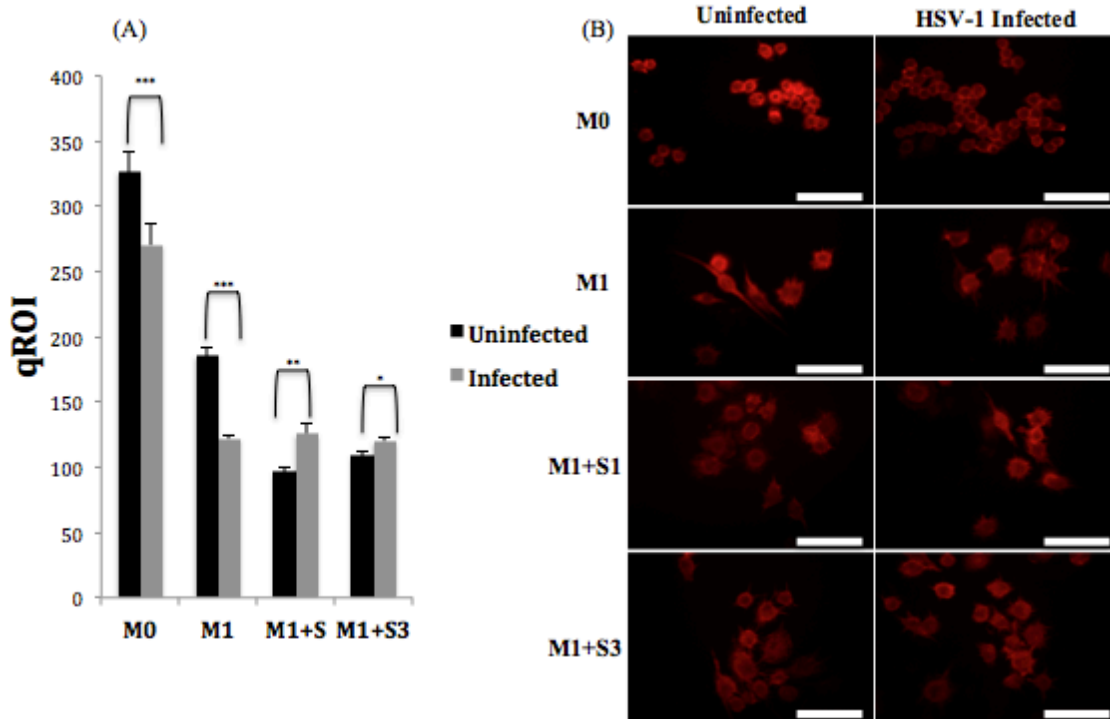
**Figure 22. F-actin immunofluorescence intensity and morphological changes in IL-10 polarized uninfected and HSV-1 infected IL-10 polarized M2c at 2h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2c cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2c cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)



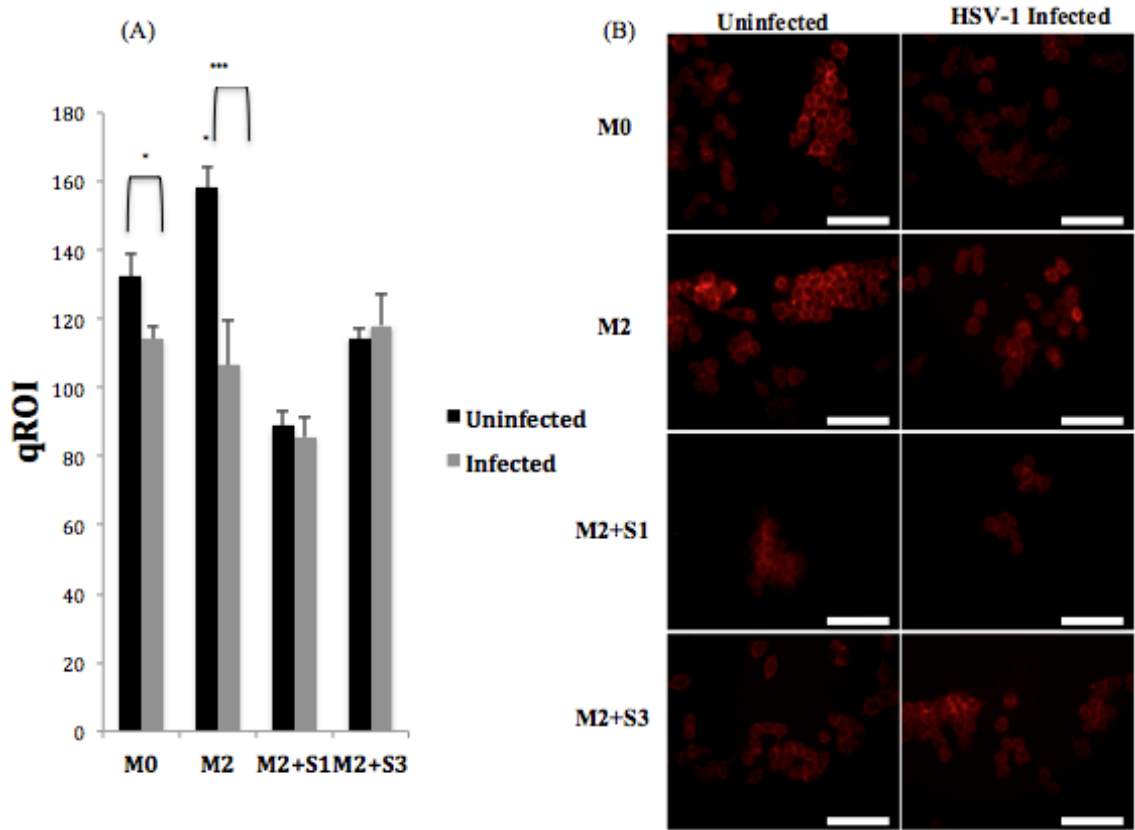
**Figure 23. F-actin immunofluorescence intensity and morphological changes in IL-10 polarized uninfected and HSV-1 infected IL-10 polarized M2c at 4h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2c cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2c cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images were captured at 500X oil magnification, scale bar = 50 $\mu$ m) (P<0.05 \*, P<0.01 \*\*, P<0.001 \*\*\*)



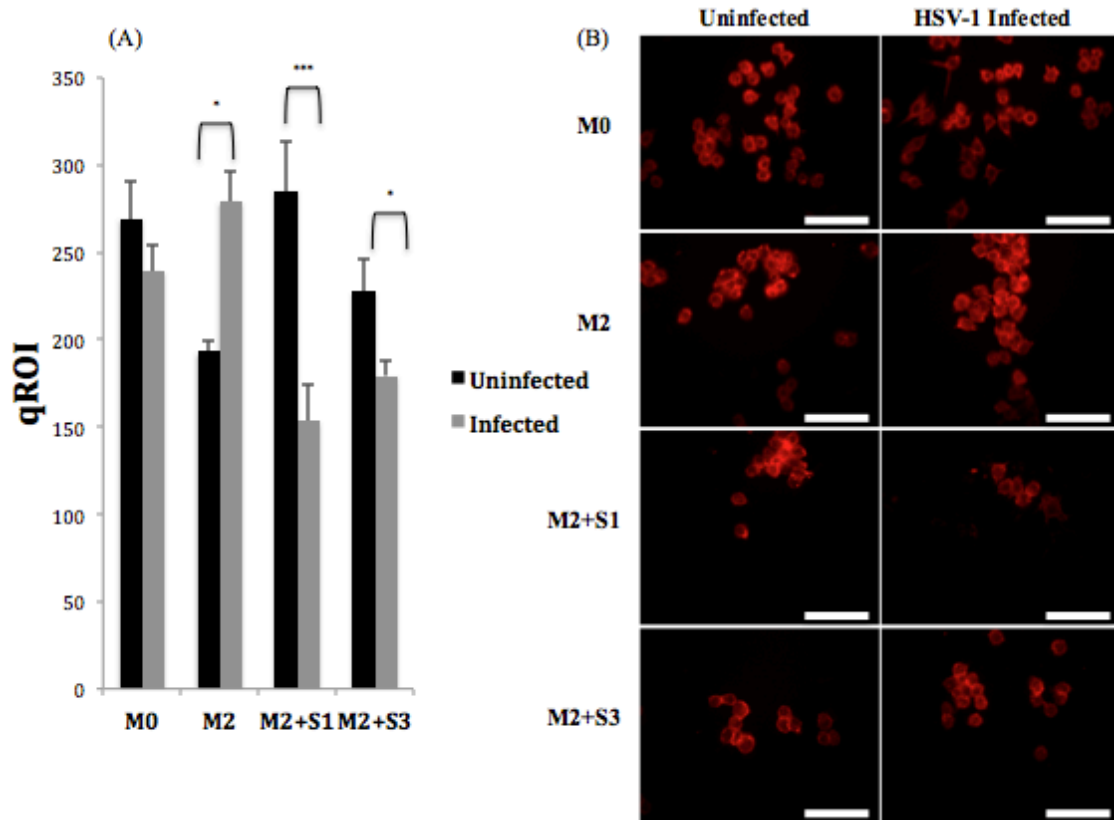
**Figure 24. F-actin immunofluorescence intensity and morphological changes in IL-10 polarized uninfected and HSV-1 infected IL-10 polarized M2 at 6h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images captures at 500X oil magnification, scale bar = 50 $\mu$ m)



**Figure 25. F-actin immunofluorescence intensity and morphological changes in polarized uninfected and HSV-1 infected M1 at 24h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M1 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M1 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images captures at 500X oil magnification, scale bar = 50 $\mu$ m)



**Figure 26. F-actin immunofluorescence intensity and morphological changes in IL-4 polarized uninfected and HSV-1 infected IL-4 M2a at 24h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images captures at 500X oil magnification, scale bar = 50µm)



**Figure 27. F-actin immunofluorescence intensity and morphological changes in IL-10 polarized uninfected and HSV-1 infected IL-10 M2c at 24h.** (A) Level of F-actin expression. The stars on black bars shows the significant of uninfected M2 cells compared to uninfected M0. The stars on gray bar demonstrate the significant of HSV-1 infected M2 cells compared to HSV-1 infected M0 cells. U shape compares the significance between each uninfected group to the same group with virus infection. (B) Representative images of all groups (Images captures at 500X oil magnification, scale bar = 50 $\mu$ m)

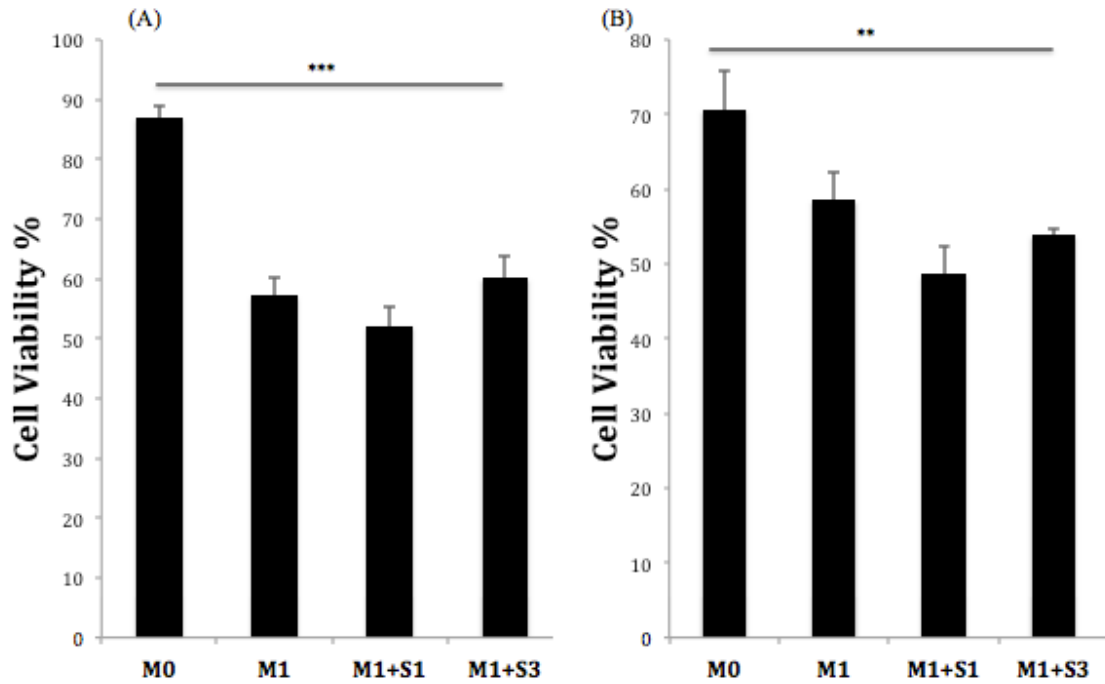
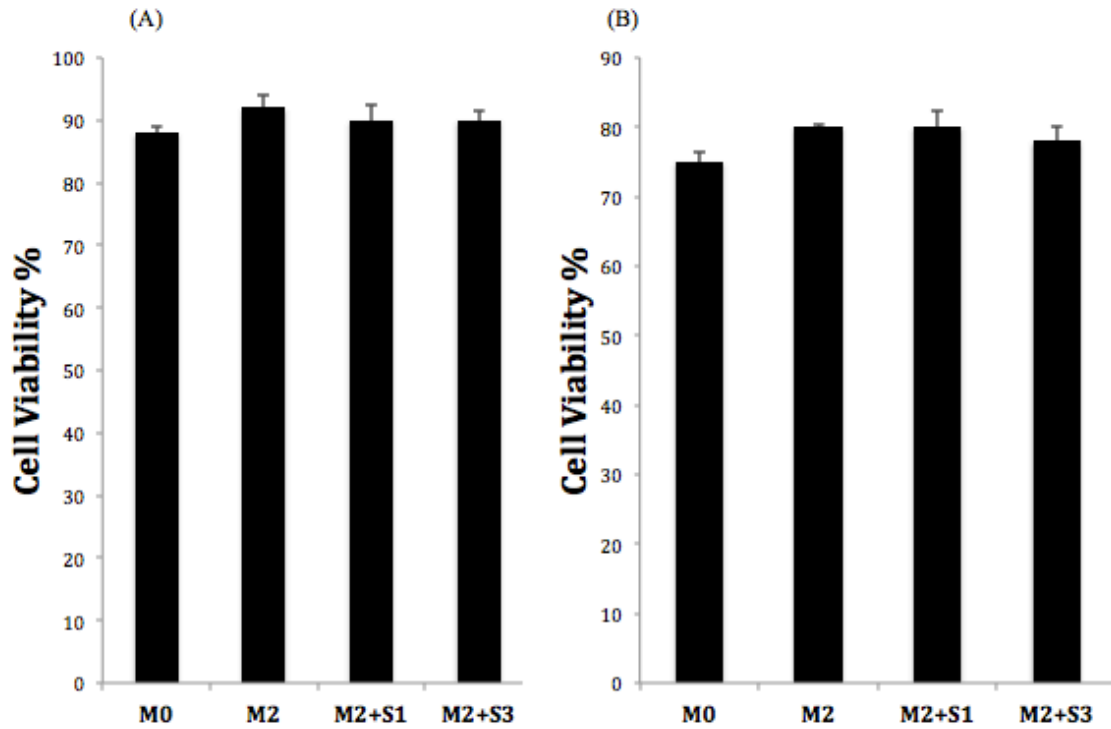


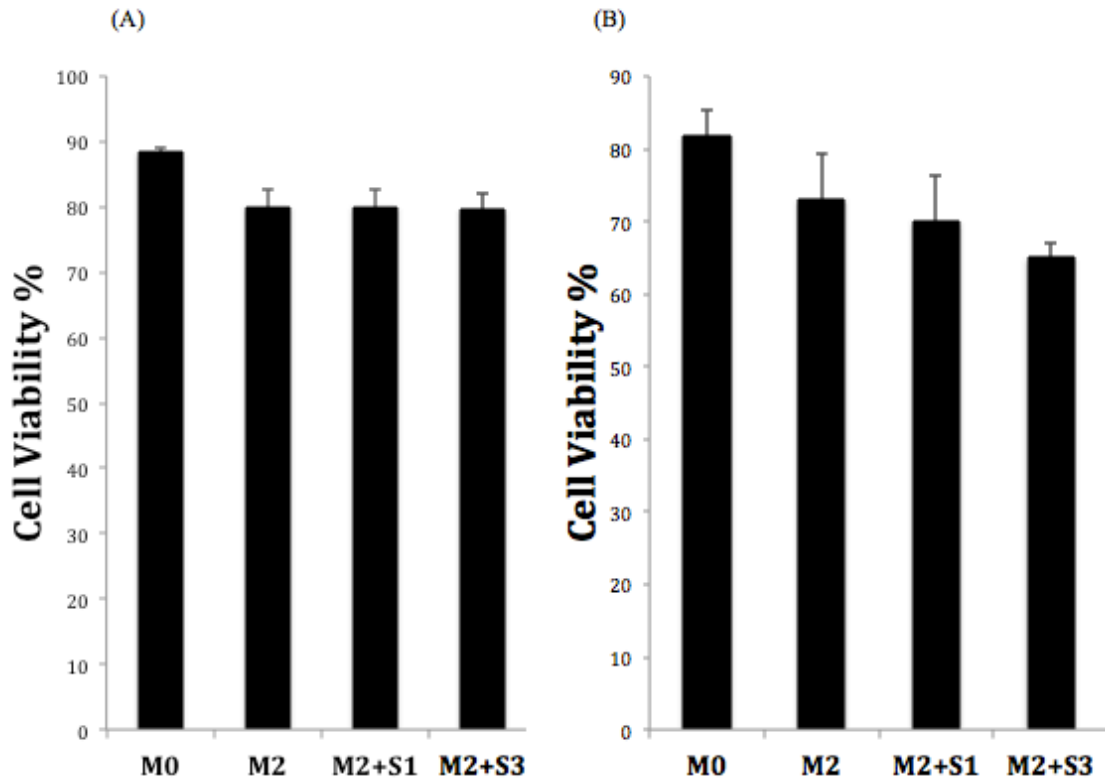
Figure 28. Cell viability for uninfected and HSV-1 infected M1 macrophage. (A)

Uninfected M1 (B) HSV-1 infected M1. Error bar represents SEM.





**Figure 29. Cell viability for uninfected and HSV-1 infected IL-4 polarized M2a macrophage. (A) Uninfected M2 (B) HSV-1 infected M2. Error bar represents SEM.**



**Figure 30. Cell viability for uninfected and HSV-1 infected IL-10 polarized M2 macrophage. (A) Uninfected M2 (B) HSV-1 infected M2. Error bar represents SEM**

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