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The Response of M0, M1, and M2 RAW246.7 Macrophage Cell Line to HSV-1 Infection in vitro

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THE RESPONSE OF M0, M1, M2 RAW 246.7 MACROPHAGE CELL LINE TO HSV-1 INFECTION IN VITRO

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

by

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amani Mohammed Alhazmi ENTITLED The Response of M0, M1, and M2 RAW246.7 Macrophage Cell Line to HSV-1 Infection in vitro BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Alhazmi, Amani Mohammed. M.S, Microbiology and Immunology, Wright State University, 2019. The Response of M0, M1, and M2 RAW246.7 Macrophage Cell Line to HSV-1 Infection in vitro

Herpes Simplex Virus Type 1 (HSV-1) infection occurs through the epithelial cells of the skin or mucous membranes. The beginning of the primary infection is rapid and is characterized by pain in the mouth, salivation, and submandibular lymphadenitis. The infected mucosa produces numerous, small and red lesions known as cold sores, however, many cases are asymptomatic. After the primary infection HSV-1 moves through the nerve to stay in trigeminal ganglia and to cause a recurrent infection from time to time. In the early hours of the HSV-1 infection, the cytokines produced by infected cells are critical in the stimulation of the innate immune response to the infection. One of the innate immune cells responded to the infected cells is macrophages. So, macrophage recruitment and differentiation are essential for effective control and clearance of viral infections.

To mimic the in vivo role of three types of macrophages against HSV-1 infected epithelial cells (PAM 212), M0, M1, or M2 RAW246.7 macrophages were added at 2 and 4 hours after an initial established infection. These times were selected to represent the influx of macrophages to the infection site within the first few hours of exposure to HSV-1 virus. In all experiments, we performed cell viabilities and virus titers at 24, 48, and 72 hours after the initial infection. After the HSV-1 infection, a morphological change was observed among all types of macrophages where most of it appeared round and granulated. This change makes it challenging to differentiate M1 from M0 or M2.

Importantly, all phenotype of macrophages showed an

essential role against the HSV-1 replication in PAM-212 keratinocytes. However, the addition of M1 Macrophages to HSV-1 infected PAM212 keratinocytes significantly decreased the percentage of the viable cells by more than 80% and restricted the HSV-1 replication more effectively than M0 and M2 macrophages. The virus replication pattern was similar in a different type of M2 macrophages (M2 a and M2 c) which was low at 24 h, then increased significantly 48 hpi then decreased significantly 72 hpi.

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LIST OF ABBREVIATIONS

3-O-HS = 3-O-Heparan Sulfate

Hpi = hours post infection

HS = heparan sulfate

HSV-1 = Herpes Simplex Virus Type 1

HVEM = Herpes Virus Entry Mediator

HSPG = Heparan Sulfate Proteoglycan

HVEM = herpes virus entry mediator

HSPGs = heparan sulfate proteoglycans

IFN-1 = type I interferons

IFN- α/β = Interferon – α/β

IL-12 = IL-12

MHC = Major Histocompatability Complex

MOI = Multiplicity of Infection

NO = Nitric Oxide

PAM-212 = Keratinocyte cell line

PFU = Plaque Forming Unit

PRR = Pattern Recognition Receptor

RAW (264.7) = Macrophage cell line

ROS = Reactive oxygen species

TNF-α = Tumor Necrosis Factor – α

TLRS = Toll like Receptors

LPS= lipid polysaccharide

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HYPOTHESIS

In mimicking the immune response of unpolarized M0 as well as M1 or M2 polarized RAW 264.7 macrophages to HSV-1 infection of PAM 212 murine epithelial cells in vivo, I predict that M2 macrophages will exhibit higher viability than either M0 or M1 polarized macrophages over a 72-hour time span in co-culture experiments, but M1 macrophages will restrict the virus replication better than M0 and M2.

AIM

The aim of this project is to mimic the in vivo role of M0, M1, and M2 RAW246.7 macrophages against HSV-1 infected epithelial cells (PAM 212) after 2 and 4 hours post the initial establishment of the co- cultures. These times were selected to represent the influx of macrophages to the infection site within the first few hours of exposure to HSV-1 virus. In all experiments, we performed cell viabilities and virus titers at 24, 48, and 72 hours after initial infection in triplicate.

INTRODUCTION

Herpes Simplex Virus Type One (HSV-1) is a widespread virus that infects more than 60% of people around the world (Rogalin & Heldwin, 2016). HSV-1 infects different cells including nerve cells, lymphoblasts, and keratinocytes (Nicola AV, 2016). Immune control of HSV-1 infection and replication occurs at the level of skin or mucosa during initial or recurrent infection and within the dorsal root ganglion, where immune mechanisms control latency and reactivation.

The early non-specific antiviral defense mechanism is considered the key player by exhibiting the least toxicity and potential interaction with other cytokines in the suppression of HSV-1 infection. In the early hours of a viral infection, the cytokines are produced by infected cells and the cells encountering viral products begin to conduct the innate immune response to the infection. In the first responses, cytokines, primarily tumor necrosis factor and type I interferons (IFN-1), are produced to function as antiviral defenses and to activate the macrophages. In the next round of responses, interleukin (IL)-12 together with INF-I, TNF, and other cytokines induce production of IFN-γ in NK cells. INF- γ activate M1 macrophages to initiate heavy antiviral protection by producing the nitric oxide (NO) and reactive oxygen species (ROS) (Anthony L.Cunningham, Russell J. Diefenbach, Monica Miranda-Saksena, et al, 2006).

Macrophages exhibit a wide variety of critically important functions, including, cytokine and chemokine secretion, tumor cytotoxicity, and phagocytosis. Macrophages can be programmed to various distinct subsets classified as M1 or M2 macrophages. M1 macrophages are effector cells against microorganisms and tumor cells and are mainly associated with pathologic type 1 inflammation. While M2 macrophages stimulate type 1

inflammatory responses and adaptive immunity, and they promote and regulate type 2 immune responses, angiogenesis, and tissue repair (Guillermo eArango Duque, & Albert eDescoteaux, 2014). Macrophage and HSV-1 interactions depend on the polarization status which influence their ability to attack the virus (Kemp, et al. 1990), however, this mechanism needs to be better understood.

This study is aimed to differentiate between the response of M0, M1, and M2 polarized macrophages after the encounter with HSV-1 infection in the keratinocytes, from the aspect of cell viability and virus infectivity.

BACKGROUND

Herpes Simplex Virus One (HSV-1)

HSV-1 is a prevalent virus infect more than 60 % of the adult's population around the world (Rogalin & Heldwin, 2016). HSV-1 infection occurs through the skin or mucous membranes followed by incubation period up to 26 days, followed by prodromal, non-pathognomonic symptoms, including irritability, malaise, chills, fever, nausea, anorexia, and headache. The beginning of the primary infection is rapid and is characterized by pain in the mouth, salivation, and submandibular lymphadenitis. The infected mucosa produces numerous, small and red lesions known as cold sores which sometimes involve tongue, posterior pharynx, buccal mucosa, and any palatal and gingival mucosae. Moderate lesions usually need 5–7 days to heal, but sever lesions may need up to three weeks and may form a scar.

HSV-1 belongs to Herpesviridae family and consists of dsDNA placed inside an icosahedral nucleocapsid which is enclosed by tegument and covered by lipid envelope loaded with various glycoproteins (Fig 1) (Roizman et al., 2007; Dakvist et al., 1995; Miller et al., 1998; Stock et al., 2001). The primary route of transmission of HSV-1 occurs during physical contact with infected individuals who are suffering from cold sores. Also, HSV- 1 may spread from site to site in one patient by touching the cold sores or the lesions and touch another part of his or her body. It has been assumed that primary herpetic gingivostomatitis is the medical sign of the primary infection in children, while mononucleosis-like disease or pharyngotonsillitis is the medical sign of primary infection in adults (Usatine, R. P., & Tinitigan, R., 2010).

Also, HSV-1 infects the cornea of the eye which can lead to blindness. In immunocompromised people, HSV-1 may lead to encephalitis and death (Cheng et al., 2000).

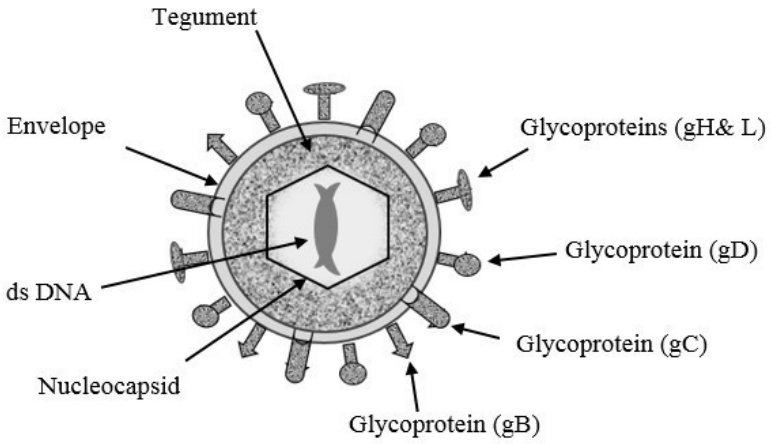

Figure 1: Herpes Simplex Virus Type One Structure: HSV-1 consist of dsDNA placed inside an icosahedral nucleocapsid which enclosed by tegument and covered by lipid envelope which loaded with various glycoproteins.

HSV-1 Entry and Infection:

The entry method of herpesvirus is a complex process. It involves several viral glycoproteins, such as gD, gB, gC, gH and gL and cellular receptor molecules including nectin-1, heparan sulfate (HS), herpes virus entry mediator (HVEM), heparan sulfate proteoglycans (HSPGs) and 3-O-Heparin Sulfate (Pertel, P., Fridberg, A., Parish, M., and Spear, P. ,2001). Entry initiates with a low-affinity attachment of the virus to the cell surface between gB or gC and HS or PILRa followed by higher affinity binding between cellular receptors nectin-1 or HVEM and viral glycoproteins D. These interactions enable fusion of viral and cellular membranes and capsid penetration (Campadelli-Fiume et al. 2012).

Then, the nucleocapsid moves through the microtubules to reach the nuclear membrane to allow the entry of viral DNA into the nucleus for replication. Proteins in the tegument are known to aid the entrance and the replication process at the end of the translation and transcription viral genome assemble within nucleocapsid to infect other cells (Rahn, E., Petermann, P., Hsu, M.-J., Rixon, F. J., & Knebel-Mörsdorf, D., 2011).

Herpes simplex virus (HSV-1) encodes seven proteins necessary for viral DNA synthesis including the origin binding proteins (UL9), the DNA binding proteins (ICP8), the polymerase proteins (UL30/UL42), and the helicase/primase proteins (UL5/UL8/UL52). Also, the viral protein ICP47 assists the virus immune evasion by inhibiting binding of viral proteins to transporter associated with antigen processing (TAP), which is necessary for major histocompatibility complex class I presentation. Also, the viral protein ICP0 enhances HSV-1 to be resistance to interferon-α and β (Campadelli- Fiume et al. 2012).

HSV-1 Infection in Keratinocytes

The primary HSV-1 infection in human initiates in keratinocytes of the epidermis of the oral mucosa or the skin. HSV-1 uses the nectin-1 receptor to enter human keratinocyte cells via a rapid plasma membrane fusion pathway at low temperature as 7°C. HSV-1 r a p i d l y entrers into human keratinocytes of maximum penetration within 5 min (Charlotte L. Sayers, Gillian Elliott, 2016). After the primary infection, herpes simplex virus enters the sensory nerve endings which carried by retrograde axonal transport to the dorsal root ganglion, where the virus develops lifelong latency, a state in which no infectious progeny is produced and only limited transcription

of viral genes occurs. If a recurrent reactivation occurs, the virus virion will transport to the skin or mucosa to shed the reborn infection.

The Immune Response to HSV-1 Infection

The early non-specific antiviral defense is considered the key player by exhibiting the minimum toxicity and essential interaction with other cytokines in the suppression of HSV-1 infection. In the early hours of the HSV-1 infection, the cytokines produced by infected cells or cells encountering viral products are critical in the stimulation of the innate immune response to the infection (Melchjorsen, et al., 2010).

The response of the macrophages to HSV-1 infection play pivotal role. A recent study describes the emergence of the macrophages in the corneas of mice before and after HSV-1 infection using anti-CD11b and anti-F4/80 antibodies (Agelidis AM, Shukla D. 2015). Before the HSV-1 infection no macrophages were found, but after the HSV-1 infection macrophages were detectable and appear to be the main immune cell recruited into the eyes of HSV-1 infected mice. This is important because macrophages have the nonspecific ability to kill viruses. In response to the viral attack the macrophages are activated to degrade and to present the pathogen to T cells (Lannello, et al., 2011). Macrophages recognize the early HSV-1 infection and release proinflammatory cytokines such as interferons (IFNs) and tumor necrosis factor (TNF-α) (Melchjorsen, et al., 2010). Also, macrophage and HSV-1 interaction depend on the polarization status which influence their ability to attack the virus (Kemp, et al., 1990), however, this still need to be better understood.

Macrophages Origin and Functions

Macrophages originates from monocyte which initiates form myeloid progenitors in the bone marrow. The interactions with proinflammatory cytokines, local growth factors, and microbial products allow the recruitment of monocytes from blood stream to peripheral tissues where they differentiate into dendritic cells or macrophage populations. The F4/80 marker can be used to differentiate between the macrophage and monocytes (Taylor P.R., 2005).

Macrophages are phagocytic cells that express many receptors to recognize the signals of danger. Pattern recognition receptors (PRRs) receive signals from external substances, dead cells and infecting cells, and are essential in the pathogen binding process in phagocytosis and in the recognition of microbial products. The PRRs are scavenger receptors that assist in the poisoning of foreign pathogens and help clear the apoptotic cells, necrotic tissues, dead red blood cells and toxic molecules from the circulation. Inflammatory cytokine receptors mediate the activation of inflammatory phenotype (Peiser, L. and Gordon, S.,2001). The activation of mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NF-kB) and interferon regulatory factors (IRFs), lead to expression of critical cytokines, chemokines, and inflammatory mediators (Gordon S., Taylor P.R,2005).

Macrophages are distributed throughout different organs and tissues and their morphology differs depending on their activity. Macrophages have been broadly classified as M1 or M2 macrophages. M1, also known as classically activated macrophages, are mainly associated with pathologic type 1 inflammation and able to present the antigens, kill tumor cells, and eliminate the infections produced by virus, fungal or bacterial factors

(Espinoza-Jiménez A., Peón A.N., Terrazas L.I,2012). M1 macrophages express most TLRs and opsonic receptors, secrete IL-12, TNF-α, IL-1β, and express inducible NO synthase (iNOS). IFN-γ is included in combination with lipid polysaccharide (LPS), bacterial endotoxin, in the activation of M1 pattern. M1 Macrophages are characterized by the secretion of co-stimulatory CD90/86 and MHC-II molecules, which are related to their capability to present the antigens (Mantovani A.et al , 2004). The polarization of macrophages increases the expression of CD64 and CD80. CD80 was found to be useful to be marker to detect the INF-γ polarized macrophages (Ambarus C.A., 2012).

M2 is also known as the alternatively activated macrophages, they are critical in the regulation of the immune response to allergic reaction and parasites (Banerjee S., 2013). They also play an important roles in tissue angiogenesis and remodeling and in tumor progression (Takeuch O., Akira S, 2011). M2 macrophages stimulate the Th2 immune response as well as the differentiation of regulatory T lymphocyte (Treg). Many cytokines are involved in the activation of M2 macrophages, such as IL-4, IL-13 or IL-10 (Locati M., Mantovani A., Sica A. 2013). Unlike M1 macrophages which display cytotoxic activity, the M2 macrophages block iNOS and produce IL-10, IL-1 receptor antagonists IL-1RA and TGF-β. M2 macrophages can be subdivided into three groups: (1) M2a, which is induced by IL-4 or IL-13; (2) M2b, which is induced by immune complexes and agonists of TLRs or IL-1 receptors; and (3) M2c, which is induced by IL-10, glucocorticoids, or TGFβ. M2 macrophages express the phagocytosis marker CD163 and CD206, scavenger receptor, which depend on the production of IL-10. The polarization of macrophage by IL-10 increase the expression of CD32, CD16, and CD163. The polarization of

macrophages by IL-4 leads to decease the expression of CD14 and to the increase the expression of CD200R and CD206 (Ambarus C.A.,t al. 2012).

The production of cytokines is an important distinguishing factor between the macrophage's phenotypes (Wang, et al., 2010). M1 macrophages increase the expression of IL-10 and decrease the expression of IL-12, whereas M2 macrophages increase the expression of of IL-10 and decrease the expression of IL-12 (Mantovani, et al., 2004).

PRELIMINARY DATA

Previous studies by Alanazi in 2018 tested the effect of different subsets of macrophages (e.g., M0, M1, M2a, or M2c) on HSV-1 replication. Unpolarized and M1, and M2 polarized RAW246.7 macrophages infected with HSV-1 for 2 hours were tested. After the infection all the cells were washed with PBS and were refreshed with suitable medium and were incubated for 24h, 48h, or 72h. At all time points, cell viability and virus infectivity were performed. These studies found that M1 phenotype of RAW 264.7 macrophages strongly suppresses the replication of HSV-1 when compared to the M0 and M2 phenotypes.

Graffagna's studies in 2018 investigated the interactions between the macrophages and HSV-1 infected HEL30 keratinocytes. The macrophages were not exposed to HSV-1 directly, but were added to assist the HSV-1 infected HEL30 keratinocytes. These co-culture models of HSV-1 infected HEL-30 keratinocytes were treated 2 hours after initial infection with unpolarized (M0) RAW 246.7 macrophages. The corresponding cell viabilities and virus infectivities were determined. M0 macrophages showed increased cell viability 24, 48, and 72 hours post infection when compared to the positive control (i.e., HSV-1 infected keratinocytes without the M0 macrophages). Also, M0 macrophages reduced the HSV-1 titer of the co-culture model at all time points when compared to the positive control.

Alradi (2018) performed similar viability and virus infectivity experiments by using PAM212, another types of keratinocyte cell line. The addition of M0 macrophages to the HSV-1 infected PAM212 was more beneficial when compared to the HEL-30 keratinocytes. M0 macrophages increased the cell viability and restricted HSV-1 by decreasing the virus titration of the of PAM-212 after 24 hpi, and the virus titers were increased after 48hpi or 72 hpi. To conclude, PAM212 keratinocytes is more susceptible to

HSV-1 infection than HEL-30 keratinocytes and macrophages phenotypes can vary the response to HSV- 1 infection.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

PAM-212 and RAW 246.7 cell lines were purchased from American Type Culture Collection (ATCC TIB-71). The cells originated from adult male of BALB/c mice.

All cell lines were handled similarly and used Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), 25- T Flasks, 75-T Flasks, 6 well plates, cell scrapers and trypsin EDTA. The cells were thawed using 25-T Flasks until the cells reached approximately 80% confluency. Then, the cells were transferred to 75-T Flasks for further splitting and experiments.

For RAW 246.7 polarization to M2 macrophages, IL-4, IL-10 or IL-13 were used by adding 20 ng/ml of these interleukins into the DMEM supplemented with 10 % FBS. To polarize the RAW246.7 toward M1 macrophage, IFN-y (20ng/ ml) and LPS (100 ng/ml) were used.

Virus

HSV-1 (syn17+) (provided by Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH), was used for infection. A plaque assay was performed to determine the virus titers in the original stock by using Vero cell line (CCL-81, American Type Culture Collection) originated from kidney epithelial cells of African green monkey.

The virus concentration in original stock was 6.8×10^7 viruses/ml.

Infection procedure of RAW 246.7 Macrophages

The RAW246.7 macrophages were seeded at density of 3 X 10^5 cells per well with 3 ml DMEM medium with 10 % FBS and incubated 24 h in 6-well plates. To polarize the RAW246.7 into M1, M2a or M2c phenotype, the interleukins were added to the medium in this step

Next, the medium was discarded and the cells were washed with 2 ml of PBS prior to infection. In each well of the 6-well plate, 500 µl DMEM was added. Then three wells were infected with HSV-1 at volume of 0.44 µl (MOI of 0.1) and the other three wells uninfected to be used as negative control. The cell cultures were incubated for 2 hours to establish the infection. Next, the medium was discarded and the cells were washed with PBS before the addition of 3 ml of DMEM supplied with 10% FBS. Three separate plates were incubated at 37° C and 5% CO_2 for 24, 48, and 72 hours. After each incubation time, RAW246.7 cells were collected using the cell scrapers into 15-ml tubes. After, centrifugation 1600 rpm at 4° C for 5 minutes, 1 ml of the supernatant from infected wells was transferred into 1.5 ml centrifuge tubes to store at –80° C for use in the plaque assay experiment. The remaining supernatant was discarded and the pellet was resuspended in 1ml of 10% FBS medium to count the viable cells.

Infection procedure of PAM-212 co-culture with either M0, M1 or M2 RAW246.7 macrophages

PAM-212 keratinocytes were seeded in 6-well plates at density of 1.5 X 10^5 cells per well for an incubation period of 24 hours. Then, the medium was discarded and all wells were washed with 2 ml of PBS prior to HSV-1 infection. In all wells 500 µl DMEM was added. Next, three wells were infected with HSV-1 (0.22 µl, MOI of 0.1) and other three wells were uninfected to be used as negative controls.

The plates were incubated for 2 hours to establish the infection. Afterward the DMEM was discarded and all cells were washed with 2 ml of PBS before the addition of 3 ml DMEM containing 10% FBS. Also, M0, M1 or M2 RAW246.7 macrophages were added to each well

at a 1:5 ratio (RAW 264.7: PAM-212) and incubated for 2 hours at 37° C with 5% CO_2. Then, another dose of M0, M1 or M2 RAW246.7 macrophages were added to each well at ratio 1:5 (RAW 264.7: PAM-212) and incubated at 37° C with 5% CO_2 for 24, 48, or 72 hours. After the incubation period, the suspension was collected from the 6-well plates individually and placed in separate 15-ml centrifuge tubes. The tubes containing the suspensions were centrifuged at 1600 rpm and 4° C for 5 minutes. An aliquot of the supernatant (1 ml) from infected cells was stored in 1.5 ml centrifuge tubes at –80 ° C to be used later for the the virus infectivity plaque assay. At the same time, trypsin was used to collect the cells. To ensure floating cells were not lost, the medium to collect the cells after trypsinization 15-ml centrifuge tubes in the previous step were reused. These reused tubes and medium were centrifuged at 1600 rpm and 4° C for 5 minutes. Then, the supernatant was aspirated, and the remaining pellet was resuspended in 1 ml DMEM containing 10% FBS to count the viable cells.

Plaque Assay

Six-wells plates were used to seed Vero cells to a density of $3x10^5$ cells/ml per-well to 90% to 95% confluency. Serial dilutions of virus were prepared in DMEM by using the stored medium (–80 ° C) that we collected previously by thawing to room temperature. Ten (10) tubes were prepared with each tube containing 900 µl of DMEM. Serial dilutions were performed by transferring 100 µl of into the first tube and repeating the dilution process by transferring 100 µl into following tube until we got 10 tubes with

dilution factors of $1x10^{-1}$ to $1x10^{-10}$. To infect cells with virus, the medium from each well was aspirated and the cells were washed with PBS once. Then, 500 µl diluted viruses were gently added into each well and incubated at 37° C with 5% CO_2 for 2 hr.

Next, the medium was aspirated from each well and the cells were washed with 2 ml PBS. Then, 2 ml of 3% agarose/growth media was added into each well. Following incubation at 37° C, 5% CO_2 for 5 to 7 days, the plaques appeared and the cells were fixed with 4 % paraformaldehyde solution for 1 hr at room temperature. Then, the plates were washed and stained by 1 % crystal violet for 2-3 minutes. Then, each well was rinsed with tap water to read the plaques. The following equation were used to find the virus concentration in PFU/ml

$$\frac{PFU}{mL} = \frac{\text{number of plaques}}{(\text{Dilution Factor})X\,(\text{mLof inculum per Well})}$$

Cell Viability

Trypan blue exclusion assay was used to determine cell viabilities. RAW246.7 or PAM212 cells were seeded in 6-well plates. Three wells were infected with HSV-1 at multiplicity of infection (MOI) of 0.1 and the other three wells were negative controls. In all experiments the cell viability was performed at 24, 48, and 72 hours. The hemocytometer was used by adding the mixture of cells and stain in the ratio of 1:2 (cells to stain). The dead cells absorb the die and appear blue when observed with a 10X objective lens of a light microscope. The whole cells were counted and the corresponding percentage of the living cells were calculated

Statistical Analysis

We conducted all experiments in triplicates. The results and the figures display the mean and standard deviation of at least three independent experiments. One-Way ANOVA was used to determine the P values by using Sigma plot 12 statistical software

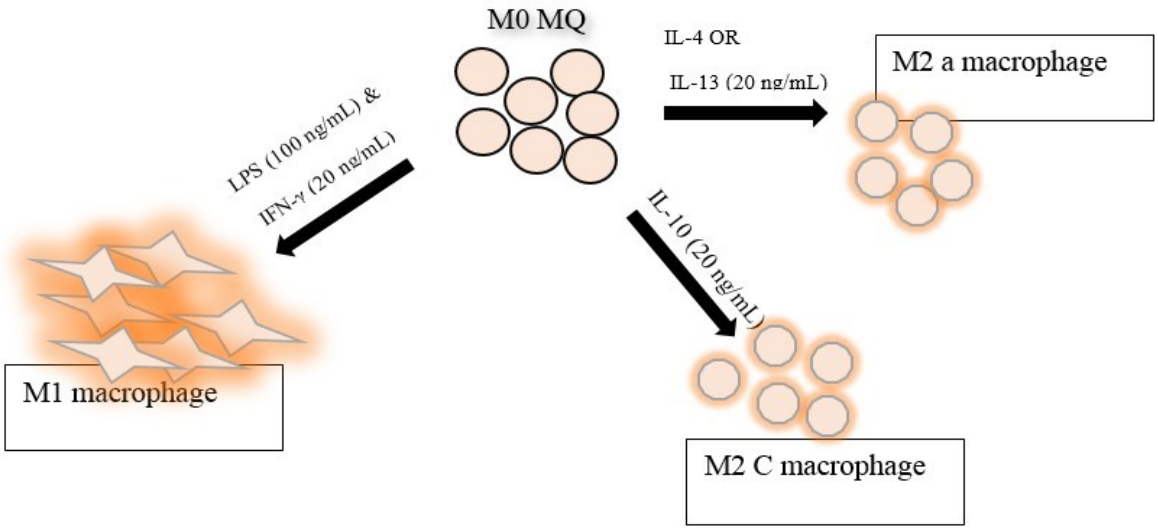

Figure 2: Macrophage Polarization

To polarize the M0 RAW246.7 toward M1 macrophage, IFN-y (20ng/ml) and LPS (100 ng/ml) were used, and to polarize M0 RAW 246.7 to M2 macrophages, IL-4, IL-10 or IL-13 were used by adding 20 ng/ml of these interleukins into the DMEM supplemented with 10 % FBS.

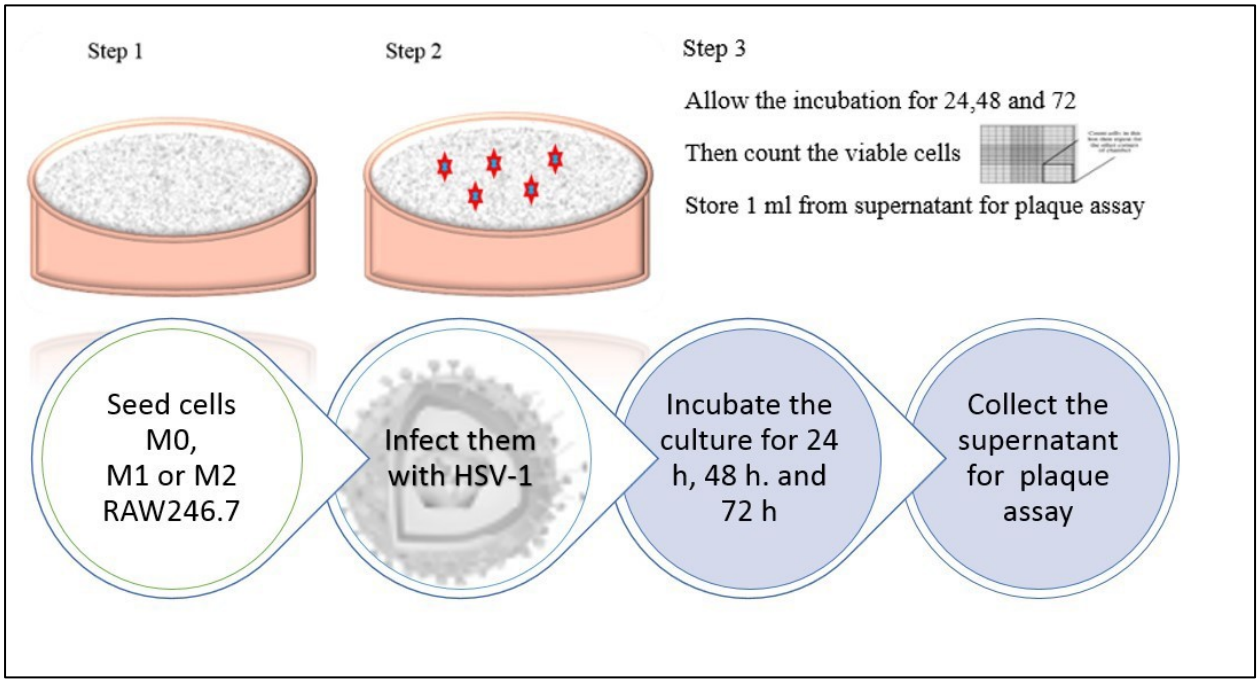

Figure 3: Infection procedure of mono-cell culture.

The RAW246.7 macrophages were seeded in 6-well plates. To polarize the RAW246.7 into M1, M2a or M2c phenotype, the interleukins were added to the medium in this step. Then three wells were infected with HSV-1 at volume of 0.44 µl (MOI of 0.1) and the other three wells uninfected to be used as negative control. The cell cultures were incubated for 2 hours to establish the infection. Next, the medium was discarded and the cells were washed with PBS before the addition of 3 ml of DMEM supplied with 10% FBS. Three separate plates were incubated at 37o C and 5% CO2 for 24, 48, and 72 hours**.**

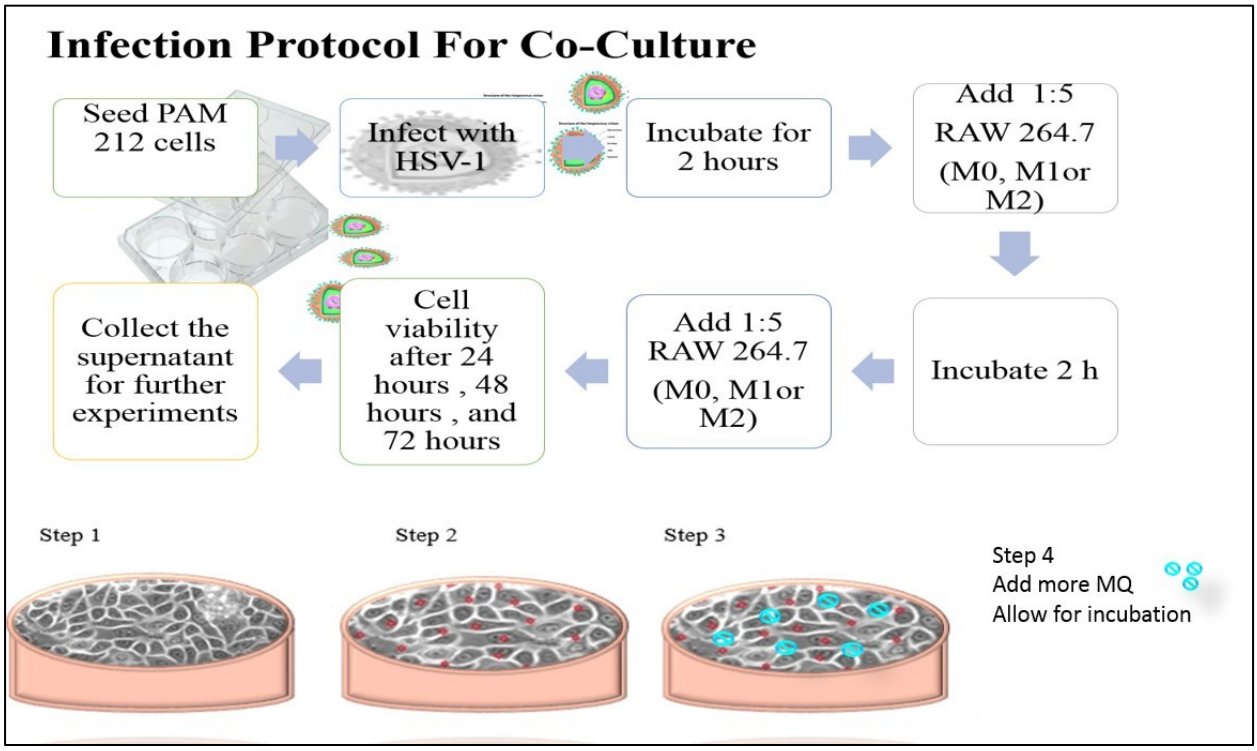

Figure 4: Infection procedure of PAM-212 co-culture with either M0, M1 or M2 RAW246.7 macrophages

PAM-212 keratinocytes were seeded in 6-well plates. Then, three wells were infected with HSV-1 and other three wells were uninfected to be used as negative controls. The plates were incubated for 2 hours to establish the infection. Afterward the DMEM was discarded and all cells were washed with 2 ml of PBS before the addition of 3 ml DMEM containing 10% FBS. Also, M0, M1 or M2 RAW246.7 macrophages were added 2 and 4 hours post infection to each well at a 1:5 ratio (RAW 264.7: PAM-212) and incubated for 24, 48, and 72 hours at 37° C with 5% CO_2.

RESULTS

The Viable Cells of PAM-212 Keratinocytes at 24, 48, and 72 Hours of HSV-1 infection:

Uninfected PAM212 keratinocytes were used as controls and were compared with the HSV-1 infected PAM212. Viable cells were counted at 24, 48, and 72 hpi using trypan blue exclusion assay. A significant decrease in viable cells was observed at all time points (e.g., 24, 48, and 72 hpi). The reduction of viable cells of the HSV-1 infected PAM212 was slightly significant at 24 ($P< 0.05$) and highly significant at 48 and 72 hpi ($P<0.001$) (Figure 5, A). The HSV-1 replication was significantly high at 48 and 72 hpi when compared to 24 hpi ($P<0.001$) (Figure 5, B)

The Viable Cells of Unpolarized M0 RAW246.7 Macrophages at 24, 48, and 72 Hours of HSV-1 infection:

Uninfected M0 RAW246.7 macrophages were used as controls and compared with the HSV-1 infected cells. The viable cells were counted at 24, 48, 72 hpi using the trypan blue exclusion assay. No statistically significant differences were observed in the viable cells at 24 and 48 hpi. At 72 hpi the viable cells of HSV-1 infected M0 RAW246.7 macrophages decreased significantly ($P<0.001$) (Figure 6, A). The HSV-1 replication was significantly higher at 72 hpi when compared to 24 and 48hpi ($P<0.001$) (Figure 6).

The Viable Cells of Polarized M1 RAW246.7 Macrophages at 24, 48, and 72 Hours of HSV-1 infection:

Uninfected M1 RAW246.7 macrophages were used as controls and were compared with the HSV-1 infected cells. Viable cells were detected 24, 48, and 72 hpi using the trypan blue exclusion assay. No significant differences existed between the infected and non-infected cells at 24, 48 and 72 hpi (Figure 7). The virus replication was not detected at all time points 24, 48, and 72 hpi

The Viable Cells and HSV-1 replication of IL-4 Polarized M2 RAW246.7 Macrophages at 24, 48, and 72 Hours of HSV-1 infection:

Uninfected M2 RAW246.7 macrophages were used as controls and compared with the HSV-1 infected cells. Viable cells were detected 24, 48, and 72 hpi using the trypan blue exclusion assay. No significant differences were observed between the infected and non-infected cells at 24 and 48 hours and significant differences among the infected and non-infected were shown after 72 hours of incubation ($P<0.001$)(Figure 8). The HSV-1 replication was significantly higher at 72 hpi when compared to 24 and 48 hpi ($P<0.001$) (Figure 8).

The Viable Cells of IL-13 Polarized M2 RAW246.7 Macrophages at 24, 48, and 72 Hours of HSV-1 infection:

Uninfected M2 RAW246.7 macrophages were used as controls and compared with the HSV-1 infected cells. Viable cells were detected 24, 48, and 72 hpi using the trypan blue exclusion assay. No significant differences between the HSV-1 infected and non-infected at 24 and 48 hours were observed and significant differences were observed among the HSV-1 infected and non-infected after 72 hours of incubation (Figure 9). The HSV-1 replication was significantly higher at 72 hpi when compared to 24 and 48 hpi ($P<0.001$) (Figure 9).

The Viable Cells of IL-10 Polarized M2 RAW246.7 Macrophages at 24, 48, and 72 Hours of HSV-1 infection:

Uninfected M2 RAW246.7 macrophages were used as controls and compared with the HSV-1 infected cells. Viable cells were detected 24, 48, and 72 hpi. using the trypan blue exclusion assay. No significant differences were detected between the HSV-1 infected and non-infected cells at 24 and

48 hours. Significant differences among the HSV-1 infected and non-infected was observed at 72 hpi ($P<0.01$) (Figure 10). The HSV-1 replication was significantly higher at 72hpi when compared to 24 and 48hpi ($P<0.001$) (Figure 10)

The Cell Viability of Uninfected and Infected Co-culture (RAW 264.7 & PAM-212) with HSV-1 after 24, 48, and 72 hpi:

PAM-212 keratinocytes were either uninfected or infected with HSV-1 at MOI of 0.1 and incubated for 2 hours. Then, either M0, M1 or M2 RAW246.7 macrophages were added at ratio of 1:5 at 2 and 4 hpi and incubated for 24, 48 or 72 h. Viable cells were detected using the trypan blue exclusion assay.

The cell viability of the co-culture model of PAM 212 with M0 RAW246.7 showed no significant differences at 24 and 48 hours and strong significant difference between the infected cells and non-infected cells at 72 hours ($P <0.001$) (Figure 6). The HSV-1 replication increased with time which was significantly lower at 24 hpi followed by 48 hpi then 72 hpi (Figure 6).

The addition of M1 macrophage to HSV-1 infected PAM212 decreased the cell's viability significantly at 48 and72 hours, but not at 24 hours (Figure 7). The HSV-1 replication in the co-culture model was low at 24 hpi followed by 72 hpi then 48 hpi (Figure7).

The addition of IL-4 M2 macrophage to HSV-1 infected PAM212 enhanced the cell viability at 24, but the cell's viability was not enhanced at 48 and 72 h (Figure 8). The HSV-1 replication in the co-culture model was lower at 24 hpi followed by 72 hpi then 48 hpi (Figure 8).

The addition of M2 RAW246.7 polarized with IL-13 to HSV-1 infected PAM212 enhance the cell viability at 24 and 48hpi, but the cell's viability did not improve at 48

and72 hpi (Figure 9). The HSV-1 replication in the co-culture model was lower at 24 hpi followed by 72 hpi then 48 hpi (Figure 9). The amount of virus detected in PAM212 decreased significantly after the addition of IL-13 M2 macrophages. When all time points are compared, the virus replication was significantly lower at 24 hpi followed by 72 hpi then 48 hpi.

The addition of M2 RAW246.7 polarized with IL-10 to HSV-1 infected PAM212 enhanced the cell viability at 24 and 48 but the cell's viability did not improve at 72 h (Figure 10). The HSV-1 replication was lower at 24 hpi followed by 72 hpi then 48 hpi (Figure 10.)

Comparison of M0, M1 M2a and M2c co-cultured with HSV-1 infected PAM212 and HSV-1 infected PAM212:

The addition of either M0, M1, M2 a or M2 c to HSV-1 infected PAM212 significantly increases the percentage of the viable cells at 24 hpi and significantly decreases the HSV-1 replication. M1 had the most effective in the suppression of HSV-1 replication followed by M2 polarized by IL-4 (Figure12, A).

At 48 hpi the addition of M1 and M2 polarized with IL-4 decreases the cell viability when co-cultured with HSV-1 infected PAM 212 keratinocytes, but the addition of M0 and M2 polarized with IL-13 or IL-10 enhanced the cell viability of HSV-1 infected PAM-212 (Figure12, B). The HSV-1 replication was significantly lower with all co-culture models, but the M1 macrophages were the best suppressors for the infection (Figure 12, E).

At 72 hpi the addition of either M0, M1 M2a or M2 c to HSV-1 infected PAM212 significantly decreases the percentage of the viable cells. The addition of M1 and M2 polarized with IL-4 reduced the viable cells when compared to other macrophage phenotypes (Figure 12, C). The HSV-1 replication was significantly lower in the all

co-culture models when compared to HSV-1 infected PAM212; however, M1 polarized macrophages were the best suppressor for the infection when compared to the other phenotype (Figure12,F).

DISCUSSION

The aim of this study was to develop a model of co-culture that could illustrate the role of the macrophages against HSV-1 infection within keratinocytes and to determine the role of unpolarized M0, and polarized M1, and M2 macrophages against the HSV-1 infection. In all experiments, cell viabilities and virus titers were performed at 24, 48, and 72 hours after the initial infection.

M0 unpolarized RAW246.7 macrophages were treated with IFN-γ and LPS to induce its polarization toward M1, and with IL-4, IL-10, or IL-13 to induce its polarization toward M2 macrophages. By examining the morphology of M0, M1 and M2 (M2a and M2c) macrophages under the light microscope, M0 RAW246.7 macrophage appeared rounded and some of it elongated. M1 macrophages appeared elongated, irregularly shapes, extended, and contained visible vacuoles. The morphology of M2 macrophages polarized by IL-4, IL-10 and IL-13 showed some elongated cells, but most of them were rounded. It was easy to distinguish between M1 from either M0 or M2 macrophages, but the morphology of M0 and M2 macrophages polarized by IL-4, IL-10 or IL-13 was almost similar.

RAW246.7 macrophages and PAM212 keratinocytes were used in mono cell culture to examine the susceptibility of each cell line to HSV-1 infection. The results showed that RAW 264.7 and PAM-212 cell lines were susceptible to the infection with HSV-1 at all time-points of 24, 48, and 72 hours (Figure 5 & 11). However, macrophages were expected to play pivotal role in preventing the HSV-1 infection.

Macrophages play critical role against herpes infection during the first hours of the attack. In the early hours of a viral infection, the cytokines produced by infected cells

mainly keratinocytes or cells encountering viral products are critical in conduction of the innate immune response to the infection. The cytokines that produced by infected cells recruit the monocytes to site of infection to be polarized to macrophages. Also, these cytokines stimulate the polarization of the macrophages to either M1 or M2. This recruitment happened as early as 2 and 7 hours post infection. (Thier, K., Petermann, P., Rahn, E., Rothamel, D., Bloch, W., & Knebel-Mörsdorf, D. d.,2017).

In the co-culture model we mimic the in vivo immune response of macrophages by seeding the PAM212 keratinocytes and allow them to grow until it reaches more than 50% of confluency. We choose to infect keratinocytes because HSV-1 infects the keratinocytes better than other cells where it induced the production of SOCS-1 protein in the infected keratinocytes to suppress the antiviral activity of the INF-γ. Then, the PAM-212 keratinocytes were infected with HSV-1 at MOI 0.1. The incubation of the virus was permitted for 2 hours which is enough time to establish the infection as stated earlier. HSV-1 entry into keratinocytes occurred with an unusual speed of maximum penetration within 5 min (Charlotte L. Sayers, Gillian Elliott, 2016). After the incubation time the cells were washed and either unpolarized M0 or polarized M1 or M2 RAW246.7 cells were added at 2 and 4 hours after the initial establishment of the HSV-1 infection. These times were selected to represent the influx of macrophages to the site of infection within the first few hours.

When the M0, M1 or M2 macrophages were co-cultured with HSV-1 infected PAM212 keratinocytes all the phenotype of the macrophages maintained the percentage of the viable cells at 24 hpi. However, at 48 hpi the M1 RAW246.7 macrophages induced the apoptosis of most of the cells in the co-culture, but M0 and M2 macrophages maintained the cell viability of the co-culture. At 72 hours the cell viability was significantly lower in all the infected co-culture except of M2 RAW246.7 macrophages that polarized with IL-13.

After the HSV-1 infection a morphological change occurred among all type of macrophages where most of the cells appeared round and granulated. This morphological change makes it difficult to differentiate M1 from M0 or M2. The viable cells of HSV-1 infected PAM212 decreased significantly at 24 hpi, but it was maintained higher in the infected RAW246.7 when compared to negative control. M1 macrophages were the better suppressor to HSV-1 replication followed by M2 polarized macrophages then M0 unpolarized macrophages. This result is equivalent with previous study showed that the replication of HSV-1 in M1 macrophages originated from J774A.1 cell line was lower than the replication of HSV-1 in M0 unpolarized macrophages and M2 polarized macrophages (Frey KG et al, 2009)(Alanazi, Y., 2018).

Unlike the M0, and M2a and M2c phenotype of macrophages, the addition of M1 macrophages to the infected keratinocytes decreased the viable cells significantly where the cell viability was about 20% at 48 hpi and 3.17% at 72 hpi (Fig12.). The virus infectivity was the lowest in the co-culture of M1 with HSV-1 infected PAM212 in all time points of 24, 48, 72 hpi.

M1 macrophages induced the apoptosis of keratinocyte and suppressed the virus replication. Previously, it was reported that M1 macrophages originated from RAW246.7 cell line secrete significant amounts of proinflammatory chemokines and cytokines when compared to M2 macrophages. One possible contributing reason is that these proinflammatory cytokines and chemokines lower virus replication in M1 polarized RAW246.7 cells than in unpolarized cells or M2 polarized cells. Another possible contributing reason is that M1 macrophage may induce growth arrest and decreases in the pH of the medium. This rationale was detected in the experiment of cell viability, when M1 macrophages exhibit a lower viable cell compared to unpolarized M0 and polarized M2 macrophages; also, the color of medium change to be more yellowish compared to M0 or M2 macrophage.

CONCLUSION AND FUTURE STUDY

All phenotypes of macrophages play a critical role in the regulation of HSV-1 infection in PAM212 keratinocytes. M0 and M2 macrophages enhanced the cell viability of the infected keratinocytes, but M1 macrophages reduced the viable cells and reduced the virus replication better than other phenotype when measured by plaque assay. The M1 polarized RAW246.7 macrophage was also the most effective in the suppression of HSV-1 either in the mono- cell culture or in the co-culture with PAM212 keratinocytes. The differences between the unpolarized M0 and polarized M1, M2a and M2c macrophages were clearly noticeable.

HSV-1 induces the production of SOCS proteins which act as suppressor of important antiviral cytokines such as INF-y. HSV-1 induced the production of SOCS proteins in the macrophages in different amount which make it susceptibility to HSV-1 infection different among different phenotype. Previous report shows a difference between the induction of SOCS1 among M0, M1 and M2 macrophages (Frey KG et al, 2009). So, it will be critical to detect the expression of SOCS proteins in the co-culture of HSV-1 infected PAM212 with either M0, M1, M2a or M2c RAW246.7 macrophages.

Further studies using the same co-culture models with determining the cytokines before and after HSV-1 infection would provide further understanding for the response of macrophage following HSV-1 challenge. Another study may test the same co-culture models using different cell line of keratinocytes such as HEL-30 or macrophage, such as J774.A1 cell line. Different cell lines may act in a different way, so it will be useful to test the same co-culture with other cell lines.

In this study we detected the HSV-1 infectivity using the plaque assay. This technique allows only the detection of infectious viruses. Other techniques such as PCR or ELIZA may use with plaque assay to allow the detection of all virus progeny regardless if the virus was infectious or not. This should be done before and after the co-culture cells encounter with HSV-1.

Figures

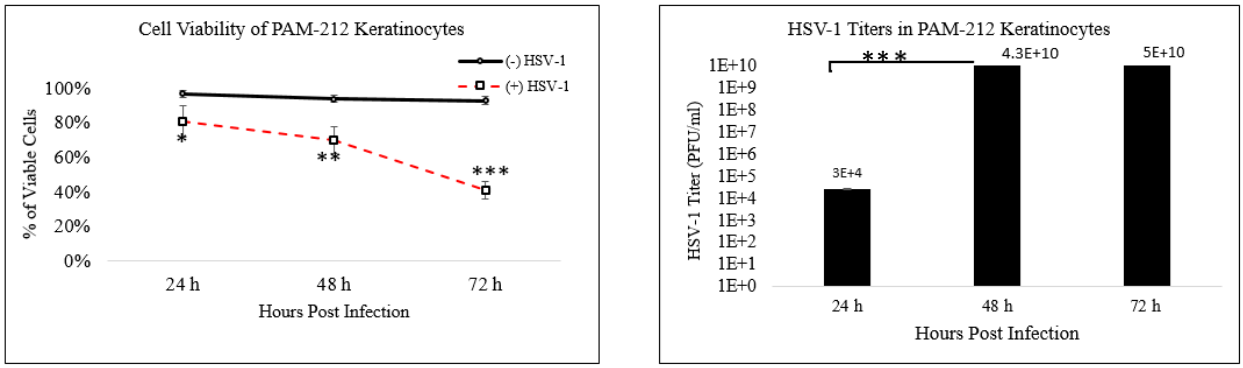

Figure 5: HSV-1 Replication in PAM-212 Keratinocytes: (A-left) The effect of HSV-1 on PAM-212 viability. Uninfected PAM212 keratinocytes were used as control and compared with the HSV-1 infected PAM212. The viable cells were counted at 24, 48, 72 hpi using the trypan blue exclusion assay. The solid line represents the uninfected cells while the dotted line represents the HSV-1infected cells. A significant reduction in viable cells was detected at all time points (24, 48, and 72 hpi). The reduction in the viable cells of HSV-1 infected PAM212 was slightly significant at 24 hpi ($P< 0.05$) and highly significant at 48 and 72 hpi. (B-right) HSV-1 infectivity by plaque assay in PAM-212 keratinocytes after the incubation periods of 24, 48, and 72 hpi. (*= $P<0.001$). *, $P \leq 0.05$; **, $p \leq 0.01$, ***, $p \leq 0.001$ Error bars represent the mean ±SD of three independent experiments.

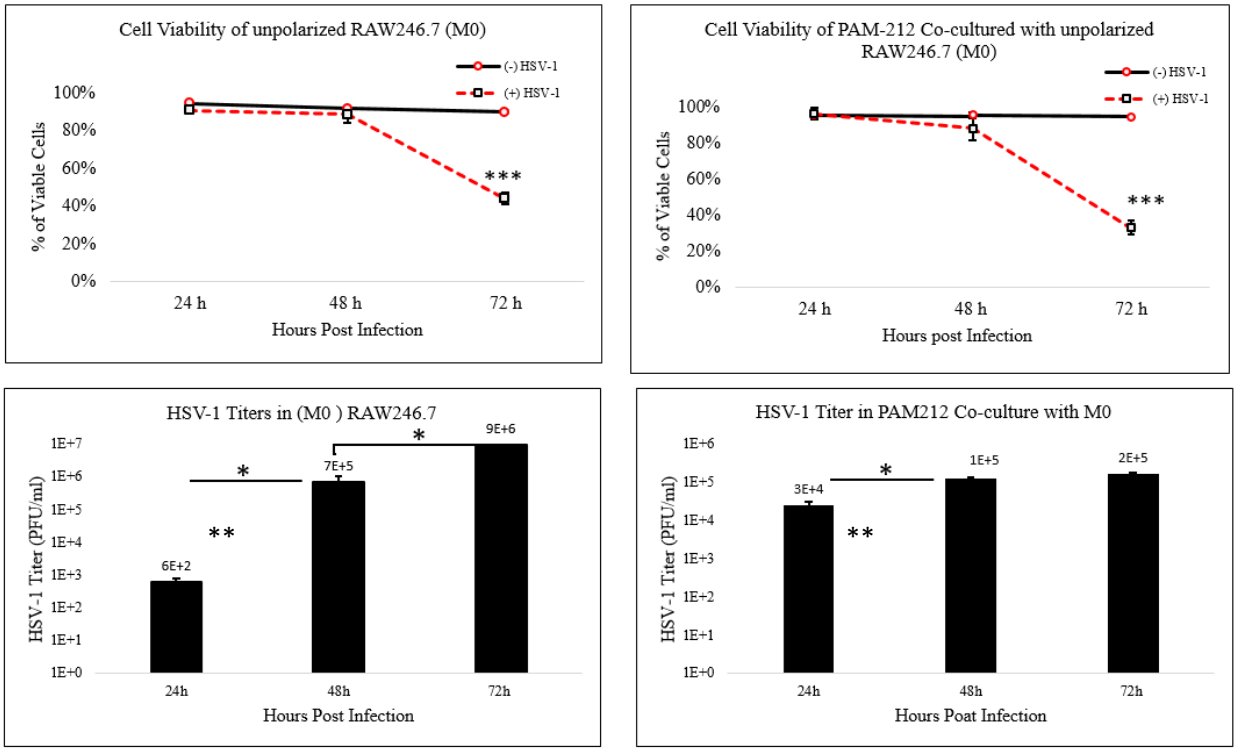

Figure 6: Effect of unpolarized M0 macrophages on HSV-1 replication: (A-top left) Percentage of Cell Viability of infected RAW246.7(M0) after 24,48, 72 hpi. RAW246.7 were seeded in six well plates. Three wells were infected with HSV-1 and the other three remained uninfected. The solid line represents the uninfected cells while the dotted line represent the HSV-1infected cells. No significant differences were seen until 72 hpi ($P<0.001$). (B-top right) Percentage of cell viability of infected PAM-212 co-cultured with M0 RAW246.7 at 24h, 48h, and 72h. PAM-212 were seeded on six wells plates and infected with HSV-1 at MOI 0.1, M0 RAW246.7 were added at 2 and 4 hpi with a ratio of 1:5 (MQ: PAM). The infected cells were collected at 24, 48, and 72 hpi**.** No significant differences were seen until 72 hpi ($P<0.001$) (C-bottom right) &(D-bottom left) represent the HSV-1 replication in (A) & (B) experiments respectively. In C) The virus infectivity was lower at 24 and 48h when compared to 72 h while D) show a significant increase in HSV-1 titer 48 and 72hpi. * means $P \leq 0.05$, ** means $p \leq 0.01$, and *** means $p \leq 0.001$.
Each bar represents the mean ±SD of three independent experiments.

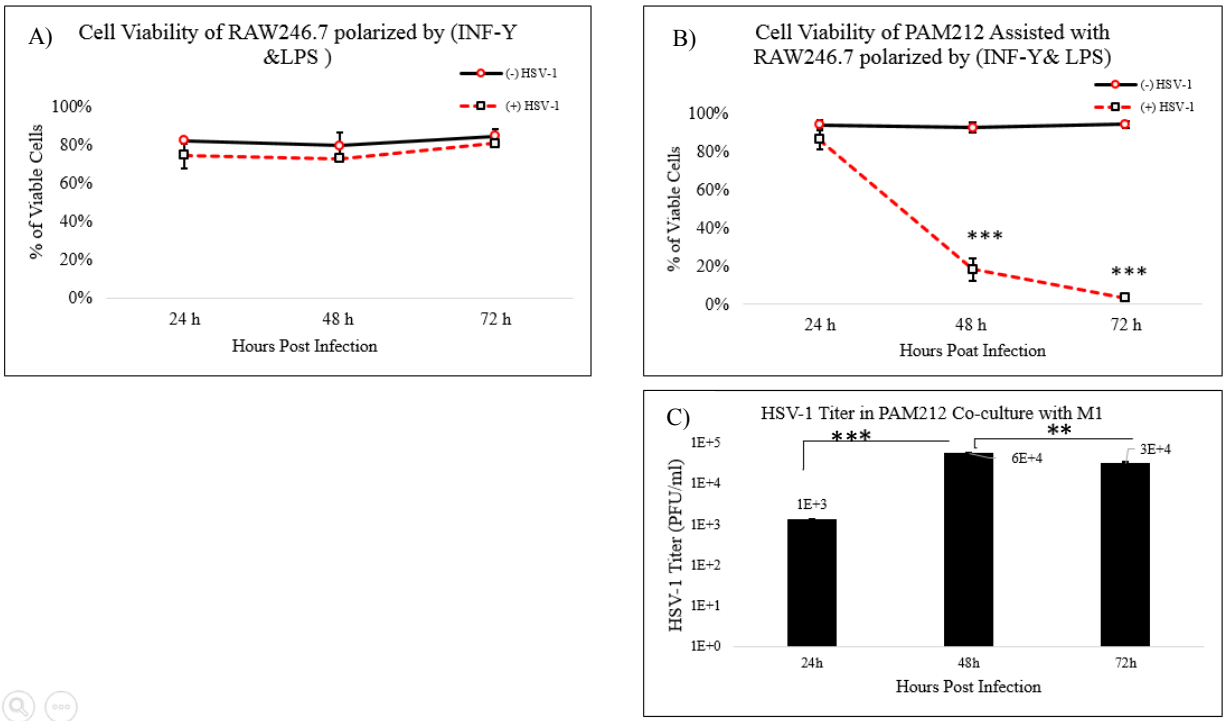

Figure 7: The Effect of M1 Polarized RAW246.7 on HSV-1 Replication:

(A-top right) Percentage of cell viability of infected RAW246.7(M1) after 24,48, and72 hpi. RAW246.7 were seeded and polarized by INF-Y& LPS 24 h before the HSV-1 infection. Three wells were infected and the other three remained uninfected. No significance difference was detected between the uninfected and HSV-1 infected cells at all time points. B-top left) Percentage of cell viability of infected PAM212 co-cultured with M1 RAW246.7 at 24h, 48h, and 72h. PAM212 were seeded on six wells plates and infected with HSV-1 at MOI 0.1, M1 or RAW246.7 were added 2 &4 hours after the infection at ratio 1:5 (MQ: PAM). Significant decrease was detected at 48 and 72 hpi. ($P<0.0001$)**.** The solid line represents the uninfected cells while the dotted line represents the HSV-1infected. (C-bottom left) represent the HSV-1 replication of the co-cultured models (PAM +(M1-MQ) a significant increase in HSV-1 titer 48 hpi then significant reduction at 72 hpi. * means $P \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$

Each par represents the mean ±SD of three independent experiments.

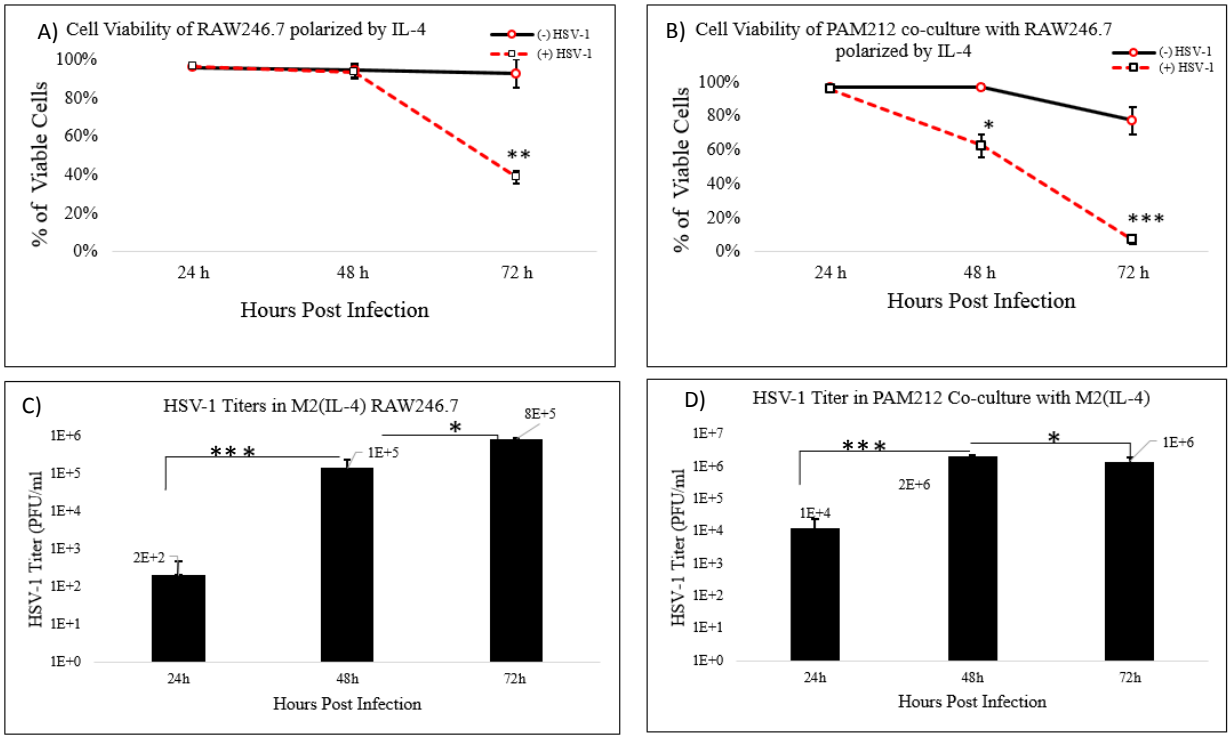

Figure 8: The Effect of M2 RAW246.7 Polarized by IL-4 on HSV-1 Replication: (A) Cell viability of infected IL-4 M2RAW246.7 after 24,48, 72 h. RAW246.7 were seeded in six well plates and polarized toward M2 by IL-4. Three wells were infected with HSV-1 and the other three remained uninfected. The solid line represents the uninfected cells while the dotted line represents the HSV-1infected cells. No significant differences were seen until 72 hpi where the cell viability was significantly lower in the infected cells when compared to uninfected cells. (B) Cell viability of infected PAM212 that assisted with IL-4 M2 RAW246.7 at 24h, 48h, and 72h. PAM212 were seeded on six wells plates and infected with HSV-1 at MOI 0.1. M2 RAW246.7 were added 2 and 4 hours after the infection at ratio 1:5 (MQ: PAM). The cell viability of infected co-culture decreased significantly at 48 ($P<0.05$) and 72 hpi ($P<0.001$). C) and D) represent the HSV-1 replication in (A) and (B) experiments, respectively. C) The virus infectivity was lower at 24 and 48 h compared to 72 h D) Lower virus titer at 24 hpi and significant increase in HSV-1 titer 48h post-infection followed by a significant decrease 72 h post infection. * mand $P \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$. Each bar represents the mean ±SD of three independent experiments.

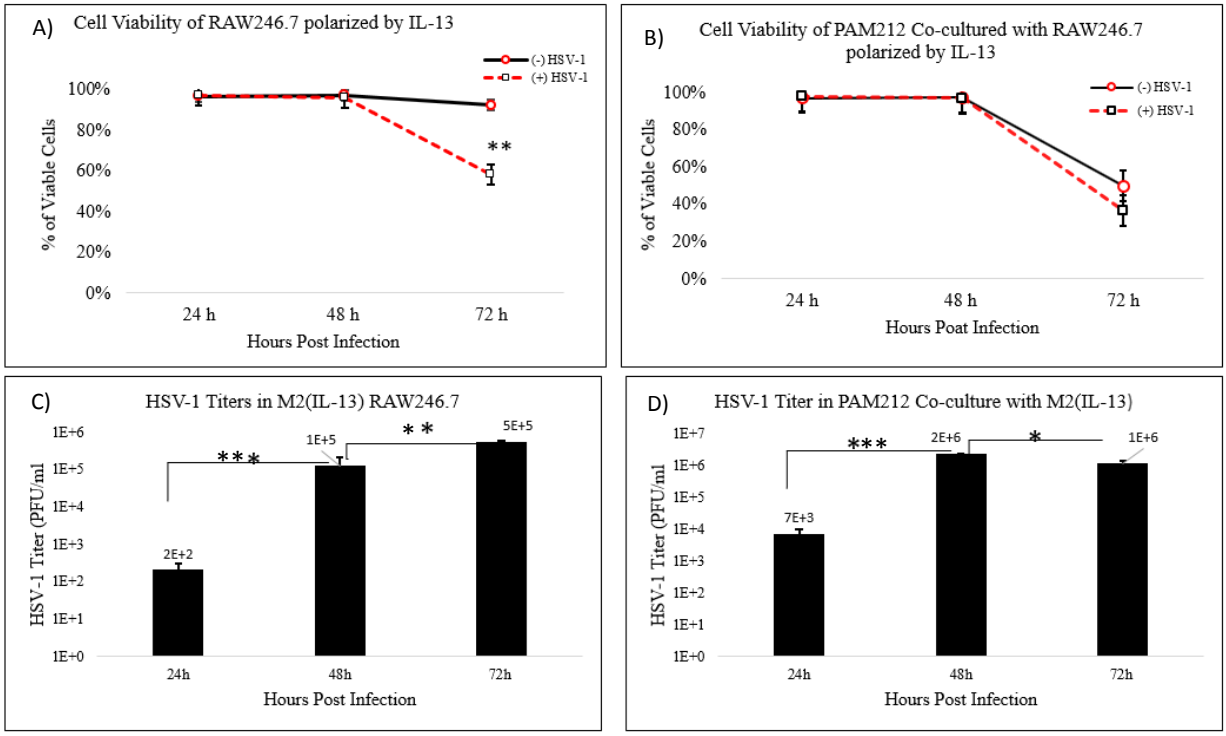

Figure 9: The Effect of M2 RAW246.7 Polarized by IL-13 on HSV-1 Replication:

A) Percentage of cell viability of infected IL-13 M2RAW246.7 after 24,48, 72 h. RAW246.7 were seeded in six well plates and polarized toward M2 by IL-13. Three wells were infected with HSV-1 and the other three remained uninfected. No significant differences were seen until 72 hpi (B) Percentage of cell viability of infected PAM212 that assisted with IL-13 M2 RAW246.7 at 24h, 48h, and 72h. PAM212 were seeded on six wells plates and infected with HSV-1 at MOI 0.1, M0 RAW246.7 were added 2 and 4 hours after the infection at ratio 1:5 (MQ: PAM). No significant differences were found at all time points. The solid line represents the uninfected cells while the dotted line represents the HSV-1infected cells. (C) and (D) represent the HSV-1 replication in (A) and (B) experiments, respectively. In C) HSV-1 increased with time. D) showed low virus titer at 24 hpi and significant increase in HSV-1 titer 48h post-infection followed by significant decrease 72 h post infection. * means $P \leq 0.05$, **means $p \leq 0.01$, *** means $p \leq 0.001$.

Each bar represents the mean ±SD of three independent experiments.

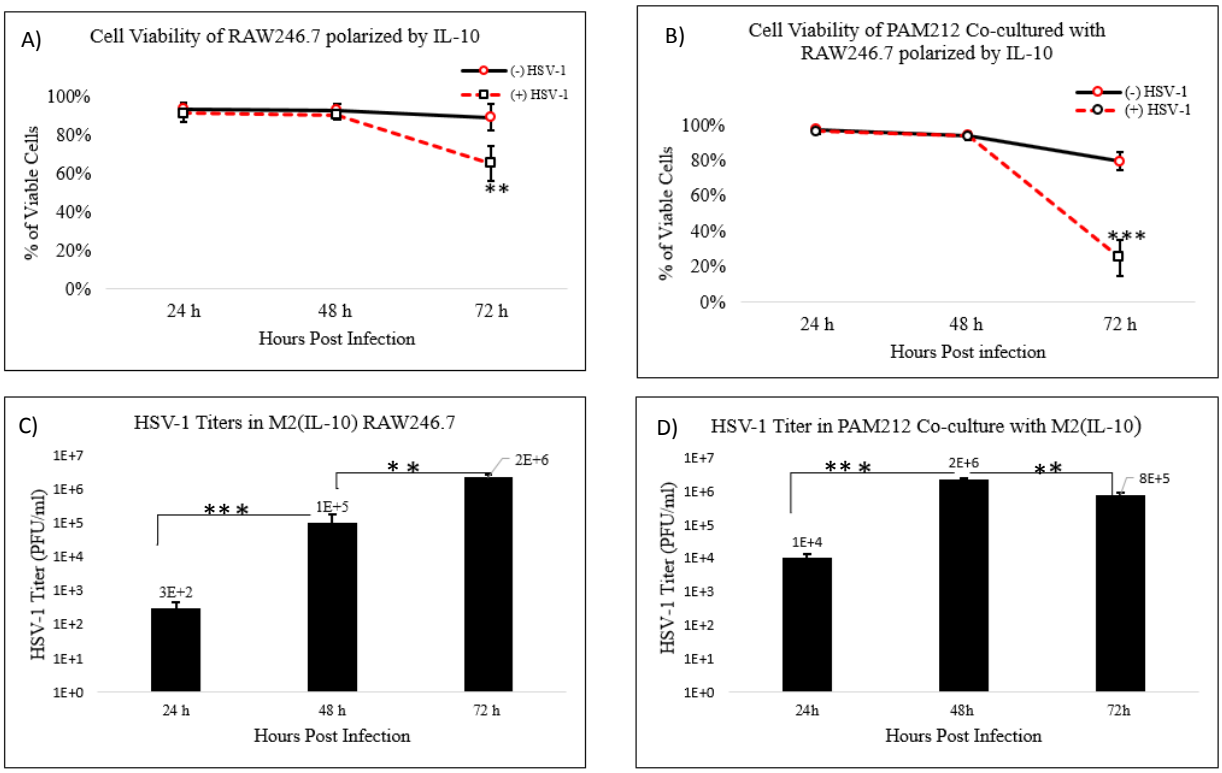

Figure 10: The Effect of M2 RAW246.7 Polarized by IL-10 on HSV-1 Replication

(A) Percentage of cell viability of infected IL-10 M2RAW246.7 after 24,48, 72 h. RAW246.7 were seeded in six well plates and polarized toward M2 by IL-10. Three wells were infected with HSV-1 and the other three remained uninfected. The solid line represents the uninfected cells while the dotted line represents the HSV-1infected cells. (B) Percentage of cell viability of infected PAM212 that assisted with IL-10 M2 RAW246.7 at 24h, 48h, and 72h. PAM212 were seeded on six wells plates and infected with HSV-1 at MOI 0.1, M0 RAW246.7 were added 2 and 4 hours after the infection at ratio 1:5 (MQ: PAM). The infected cells were collected at 24, 48, and72 hpi.

In (A and B) No significant differences were seen until 72 hpi (A= $P<0.001$, B=$P<0.0001$) (C) and (D) represent the HSV-1 replication in (A) and (B) experiments, respectively. In C) HSV-1 increase with time D) showed low virus titer at 24 hpi and significant increases in HSV-1 titer 48 h post-infection followed by significant decrease 72 h post infection.

*means $P \leq 0.05$; ** means $p \leq 0.01$, *** means $p \leq 0.001$

Each bar represents the mean ±SD of three independent experiments.

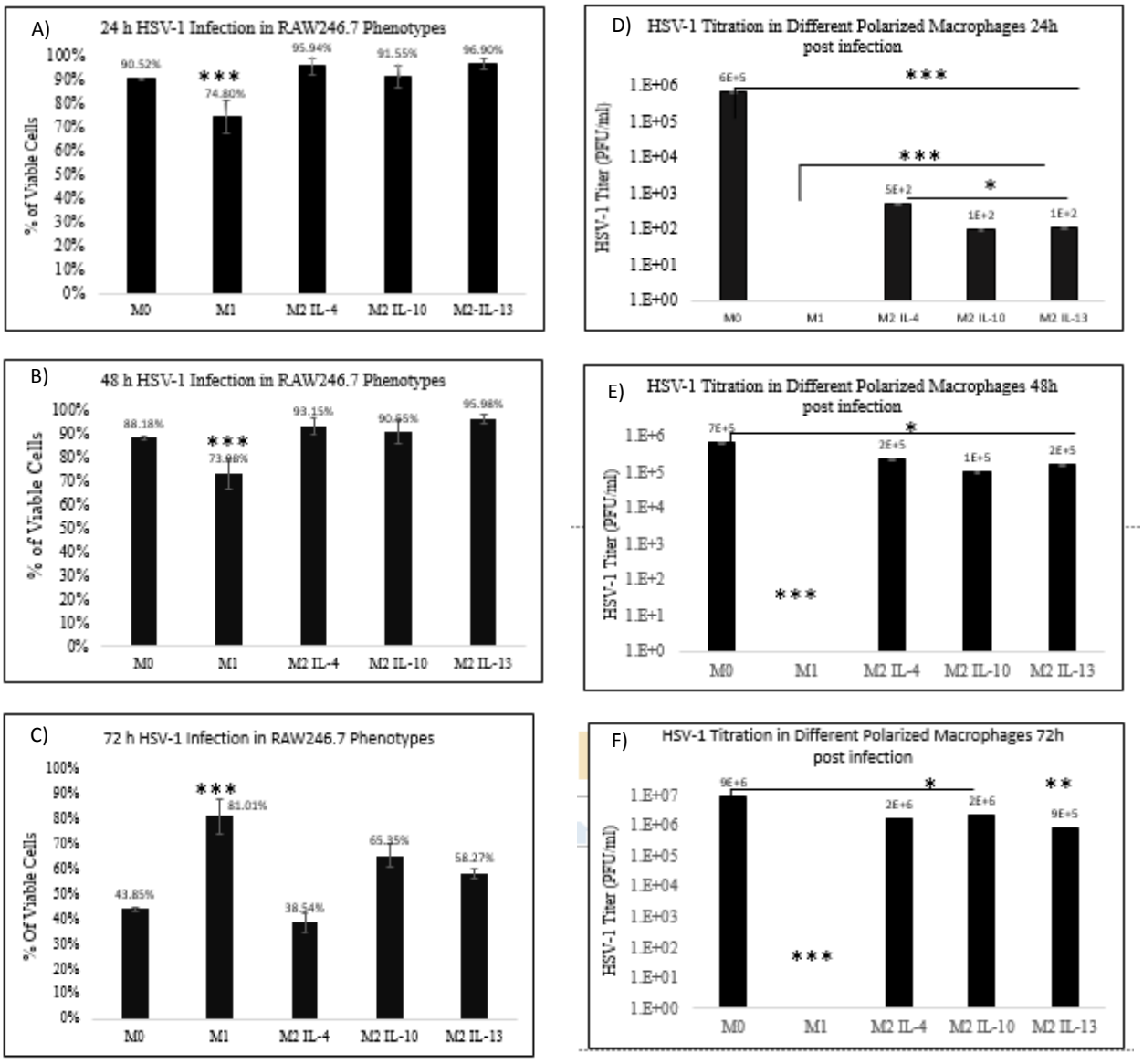

Figure 11: Comparison of RAW246.7 phenotypes Infected M0, M1, M2IL-4, M2IL-10 and M2 IL-13 RAW246.7. Cell viability represented in A) 24 hpi B) 48 hpi and C)72 hpi, and Plague assay in D) 24 hpi h E) 48 hpi and F) 72 hpi. The co-culture of M1 with infected PAM was most effective among all phenotypes.

means $P \leq 0.05$, ** means $P \leq 0.01$, *** means $P \leq 0.001$.

Each bar represents the mean ± SD of three independent experiments.

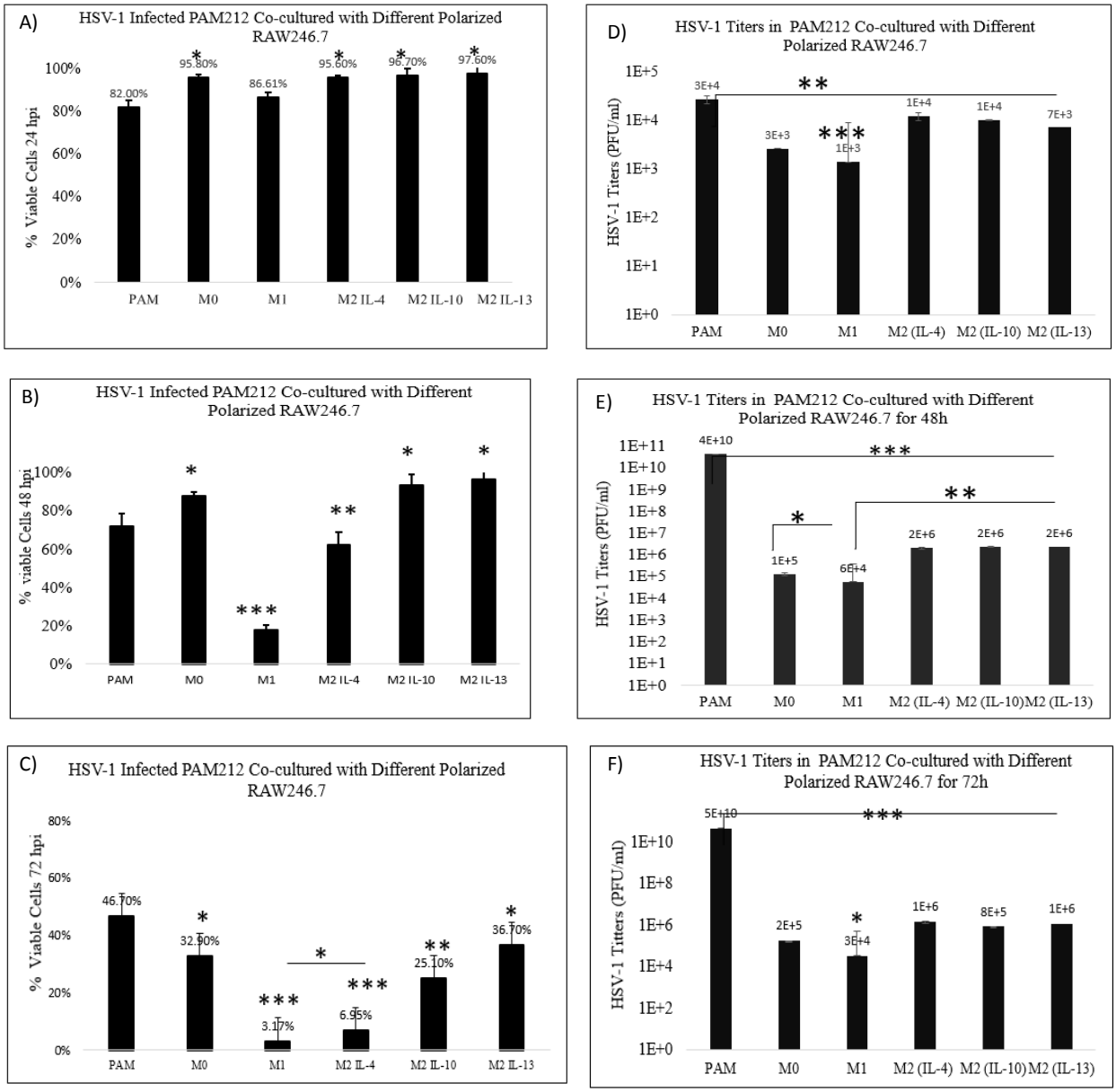

Figure 12: Comparison between the HSV-1 Infected Co-culture models: HSV-1 Infected PAM-212 alone or co-cultured with either M0, M1, M2IL-4, M2IL-10, and M2 IL-13. Cell viability represented in A) after 24 h B) 48 h and C)72h. Plague assays are displayed in D) after 24 h E) 48 h and F) 72 h. The co-culture of M1 with infected PAM was most effective among all phenotypes. * means $P \leq 0.05$, ** means $P \leq 0.01$, and *** means $P \leq 0.001$.

Each bar represents the mean ± SD of three independent experiments.

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